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Equinatoxins: A Review

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Abstract

Equinatoxins are basic pore forming proteins isolated from the sea anemone *Actinia equina*. Pore formation is the underlying mechanism of their hemolytic and cytolytic effect. Equinatoxin concentrations required for pore formation are higher than those causing significant effects in heart and skeletal muscle. This means that other mechanisms must also be involved in the toxic and lethal effects of equinatoxins. Effects of equinatoxins have been studied on lipid bilayers, several cells and cell lines, on isolated organs and in vivo. Different cells have distinct susceptibilities to the toxin, ranging from <1 pM up to >100 nM. The cells are swollen after a prolonged treatment with low concentrations of equinatoxin II, or rapidly when 100 nM or higher concentrations of the toxin are used. Equinatoxins increase cation-specific membrane conductance and leakage current, affect the function of potassium and sodium channels in nerve, muscle and erythrocytes, increase intracellular Ca^{2+} activity, and cause a significant increase of cell volume. In smooth muscle cells and in neuroblastoma NG108-15 cells, an increase in intracellular Ca^{2+} activity is observed after exposure to 100 nM equinatoxin II. The large difference in toxin concentrations needed for the pore formation and other effects suggest that equinatoxins exert their effects through at least two different mechanisms. It is well known that lipid environment is important for the proper functioning of membrane channels and other membrane proteins. It is possible that toxin monomers disturb local conditions around ionic channels and/or receptors by binding in the vicinity of those structures, thus altering their function.

Introduction

Synthesis of toxins by toxic live organisms is energetically demanding and it is reasonable to assume that toxins must provide a kind of benefit for survival. Some toxic substances have regulatory roles in the life cycle of an organism, such as cyanotoxins in cyanobacteria. Other toxins provide either defense against predators or serve as a "weapon" in hunting a prey. Some toxins, such as cytolytic toxins, may serve several roles – they can help catch and digest the pray, or they serve as defense chemicals and substances that help secure their territory in interspecies competition.

Anthozoans and Their Toxins

Cnidarians comprise four classes: Anthozoa, Cubozoa, Hydrozoa and Scyphozoa. Tentacles of all cnidarians contain specialized cells, nematocytes, organized in nematocysts. This is an organelle capable of producing and delivering their toxins into a prey. Several Anthozoans can also release their toxins from the digestive cavity. Although all cnidarians produce powerful toxins and all of

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Fig. 1 *Actinia equina* in its natural environment on a rock in shallow water. Adriatic sea, island Vis, July 2013

them possess nematocysts not all of them are harmful to humans. Nematocysts of several Anthozoans, including *Actinia equina* (Fig. 1), are not capable of penetrating human skin.

A Brief History on Equinatoxin Research

In the early seventies of the previous century a toxic protein, equinatoxin has been found in the tentacle extract from the sea anemone *Actinia equina*. The toxin has been purified and characterized by Lebez and Ferlan a year later; in 1973. The isolated and purified toxin was a polypeptide with isoelectric point (pI) of 12.5 and molecular weight (m.w.) of roughly 20 kDa. A remarkable property of the toxin was the fact that it contained no cysteine; therefore there were no disulphide bonds in that protein (Ferlan and Lebez 1974). At that time this was the only sea anemone toxin with the m.w. of 20 kDa; other coelenterate toxins were either larger (>70 kDa) or smaller (<15 kDa).

The toxin was lethal to rats with the $LD_{50} = 33.3$ μg/kg. The animals died because of cardio-respiratory arrest (Sket et al. 1974), but animals that have survived the intoxication showed tacyphylaxis – a larger dose was needed to cause apnea and cardiac arrest. On the contrary, it has been shown that equinatoxin has a cardio-stimulatory effect on guinea-pig atrium. These controversial findings might be explained by higher resistance of the atrial myocytes to the effects of equinatoxin, and less so to different concentrations of the toxin used in experiments. In fact, minute concentrations of equinatoxin can be cardiostimulatory due to the entry of calcium into a cell. However, the initially elevated arterial blood pressure (aBP) after injection of equinatoxin is always followed by a profound fall of aBP within a few minutes. Respiratory arrest caused by equinatoxin is poorly understood, but efferent impulses from the respiratory centre were absent after application of the toxin. Direct stimulation of the phrenic nerve still caused contractions of diaphragm. A detailed study of the effects of equinatoxin on isolated lungs showed that the vascular resistance increases and lungs become edematous when perfused with 50–100 nM equinatoxin (Lafranconi et al. 1984). This may explain breathing disturbances such as restriction of ventilation and decreased alveolar ventilation, but not necessarily also the complete cessation of breathing.

Hemolytic activity was the most evident and documented action of equinatoxin. A very small concentration of the toxin, only 0.1 nM, was needed for complete hemolysis of erythrocytes (Macek and Lebez 1981). Hemolysis was increased in the presence of calcium ions. An important finding was that the action of equinatoxin on erythrocytes is inhibited in the presence of sphingomyelin. The importance of reactions between some pore forming toxins from sea anemones and sphingomyelin has been described by Bernheimer and Avigad who demonstrated that pre-incubation of a cytolysin

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Table 1 Basic characteristics of equinatoxins

	EqT I	EqT II	EqT III
Isoelectric point (pI)	9.80	10.50	10.50
LD50 mice (μg/kg)	23.00	35.00	83.00
LD50 window	±30 % of LD_{50}	±30 % of LD_{50}	±30 % of LD_{50}
Time to death	5 min	5 min	5 min to 12 h
Time to half hemolysis (min)	1.6	0.8	0.5
Time to half hemolysis relative to EqT I (%)	100	49.38	30.86

from *Stichodactyla helianthus* with sphingomyelin abolishes its hemolytic activity. This lead them to the conclusion that sphingomyelin is the acceptor for the toxin in the erythrocyte membrane (Bernheimer et al. 1982). This finding is also important for the present research of membrane rafts, the sphingomyelin and cholesterol rich cell domains, as labeled equinatoxins can be used for tagging these membrane domains. In 1988 Kem named the whole class of sea anemone toxins "actinoporins" due to their ability to bind to sphingomyelin and to porate cell membranes (Kem and Dunn 1988).

Potential use of equinatoxin as anti-neoplastic agent has been studied on mice bearing the ascitic form of Ehrlich carcinoma. Equinatoxin increased the survival time of those mice. In vitro it was cytotoxic to tumor cell lines in 0.1 nM concentrations, but the resistance of the cells increased after several administrations of the toxin. Lung macrophages were resistant to the action of equinatoxin. Toxic effects of equinatoxin and very steep dose/response curve made equinatoxin a poor candidate for clinical use (Giraldi et al. 1976).

"Equinatoxin" Comprises a Group of Three Isotoxins with Similar Action

Soon after the isolation of equinatoxin a number of basic polypeptide toxins from several sea anemones have been isolated. All of them were cytolytic, and their action was inhibited by sphingomyelin. Reinvestigation of the venom from the sea anemone *Actinia equina* yielded three isotoxins with similar structure and function (Macek and Lebez 1988). All of them were proteins devoid of cysteine with m.w. roughly 20 kDa and pI ≈ 10. They are stable proteins although their structure is not fixed with disulphide bonds. The major constituent of the hemolytic fraction of the venom was equinatoxin II (EqT II), and two smaller fractions were named equinatoxin I and III (EqT I and EqT III respectively). One gram of wet weight of the sea anemone yielded 61.3 μg of EqT I, 288 μg of EqT II, and 108.9 μg of EqT III (Macek and Lebez 1988).

The most toxic isotoxin is EqT I with LD_{50} 23 μg/kg in mice, followed by EqT II (LD_{50} 35 μg/kg) and EqT III (LD_{50} 83 μg/kg). Contrary to its toxicity, EqT III is roughly three times more efficient in producing hemolysis of sheep erythrocytes than EqT I and nearly two times more efficient than EqT II (see Fig. 1 and Table 1). EqT II is present in the tentacles and the body of the sea anemone while EqT I and EqT III seem to be present only in the body of the animal. Equinatoxin has been shown to increase leakage current before the lysis of skeletal muscle cells in sub-nanomolar concentrations (Šuput 1986).

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Cloning, Sequencing and Expression of Equinatoxins

Soon after isolation of three isotoxins from *Actinia equina* the most abundant isotoxin, EqT II, was cloned and expressed in the cytoplasm of *Escherichia coli* (Anderluh et al. 1996). The cloned and expressed EqT II was a 20 kDa protein that reacted with polyclonal antibodies against the wild type EqT II. It had identical hemolytic activity as the natural EqT II. Like EqT II, the cloned and expressed EqT II consists of 179 amino acids (a.a.). Precursor protein consisted of 214 a.a., the first 35 a.a. being pre-propeptide. The pro-part was not important for the proper folding of the protein. Cloning of EqT II showed that serine at position 177 was replaced by threonine, but this had no effect on the hemolytic activity of the toxin.

Three isotoxins can be isolated and purified from the *Actinia equina*. A detailed analysis of the wild types of EqT I and EqT III was needed before the comparative analysis of all equinatoxins encoded by the sea anemone's genome could be performed. It was shown that EqT I consists of two forms, EqT I' and EqT I", differing in only one a.a. Similarly, isoforms of EqT II have also been determined while only one form of EqT III has been found. Cloning of equinatoxins revealed that more than three equinatoxins are encoded in the animal's genome (Anderluh et al. 1999). Two of them, EqT IV and EqT V have also been cloned and then expressed in *Escherichia coli*. The N-terminal sequence of EqT IV showed high homology with EqT III, and the N-terminal sequence of EqT V was identical to that of EqT II and EqT I'. It is very interesting that the sequence of EqT II is identical to the sequence of tenebrosin C isolated from the sea anemone *Actinia tenebrosa*, and N-terminal of tenebrosin B is identical to that of EqT I".

Equinatoxin II as a Model Molecule to Study Mechanism of Action of Actinoporins

Several bacterial pore forming proteins have been studied in detail, but pore forming toxins from sea anemones represent a completely new type of pore forming proteins, and they were among the first pore forming toxins found in eukaryotic organisms. Several tens of pore forming cytolytic toxins have been isolated from over 20 species of sea anemones. They are all basic proteins with pI > 9 and m.w. close to 20 kDa. Their pore-forming activity can be reduced or abolished with pre-incubation with sphingomyelin. On the other hand, these toxins preferentially or exclusively bind to lipid bilayers and cellular membranes containing sphingomyelin, which seems to be the low affinity receptor for actinoporins. EqT II is thoroughly studied actinoporin, and the mechanism of its pore-formation has been elucidated. It can be considered as a model to study the mechanisms of action of all actinoporins.

Sphingomyelin is the Acceptor for Equinatoxins and Other Actinoporins

All actinoporins have a similar structure and they belong to a highly conserved family of proteins. Even more so, several living organisms have been shown to produce proteins with at least 60 % homology to actinoporins. They have been found in a marine gastropod *Monoplex echo* (25 kDa echotoxin 2) and fungus *Xerocomus chrysenteron* (143 a.a. fungal lectin XCL). Echotoxin 2 is hemolytic and lectin XCl is not as it lacks the N-terminal region.

Cristal structure (Athanasiadis et al. 2001; Mancheno et al. 2003) and 3D structures of EqT II form *Actinia equina* and sticholysin II from *Stichodactyla helianthus* determined in solution by means of NMR spectroscopy (Hinds et al. 2002; Castrillo et al. 2009, 2010; Drechsler et al. 2010) revealed that they are composed of a core of ten tightly packed β-sandwiches and two α-helices on its sides.

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For EqT II it has been shown that the loose N-terminal helix part is composed of 30 a.a. and it is attached to the core of the protein only at its C-end while the other α-helix is attached at both ends. The N-terminal region can undergo structural changes independently of the conformation of the core of the protein and it is the crucial part of the molecule for the formation of the pore after oligomerization of at least three molecules of EqT II (Belmonte et al. 1994).

The protein core has extensive hydrophobic interfaces formed by residues projecting mostly from the internal faces of two β-sheets. A crucial characteristic of the toxin needed for the binding of the toxin to membrane is a patch of a.a. at the bottom of the core of the molecule that can serve as a binding site for sphingomyelin. It typically consists of four exposed a.a. coming from the loop between strands 6 and 7 of the β sheet core, and three a.a. from α helix (Athanasiadis et al. 2001; Hinds et al. 2002). Cocrystallization of phosphocholine (POC) with Sticholysin II revealed that POC binds to a partly hydrophobic and partly hydrophilic cavity of the toxin with dimensions of 9 × 11 × 13 Å. The cavity composed of the hydrophobic, non polar a.a. (Val85 Pro105 and side chains of Tyr131 and Tyr135), and hydrophilic, polar a.a. (Ser52, Ser103, Tyr 111, Tyr131, Tyr135 and Tyr 136), forms the POC binding site (Mancheno et al. 2003; Castrillo et al. 2010) and these a.a. residues are conserved in nearly complete actinoporin family (Alvarez et al. 2009). In EqT II two additional aromatic a.a., Trp112 and Tyr113 are required for POC, the head of sphingomyelin, recognition and binding (Turk et al. 1992; Bakrac et al. 2008). The positive charge of the choline is positioned within the cavity by means of cation–π interactions with the aromatic rings of Tyr111 and Tyr135. Phenolic hydroxyl groups of Tyr111 and Tyr136 of the toxin interact with the phosphate group of sphingomyelin which additionally secures the molecule in place within the cavity. This composition of a.a. of the POC binding cavity is a perfect match to the 3D structure found in EqT II when corrected for EqT II versus Sticholysin II numbering.

Cnidarians produce toxins that destroy cellular membranes. How can they survive? Interestingly, sea anemone sphingolipids do not contain sphingomyelin. Instead they are largely composed of phosphonosphingolipids that do not bind actinoporins. This is a protective mechanism that shields coelenterates against the action of their own cytolysins.

Binding of actinoporins to sphingomyelin is facilitated in the presence of cholesterol in the phospholipid bilayer. Cholesterol alters the physical state of the membrane forming domains where phosphorylcholine groups are exposed and easily accessible to the action of actinoporins.

Mechanism of Pore Formation with EqT II

Several well studied bacterial pore forming toxins such as α toxin from *Staphylococcus aureus* form stable β barrel pores in the membranes. Contrary to that, actinoporins and several other toxins form pores consisting of α helices. These pores are usually less stable than the β barrel pores. Binding of EqT II and other actinoporins to sphingomyelin in cellular membranes is merely the first of at least three steps leading to the pore formation. As mentioned before, at least three to four molecules of EqT II must oligomerize to form a pore.

The first step of pore formation by actinoporins, the binding to sphingomyelin in phospholipid bilayer, has been described above. This step is necessary, but binding to sphingomyelin does not warrant conformational changes leading to the pore formation.

The second step involves conformational change allowing the N-terminal α helix to move from the core of the molecule and to bind to the membrane at the membrane/water interface (Hong et al. 2002) with hydrophobic a.a. in the bilayer and polar a.a. facing the solution. The terminal five a.a. on the α helix of EqT II serve as an anchor. Lipid phase separation, coexistence of ordered and disordered lipid phases in sphingomyelin/phosphatidylcholine/cholesterol mixtures favors membrane insertion of EqT II. The presence of raft-like liquid ordered lipid domains in a membrane

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promotes binding of EqT II and pore formation (Schon et al. 2008). The second step of pore formation has been confirmed by studies of reduced and oxidized cysteine mutants of EqT II (Hong et al. 2002; Rojko et al. 2013). It has also been shown that this step must precede the oligomerization of EqT II molecules and formation of the pore. As the second step does not involve a conformational change in the β sandwich it is clear that the N terminal α helix must be loose. It seems that α helix becomes completely folded and elongated only after insertion into the phospholipid membrane. This is important event allowing N-terminal α helix to reach the trans- side of the membrane. In fact, this is the only part of the toxin, composed of 23 a.a., capable of forming the pore as it is also the only part that spans the membrane reaching its trans- side (Drechsler et al. 2006).

The third and at the same time the last step is oligomerization of the inserted EqT II molecules and formation of a functional pore with the diameter of at most 2 Å. The pore has a funnel shape with a constriction; best fit gives the size of 1.3 Å. The size of the pore itself suggests that formation of pre-pore, like in bacterial barrel pores composed of β sheet hairpins, is impossible. The small size of the pore and pore complex does not give enough space to allow translocation of four N-terminal helices after oligomerization. This confirms the observation that the second step, the binding of N-terminal α helix must occur before the oligomerization.

The walls of the toroidal pore (Anderluh et al. 2003) are composed of N-terminal α helices and lipids. The models of the pore show four α helices and lipid forming the pore. How is the cation-selectivity achieved?

Characteristics of the Pore

As mentioned before, the size of the pore is about 2 Å. Soon it became evident that the pore is cation selective, and experiments on various types of cells have shown that EqT II causes an influx of Ca^{2+} thus increasing intracellular Ca^{2+} activity. This effect is responsible for several toxic effects observed on isolated cells, organs and in vivo. In order to understand the basis of cation selectivity one must have another look at the pore. N-terminal α helix is mostly composed of aromatic a.a. that make the contact with membrane phospholipids. The hydrophilic a.a. faces the pore lumen. The conductance and selectivity of the pore depends on its size and charges. The negatively charged a.a. and phospholipids provide the basis for the cation selectivity of the pore. EqT II mutants showed that Asp10 is most probably positioned at the constriction part of the pore as the replacement of Asp10 with another a.a. had the highest influence on the conductivity of the pore (Kristan et al. 2007). Reduction of the negative charge by replacing negatively charged a.a. with neutral or positively charged decreased cation selectivity, and introducing negatively charged amino acids increased the selectivity.

The measured size of the pore formed by four EqT II molecules is too large to be formed only by four α helices; it must also incorporate membrane phospholipids thus forming a toroidal pore. This is in agreement with the finding that negatively charged lipids in membranes increase cation selectivity of the pore.

Pore formation in cellular membranes causes osmotic imbalance and lysis of cells. For the sessile organisms such as Anthozoans it is advantageous to produce an effective and rapidly acting toxin that also contributes to the first steps of digestion by lysing the cells.

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Table 2 A summary of the effects of equinatoxins

Target	Effect	Source
Erythrocytes	Lysis	(Macek and Lebez 1981)
Erythrocytes	Activation of K_{Ca}^{2+} channels	(Šuput and Schwarz 1992)
Erythrocytes	Pore formation	(Belmonte et al. 1993)
Skeletal muscle	Lysis	(Šuput 1986)
Myelinated nerve fiber	Increased leakage current	(Suput et al. 1987)
Node of Ranvier	Swelling	(Benoit et al. 2002)
Lactotrophs	Increased intracellular Ca^{2+}	(Zorec et al. 1990)
NG108-15 cells	Increased intracellular Ca^{2+}	(Meunier et al. 2000)
NG108-15 cells	Increased volume	(Meunier et al. 2000)
Guinea pig atrium	Positive inotropic effect	(Ho et al. 1987)
Isolated rat heart	Negative inotropic effect	(Bunc et al. 1999; Suput et al. 2001)
Smooth muscle	Contraction	(Suput et al. 2001; Frangez et al. 2008)
Endothelial cell line ECV-304	Increased cell volume and lysis	(Bunc et al. 2002)
Skeletal muscle fiber	Decreased sodium current[a]	(Šuput 1986)
Myelinated nerve	Decreased potassium current[b]	(Suput et al. 1987)
Thrombocytes	Aggregation	(Teng et al. 1988; Suput 1994)

[a]The effect due either to interactions of the toxin with the lipid at the channel or due to impurities?
[b]The effect due either to the interactions of the toxin with the lipid at the channel or due to other substances from the sea anemone?

Pore Formation Explains Several Effects of Equinatoxins on Cells and In Vivo

Hemolysis induced by nM concentrations of EqT II has been studied in detail, and it was a consequence of osmotic imbalance caused by EqT II pore formation. Patch-clamp experiments on human erythrocytes showed an increase in small conductance calcium activated channel ($K_{Ca)}$ activity that was inhibited by melittin. Most probably the activation of K_{Ca} channels was the result of calcium entry into the erythrocytes. It was reasonable to assume that pore formation and calcium entry will also occur in other cells, and that this will trigger several secondary effects.

First experiments performed in vivo by using equinatoxin showed that the efferent impulses from the respiratory centre to the diaphragm disappeared after intravenous application of the toxin. The effect was poorly understood, but it was known that the phrenic nerve itself was not affected. First experiments with EqT II aimed at understanding the effects of the toxin on the afferent sensory nerve fibers, efferent motor nerve fibers and skeletal muscle. 100 pM EqT II rapidly destroyed the outer layers of the skeletal muscle from frog indicating that its binding to cellular membranes is faster than its diffusion to the deeper layers of the muscle. Muscle fibers rapidly increased their volume, showed blebs on cell surface and underwent lysis within few minutes. Myelinated nerve fibers were more resistant to the action of the toxin; presumably because the myelin sheath had bound most of the toxin and the nerve fiber itself in the node of Ranvier was then exposed to lower concentration of the toxin. Even 30 min. long exposure of myelinated nerve fibers to 10 nM concentration of EqT II left the nerve fibers unaffected. Higher concentrations of EqT II increased leakage current in myelinated nerve fibers. A summary of the effects of equinatoxins is presented in Table 2.

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Effects of Equinatoxins In Vivo and on Isolated Organs

Cardiorespiratory arrest was the first reported in vivo effect of EqT (Sket et al. 1974), but understanding of the mechanism of this lethal effect was incomplete.

Application of 3 LD_{50} of either EqT II or EqT III causes a rapid cessation of respiration within a minute after the intravenous (i.v.) application of the toxins. Cardiovascular effects follow a typical pattern: the aBP initially increases and oscillates for up to 4 min and then steadily decreases until it reaches the mean circulatory filling pressure within the next 2 to 4 min. During the positive inotropic phase the EKG shows initial tachycardia that reverses to bradycardia within 30 s. This is then followed by ventricular extrasystoles and ventricular fibrillation. At that time the aBP declines irreversibly. Several mechanisms of EqT cardiotoxicity can be envisaged: hyperkalemia, apnoe, direct cardiotoxic effects of equinatoxins and release of vasoactive substances from blood cells.

The most tempting explanation can be derived from the fact that EqTs porate erythrocyte membrane releasing potassium into blood. Elevated extracellular potassium concentration affects all excitable membranes. The membrane potential drifts towards the excitation threshold for many voltage gated channels, and at the same time the prolonged depolarization inactivates voltage gated channels. Elevated potassium concentration is arrhytmogenic. In severe cases it leads to ventricular fibrillation, a phenomenon also observed after injection of a lethal dose of EqT II or III. This is the simplest and most straightforward explanation of EqTs' cardiotoxic effects, but direct evidence shows that this cannot be the (only) explanation (Bunc et al. 2000). Injection of KCl to experimental animals in a sufficient amount to rise the plasma concentration to 15–20 mM, which is the concentration usually observed in plasma of experimental animals that received lethal dose of an equinatoxin, caused only transient arrhythmia without lethal outcome (Bunc et al. 2000; Suput et al. 2001).

Anoxia due to the cessation of breathing is also not the mechanism of cardiotoxicity of EqT II as artificial respiration does not prevent the changes in EKG and pumping insufficiency. Experiments on isolated rat heart show that coronary blood flow and left ventricular pressure drop before the changes of heart rate or release of lactate dehydrogenase from heart muscle cells. This suggests that vasoconstriction and direct cardiotoxic effects are responsible for the observed effects in vivo, but a combined effect of EqT II and hyperkalemia cannot be ruled out.

Using calcium-free solutions prevents the EqT II induced vasoconstriction, but the use of L-type calcium channel blockers has only minor effect. This confirms the role of pore formation in cellular membranes in the toxic effects of EqT II. Vasoconstriction can also be only partly reduced by nitroglycerin or in the presence of Tezosentan, a non-selective endothelin receptor antagonist. To understand the effects of EqT II in vivo it is necessary to understand its effects on cell membranes and cells.

Pore Formation is Responsible for the Contraction of Smooth Muscle

EqT II and EqT III induced smooth muscle contraction is responsible for the vasoconstriction observed on isolated heart. It may be produced by hemolysis or by EqT II induced release of vasoactive substances from blood cells, by direct effects on the endothelial cells, by direct action on the vascular smooth muscle cells or by a combination of these mechanisms. In animals that received lethal doses of EqT II or EqT III the serum potassium concentration reached 12–19 mM (Bunc et al. 2000). On isolated rings from coronary arteries maximal contraction has been achieved with 60 mM KCl, and 20 mM KCl increased the tension of the vascular ring to only 50 % of the maximal contraction. 50 nM EqT III was even less effective, the contraction reached only 40 % of the maximal contraction, but it lasted up to 30 min. Combined action of EqT III and increased potassium concentration had a potentiating effect. Exposure of EqT II pretreated vascular rings to 20 mM KCl

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caused a sustained smooth muscle contraction reaching 300 % of the contraction caused by 20 nM KCl alone. This supports the view that EqT II and III cause vasoconstriction by a combination of direct actions of equinatoxins and by elevation of extracellular potassium concentration caused by cation selective pore formation in erythrocytes.

Electron paramagnetic resonance (EPR) studies on vascular smooth muscle cells revealed at least three regions of membrane with different membrane fluidity. Binding of EqT II to vascular smooth muscle cells increases the portion of membrane with lower fluidity which is in agreement with the observation that EqT II induces aggregation of ordered membrane nanodomains into microdomains (Sentjurc et al. 1996). Patch clamp studies also showed that EqT II increased leakage current in the smooth cell plasmalemma indicating formation of pores in the membrane of smooth muscle cells.

The role of pore formation in smooth muscle cells has been elucidated using EqT II on isolated smooth muscle cells from taenia coli (Frangez et al. 2008). Ten to five hundreds nM EqT causes irreversible contraction of smooth muscle cells and an increase of intracellular Ca^{2+} activity. The rate of changes depends on the concentration of the toxin used. The incubation time before the rise of intracellular Ca^{2+} activity follows a sigmoid curve; it shortens steeply in the concentration range from 10 nM (350 s) to 50 nM EqT II (22 s.) and with further increase of EqT II concentrations the incubation time does not change significantly. Time to peak, measured from the first change of intracellular Ca^{2+}, is also concentration dependent in the range from 10 to 50 nM (135 and 51 s, respectively). Considering the proposed mechanism of pore formation by EqT II it may be assumed that the time needed for pore formation in smooth muscle cells at optimal concentration of EqT II needs at least 20 s.

The origin of Ca^{2+} rise in the cell is the influx of $Ca^{2'}$ as removal of Ca^{2+} from the bathing solution prevents the EqT II induced rise in intracellular Ca^{2+}. In smooth muscle the large conductance voltage gated calcium channels (L-type Ca^{2+} channels) are responsible for Ca^{2+} influx. Depolarization of the cellular membrane leading to inactivation of these voltage activated channels or a block of those channels by L-type Ca^{2+} channel blocker verapamil had no influence on the EqT II induced rise in intracellular Ca^{2+}. This means that only pore formation is responsible for Ca^{2+} influx in smooth muscle cells (Frangez et al. 2008). Intracellular Ca^{2+} binds with calmodulin leading to activation of myosin light chain kinase, activation of myosin ATPase and muscle concentration. This explains the vasoconstrictory effects of equinatoxins.

Pore Formation Increases Intracellular Calcium Concentration, Cell Volume and Causes Lysis of Cells

In bovine lactotrophs an increase in the intracellular Ca^{2+} activity was observed after exposure to at least 230 nM EqT. This was attributed to the formation of calcium selective pore in the membrane of lactotrophs. Latter the cell membrane became leaky and the calcium indicator fura-2 diffused out of the cells (Zorec et al. 1990). An increase in intracellular [Ca^{2+}] is probably also responsible for thrombocyte aggregation and degranulation (Teng et al. 1988; Suput et al. 2001) and for degranulation of neutrophil and basophil granulocytes with subsequent lysis. Eosinophil granulocytes remained intact.

Cytolysis is always the end results of exposure of cells to sufficient concentration of EqT II. This was studied in detail on mouse neuroblastoma/rat glioma NG108-15 hybrid cells (Frangez et al. 2000). The volume of the cells increased within 15 min. using 100 nM EqT II, and the cells showed no lysis for up to 2 h after exposure to the toxin. Removal of Ca^{2+} from the medium prevented the increase of cell volume. Similarly, replacement of Na^{+} in the bathing solution with sucrose prevented the increase of cell volume after exposure to the toxin. This indicates that not only Ca^{2+} but also Na^{+} or Cl^{-} is involved in the mechanism of cell swelling. Replacement of NaCl with

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Na-isethionate had no effect on EqT II induced cell swelling demonstrating that Na^+ and not Cl^- is involved in the swelling of cells.

Intracellular Ca^{2+} concentration increased when the cells were exposed to the threshold concentration of 10 nM EqT II. Pretreatment of cells with thapsigargin that releases calcium from intracellular stores had no effect on EqT II induced rise of intracellular Ca^{2+} concentration but intracellular Ca^{2+} increase was abolished by removal of Ca^{2+} from the bathing solution. This shows that only Ca^{2+} entry into the cell is responsible for the increase in intracellular Ca^{2+} concentration. Ca^{2+} can enter a cell either through the newly formed pores or through the existent Ca^{2+} channels, or both. Neither Ca^{2+} channel blockers nor depolarization of the cellular membrane had any effect on Ca^{2+} entry which gives a clear answer that the newly formed pores in the membrane are the only structure that allowed Ca^{2+} to enter the cell, a process driven by the concentration gradient.

In the node of Ranvier 100 nM concentration increases the nodal volume. The mechanism of this effect is similar as in the NG108-15 cells. The presence of both Ca^{2+} and Na^+ is needed for the increase of volume. Sodium channel blocker tetrodotoxin (TTX) had no effect on the rate and amplitude of nodal volume increase supporting the view that Na^+ enters the cell in exchange for Ca^{2+} (Benoit et al. 2002).

EqT II has similar effects also on the endothelial cell line ECV-304. In 1 nM concentration it causes a huge increase in cell volume (Fig. 2) followed by cell lysis within 1 h after exposure of cells to the toxin. The dose–response curve is very steep; 0.5 nM concentration has no effect even after several hours of exposure of cells to the toxin (Bunc et al. 2002).

Considering these data the following mechanism of cell volume increase may be proposed: EqT II binds to cellular membranes. The rate of this process and the rate of pore formation depend on the concentration of EqT II in the medium. Once the pores are formed a rapid inflow of Ca^{2+} and increase of its intracellular concentration cause the Na/Ca exchanger to exchange intracellular Ca^{2+} for extracellular Na^+. Increased intracellular Na^+ concentration then causes an increase in osmotic pressure across the cell membrane. A rapid inflow of water molecules follows. This leads to increased cell volume and eventually osmotic lysis of cells exposed to the toxin. At least in some

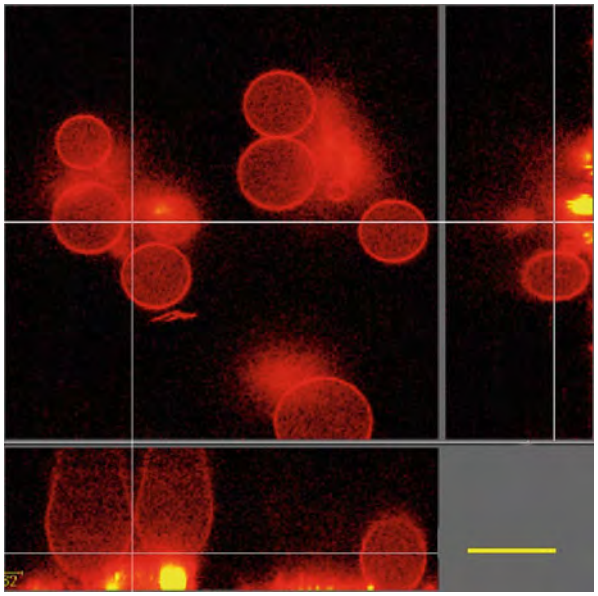

Fig. 2 Equinatoxin II causes blebbing of endothelial cells. Endothelial cells were exposed to 1 nM EqT II

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cells degradation of phosphatidylinositol 4,5-bisphosphate (PIP2) and inhibition of endocytosis may promote cell volume increase by formation of blebs (Garcia-Saez et al. 2011).

Understanding the effects of EqT II on cells and cell membranes, summarized in Table 2, also completes the understanding of its effects in vivo. Injection of a lethal dose of EqT II into a vein of an experimental animal causes hemolysis, release of potassium into the blood and release of vasoactive substances from blood cells. A rapid aggregation of trombocytes blocks the blood flow through microcirculation. This effect is enhanced by swelling of endothelial cells that may release endothelin. Endothelin, increased potassium concentration and direct effects of EqT II cause persistent contraction of vascular smooth muscle leading to vasoconstriction. This raises vascular resistance and obstructs pulmonary and coronary blood flow. Ensuing hypoxemia and direct cardiotoxic effects damage cardiomyocytes leading to a complete cardiac arrest and death of experimental animals.

Equinatoxin II Causes Reorganization of Membrane Lipid Structure

Equinatoxin/lipid interactions are complex. Not only that the presence of cholesterol and physical properties of a membrane determines the kinetics of binding of EqT II and subsequent pore formation, but binding of EqT II also changes membrane properties. It induces formation of sphingomyelin enriched phases in lipid vesicles. Electron microscopy showed formation of a honeycomb structure in multilamellar vesicles. Most probable explanation of this phenomenon comes from the fact that EqT II perturbs membrane lipid bilayer structure by inducing toroidal pore formation composed of EqT II α helices and lipid. N-terminal α helix is always inserted on the membrane/water interface either laying on the surface or penetrating to the trans-side. Different combinations of organization of this structure can lead to formation of compartments in the multilamellar vesicles (Anderluh et al. 2003). Membrane perturbation also occurs in cells. Colocalization of EqT II with raft- and non-raft membrane proteins clearly showed that EqT colocalizes with raft membrane proteins. Furthermore, EqT II causes redistribution of those proteins into new microscopic microdomains. Normally rafts tend to cluster and grow from “nano”- to “micro”- domains, but in real life this does not happen as several cellular processes, including endocytosis, remove clusters of nano-domains when they grow too large. It seems that EqT II overcomes these processes by inhibiting endocytosis (Garcia-Saez et al. 2011). Inhibition of endocytosis is independent of intracellular Ca^{2+} increase and independent of PIP2 degradation, but it requires binding of an active toxin to cell membranes. EqT II cysteine mutants capable of binding to sphingomyelin, but incapable of pore formation are also ineffective in rafts redistribution and endocytosis inhibition. A tempting explanation for this observation is that pore formation is needed to produce these effects, but the correct understanding of these phenomena is still elusive. The tested EqT II mutant was capable of binding to sphingolipids, but its N-terminal was fixed with cysteine-cysteine bonds so that it could not move to the membrane/water interface. It is quite possible that not the pore formation but N-terminal insertion is sufficient for membrane lipids perturbation. Atomic force microscopy and differential scanning calorimetry have modified parts of the present scheme of sticholysin/lipid interactions. As sticholysins, like equinatoxins, belong to actinoporins it may be assumed that equinatoxins act in a similar way. The first step of actinoporin binding to the membrane requires favorable physicochemical properties of the membrane such as the presence of sphingomyelin and lipid ordered/disordered phase separation in the bilayer. Binding of proteins to the ordered phase may be hindered by tight molecular packing and, like most detergents, proteins preferentially bind to organized/disorganized phase interfaces. Binding of

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actinoporins then lowers the line tension between the ordered and disordered membrane phases and promote formation of disordered lipid regions. Membrane rafts are defined by separation of ordered and disordered membrane phases, cholesterol in the ordered phase acting as "dynamic glue." The latest concept suggesting that sticholysins promote membrane lipids mixing (Ros et al. 2013) is seemingly, but not necessarily, in contrast with colocalization of EqT II with membrane rafts and coalescence of "nano" domains into "micro" domains observed by other investigators. It is quite possible that disorganization of the lipid bilayer actually promotes lateral diffusion and oligomerization of actinoporins. The final step, the pore formation, would then locally again organize membrane within the toroidal pore and around it. It is not known how this step, if at all, relates to de-novo membrane organization of cell membranes in the observed microdomains. Explanation of actinoporin/raft association became elusive again and needs further studies. Nevertheless, it seems that lipid perturbation before the pore formation can severely alter membrane domains and possibly the function of several membrane proteins.

A Wide Range of Effects of EqT II on Regulatory Membrane Proteins may be Explained by Its Effect on the Lipid Bilayer Reorganization

Ion channels, transporters, pumps and other regulatory proteins are selectively transported in lipid rafts by complex protein trafficking systems within the cell. Insertion of these proteins into membrane rafts defines their function in the membrane. As binding of actinoporins to the lipid bilayer causes lipid perturbation leading to a more disorganized lipid environment it is reasonable to assume that this could alter the function of membrane proteins, including ion channels. After oligomerization and formation of functional channels EqT II reorganizes the membrane in such a way that the organized lipid bilayer is preferred, and lipid rafts coalesce forming larger microdomains. This may also alter the function of membrane proteins in these microdomains. The effects may be widespread and unspecific, different form cell to cell, but important for cell and organ functioning.

In smooth muscle cells from rabbit pulmonary artery EqT II and other permeabilizing agents caused removal of inward rectification in large conductance Ca^{2+} – activated potassium channels that could not be explained by pore-formation and cell permeabilization (Snetkov et al. 1996). It is well established that pore formation in erythrocytes requires at least 0.1 nM, but in most other cells at least 100 nM EqT II. Several effects of equinatoxins have been observed in several orders of magnitude lower concentrations of EqT and EqT II. Thrombocyte aggregation has been observed in pM concentration (Teng et al. 1988). In isolated guinea-pig heart a positive inotropic effect has been observed in <1 pM concentration of EqT II, and in skeletal muscle from frog 1 pM EqT II caused initial decrease of sodium currents without an effect on the reversal potential (Šuput 1986). Only after several minutes the reversal potential started to drift towards 0 mV, and the leakage current increased. Although the putative effects of membrane reorganization are difficult to assess they may have an important effect in vivo. This is supported by the fact that EqT I is the most toxic, but least hemolytic of all equinatoxins, and the most hemolytic EqT III is the least toxic (Fig. 3).

What are Equinatoxins and Other Actinoporins Good for?

Studying a substance is usually performed to satisfy the curiosity and to find a use of the substance for the benefit of humankind. Equinatoxins have been shown to inhibit the growth of implanted

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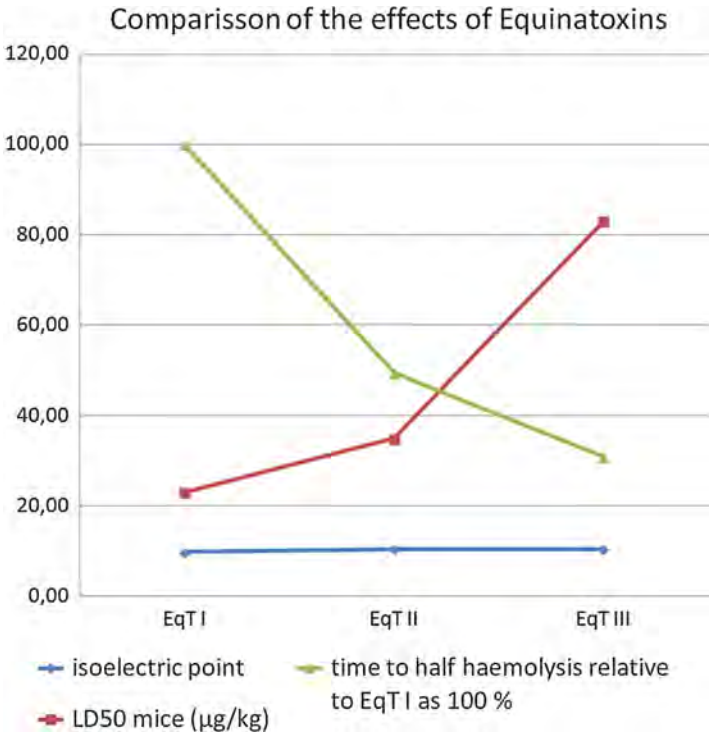

Fig. 3 Lethality and hemolytic effects of equinatoxins. EqT II is the least hemolytic but the most lethal isotoxin. It has the lowest pI value, 9.8, as compared to 10.5 for EqT II and III

tumors in mice (Giraldi et al. 1976), but very steep dose–response curve and potent lethal effects make direct use of equinatoxins in oncology impossible. A more promising approach for treatment of various diseases is development of immunotoxins. An advance has been done by constructing immunotoxins against *Giardia duodenalis* (Tejuca et al. 1999) and against colon cancer antigen (Tejuca et al. 2004). An interesting approach in developing antineoplastic agents was a construction of EqT II conjugate with a protein containing cleavage site that could be activated by cathepsin B and by matrix metalloproteases (Potrich et al. 2005) released from tumor cells. Cytotoxic effects of EqT II were induced by these enzymes secreted from fibrosarcoma and breast carcinoma cells, but the promise of use of actinoporins as drugs is still distant.

The most important use of equinatoxins is in research. In the past EqT II was a crucial substance that helped to resolve the interaction of actinoporins and some other proteins with sphingomyelin, in particular with POC. Research in this field lead also to a discovery of a novel mechanism of pore formation and several interactions of proteins with membrane lipids.

Presently, and also in the future, EqT II promises to be an important tool in the research of membrane rafts and association of membrane proteins with the rafts

Conclusion and Future Directions

Equinatoxins, especially EqT II, have greatly contributed to the understanding of protein/lipid interactions, in particular to the toroidal α helix pore formation. As in most cells the pore formation requires relatively high EqT concentrations II, another mechanism may be responsible for the variety of the toxin effects on different cells and organs. It may be reasonable to assume that equinatoxins

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affect the membrane ionic channels and/or receptors by changing the lipid phases in the vicinity of those structures, since no protein receptor has been discovered for these toxins.

Recent advances in actinoporin and lipid bilayers research promise new insights in the mechanism of their actions and better understanding of membrane rafts dynamics and function. Membrane rafts belong to the ordered membrane lipid phase, and due to the height difference between the rafts and the rest of the membrane lipid sphingomyelin heads are probably more exposed to POC binding site on actinoporins. At the same time the hydrophobic mismatch between the two phases increases the line tension promoting formation of larger rounded rafts. Actinoporins have been shown to preferentially bind to the borderline between the ordered and disordered lipid phases. EqT II binds to the vicinity of rafts and colocalizes with raft membrane proteins and it promises to become an important tool to study these structures.

Recent finding that another actinoporin, sticholysin II causes lipid mixing and disorganization of membrane lipids after binding to the bilayer opens new questions. Is this another step in actinoporin/membrane interactions? Is the observed effect, although unlikely, present only in sticholysin interactions with cellular membranes? Are there four and not only three steps in pore formation? How can one explain the (co)existence of two opposing observations, namely the rafts coalescence and membrane lipid mixing? It could be expected that disorganization of lipid bilayer would lead to break-up of the rafts, but binding of EqT II has an opposite effect. Future research of actinoporin and membrane interactions will certainly bring new exciting results.

Cross-References

- ► Ciguatoxin and Ciguatera
- ► Clinical and Therapeutic Aspects of Envenomations Caused by Sponges and Jellyfish
- ► Phylum Porifera and Cnidaria
- ► Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Review

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Marine and Freshwater Toxins
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Toxins Produced by Marine Microorganisms: A Mini Review

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Abstract

Structures, toxicological properties, and mechanisms of biological action of the best known representatives of the main groups of marine microbial toxins are presented. It is shown that many compounds have complex chemical structures and possess extremely high toxicities. These highly dangerous toxins are accumulated in mollusks and other kinds of sea food. They represent a serious health and ecological problem.

Introduction

Some terrestrial animals, higher plants, and microorganisms have been considered as main sources of toxins for a long time. However, since the middle of the twentieth century, many groups of marine organisms have been identified as producers of natural toxic products. It is well known that marine organisms not only contain many hundreds of toxic secondary metabolites but that a large number of these compounds demonstrate extremely high toxic activities (Yasumoto and Murata 1993; Stonik and Stonik 2010).

Studies on marine toxins became an important scientific direction, developing in the frameworks of marine bio-organic chemistry, pharmacology, marine biology, and several other scientific fields. The corresponding investigations have played an outstanding role in helping identify the structural diversity and chemical peculiarities of toxins, as well as their biological functions, mechanisms of action, and significance for the survival of some species of marine organisms and maintenance of marine communities. Medical, economic, biochemical, toxicological, and other aspects of marine toxinology are also very significant. Herein, we give a short review of the main achievements in the study of marine toxins from microorganisms, with particular attention to results obtained during last 10 years.

Tetrodotoxin

Toxins produced by marine microorganisms attract special attention because they may be transferred through food chains into various shellfish, fish, and other macroorganisms. Accumulation of toxins in these marine animals can make them dangerous for human consumption. Tetrodotoxin (TTX), isolated in 1909 in Japan by Tahara (for review see, Halstead and Courville 1967), was found to be of microbial origin, was the first chemically studied and purified marine toxin. Structurally, the toxin

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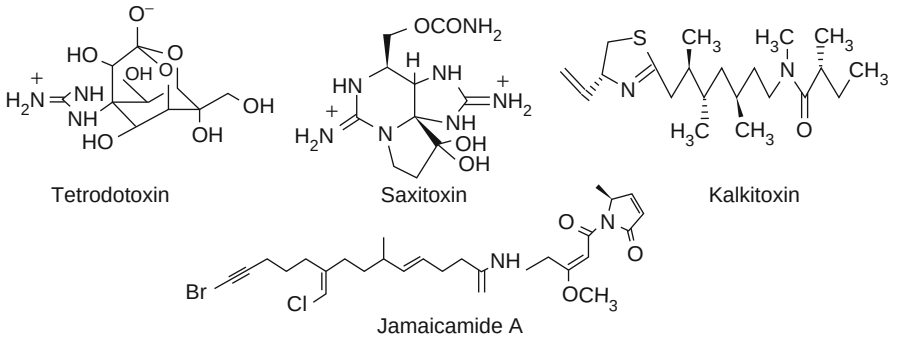

Fig. 1 Some marine toxins of microbial origin: blockers of sodium channels

represents a polyhydroxylated polycyclic bipolar ion with orthoester and guanidine fragments (Fig. 1). Its structure was established by the classic works of Woodward (Woodward 1964) and two groups of Japanese scientists (Tsuda and Goto-Hirata) in the 1960s (for reviews see Halstead and Courville 1967; Suehiro, 1994). The first asymmetric synthesis of TTX was carried out in the beginning of the 2000s (Onyabu et al. 2003). The toxin accumulates in the liver, gonads, and sometimes skin of the pufferfish,, which belongs to the family Tetraodontidae. Some toxic fish, for instance *Takifugu* = *Spheroides rubripes,*) are used to prepare delicious dishes in specialized restaurants in Japan, although mortal poisonings occur almost annually following human consumption.

Later, this toxin, termed tarichatoxin,, was found in rough-skinned newts belonging to the genus *Taricha*, some marine proboscis worms (phylum Nemertea), xanthic crabs, blue-ringed octopuses of the genus *Chaetognatha*, and other species, primarily in marine life (Miyazawa and Noguchi 2001; Noguchi and Arakawa 2008). For example, toxicity of the ribbon worm *Cephalothrix simula* in Hiroshima Bay, Japan, exceeded the human lethal dose per single worm (Asakawa et al. 2013). The toxin plays an outstanding role in the predator–prey relationship, especially in the marine environment.

The toxin is considered to be produced by bacteria, in particular by some strains of *Vibrio alginolyticus* and *Pseudoalteromonas tetraodonis.* This explains the isolation of TTX from taxonomically different biological sources. However, toxin identification in some microbial cultures after its transformation into degradation products, using mainly chromatographic procedures, was not absolutely reliable (Piel 2004). As result, debates concerning the origin of tetrodotoxin continue in the literature (Chau et al. 2011).

TTX is an extremely potent neurotoxin that can enter the body via ingestion of TTX-containing marine animals. It selectively inhibits the TTX-sensitive neuronal sodium channels, blocking action potentials in nerves and muscles. TTX enters the outer vestibule of channel pores and binds to amino acid residues that control permeation. After discovery of its molecular mechanism of action in the 1960s, it has become extremely useful in neurophysiological and pharmacological laboratories (Narahashi 2001; Fozzard and Lipkind 2010). Toxicity is especially high following intravenous application ($LD_{50} \sim 8\ \mu g/kg^{-1}$), but it is 40 times less toxic on peroral introduction ($LD_{50} \sim 330\ \mu g/kg^{-1}$). The main cause of human death is paralysis of the diaphragm and thus cessation of breathing.

TTX in subtoxic doses has been used on humans to alleviate pain; in clinical trials, its analgesic effect was sustained in 50 % of patients with severe cancer-related pain (Nieto et al. 2012).

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Paralytic Shellfish Poisoning (PSP) Toxins

Marine toxin saxitoxin (Fig. 1) is another of the most potent toxins and is almost as well known as TTX. As with about 20 of its analogs and derivatives described to date, including neosaxitoxin, 11α- and 11β-sulfoxy saxitoxins, gonyautoxins, etc., saxitoxin causes paralytic shellfish poisoning (PSP) following ingestion of mollusks contaminated with these toxins from blooming microalgae and cyanobacteria; they are thus known as PSP-toxins.

Saxitoxin was first isolated from the butter clam, *Saxidomus giganteus*. Later, the toxin and/or related compounds were found in many edible mollusk species and pufferfish (Shimizu 1996). PSP-toxins infiltrate into edible mollusks from dinoflagellates *Alexandrium, Gymnodinium*, and *Pyrodinium* spp. via the food chain. As a rule, toxins can accumulate in certain body components of mollusks, where they are preserved for several weeks. These toxins have also been found in *Aphanizomenon flos-aquae, Anabaena circinalis*, and other cyanobacteria. There is a suggestion that saxitoxin originates from symbiotic bacteria, such as TTX. In fact, the toxin was found in culture of the bacterium *Moraxella* sp. isolated from the dinoflagellate *Alexandrium tamarense* (=*Protogonyaulax tamarensis*) (Kodama et al. 1990).

Saxitoxin poisoning symptoms in humans include numbness in lips and gastrointestinal disorders. Death often occurs from flaccid paralysis followed by respiratory failure. Its lethal dose for mice is very low: $LD_{50} \sim 4\ \mu g/kg^{-1}$ and $\sim 330\ \mu g/kg^{-1}$ following intravenous and peroral administration, respectively.

Other Sodium Channel Blockers

Several other potent sodium channel blockers were found in marine cyanobacteria. As an example, the ichthyotoxin kalkitoxin, recently isolated from the Caribbean collection of the marine cyanobacterium *Lyngbya majuscula*, deserves mention. Chemically, it is a thiazoline-containing lipid with absolute configuration established after synthesis of four stereoisomeric compounds, one of which was identified as kalkitoxin (Fig. 1) (White et al. 2004). It was shown that kalkitoxin interacts with the neuronal TTX-sensitive voltage-dependent sodium channels (LePage et al. 2005). Ichthyotoxicity from kalkitoxin is exclusively potent; its lethal concentration in sea water is $600\ pg/ml^{-1}$.

Another such toxin, a blocker of voltage-gated sodium channel jamaicamide A from the same cyanobacterium species, is a novel and highly functional lipopeptide with alkenyl bromide, vinyl chloride, and betaenone functionalities and pyrrolidone ring (Fig. 1) (Edwards et al. 2004). It is known to be toxicity to fish.

A series of neurotoxins acting on nicotinic acetylcholine receptors or as inhibitors of acetylcholinesterase, being also tumor promoters and liver toxins, were also isolated from different marine cyanobacteria (Araos et al. 2010).

Amnesic Shellfish Poisoning (ASP) Toxins

Two related pyrrolidine-containing marine toxins, namely, kainic and domoic acid, were originally isolated from seaweed. Kainic acid was found in the red algae *Diegenea simplex* as early as 1953 and later shown to be an active anthelminthic. This alga was being used in traditional Japanese medicine against parasitic worms for a long time. It mimics the effect of glutamate and is known to be a

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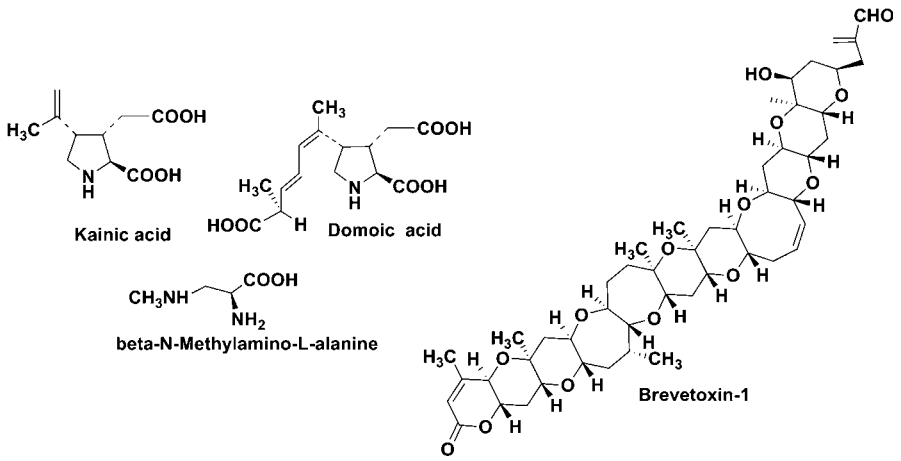

Fig. 2 Some marine toxins causing neurodegenerative decay and neurotoxic shellfish poisonings

specific agonist of one type of glutamate receptors (kainate receptors). Kainic acid was applied in animal experiments in order to model epilepsy and other neurodegenerative diseases such as Alzheimer's disease (Moloney 1998). Domoic acid (known as *domoi* in Japan) was isolated from the red algae *Chondria armata*. The compound was identified as a neurotoxic agent causing amnesic shellfish poisoning (ASP-toxicity). The first incident of the ASP poisoning in a human was documented in 1987 in Canada after consumption of contaminated blue mussels, *Mytilus edulis:* four people died, and more than 100 had different clinical symptoms of acute toxicosis. Among the most prominent clinical symptoms described was short-term memory loss, which led to its name (Pulido 2008). In comparison with women and younger men, elderly men are more susceptible to both the severity of the illness and to memory loss.

It was later found that domoic acid is produced by some strains of diatoms, such as *Pseudo-nitzschia* spp., *Nitzschia navis-varingica*, and *Amphora coffeaeformis* (Skov et al. 1999). It is of special interest that *Pseudo-nitzschia multiseries* loses its ability to produce domoic acid in axenic culture. However, the alga recovers production when bacteria from the original toxic culture are added to the culture. This suggests that association between bacteria and microalgae may be necessary for biosynthesis of domoic acid (Kobayashi et al. 2009).

Both kainic and domoic acids are excitatory amino acids and analogs of glutamate, a neurotransmitter in the brain, which activates glutamate receptors (GluRs). Domoic acid induces activation of ionotropic GluRs on both sides of the synapse, which results in almost permanent stimulation of hypothalamic neurons, ultimately leading to their destruction (Todd 1993).

Domoic acid from algal blooms may accumulate from algal blooms not only in shellfish but also in fish such as sardines and anchovies and, further, via the food chain, it can be transferred into sea birds, sea lions, and other animals, causing illness and sometimes death depending on the amount of toxic fish they eat. Moreover, even animals as large as whales may suffer toxin poisoning: domoic acid intoxication was identified as the cause of the mortality of Minke whales in southern California in April 2007 (Fire et al. 2010). Toxicosis from this substance is also called domoic acid poisoning or DAP-toxicity.

Several cyanobacteria taxa produce another toxic compound that induces the development of amyotrophic lateral sclerosis/Parkinsonism–dementia complex. The agent responsible was suggested to be the neurotoxic amino acid beta-*N*-methylamino-L-alanine (Fig. 2) (Araos et al. 2010).

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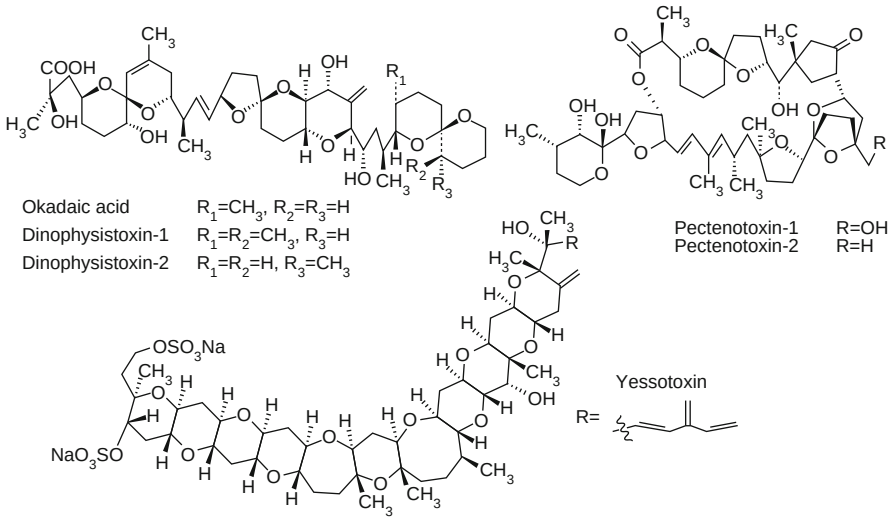

Fig. 3 Some toxins inducing diarrhetic shellfish poisonings

Neurotoxic Shellfish Poisoning (NSP) Toxins

Neurotoxic shellfish poisoning (NSP-toxicity) results from accumulation of microalgal toxins known as brevetoxins (Fig. 2) and analogous natural products in shellfish and other marine organisms. There is a link between neurotoxin production by the dinoflagellate *Karenia brevis* during algal blooms called the Florida red tide and the deaths of a variety of marine animals, such as bottlenose dolphins and sea birds, along the coast of Florida. Similar phenomena were observed following blooms of the raphidophyte microalga *Chattonella* cf. *verruculosa* in North Atlantic waters of the coast of USA (Wang 2008; Watkins et al. 2008). Brevetoxins – for example, brevetoxin-1 – belong to the group of natural polyether products, which usually bear a system of fused oxygen-containing rings (Nakanishi 1985).

Brevetoxins bind to voltage-gated sodium channels in nerve cells and activate them. This gives rise to sodium influx and membrane depolarization and induces the poisoning, which often leads to hospitalization. People ingesting toxins from contaminated shellfish do not die as a rule but exhibit gastrointestinal and neurological symptoms such as nausea, vomiting, slurred speech, loss of coordination, and paresis of facial muscles. Neurological symptoms can progress to partial paralysis. Several NSP cases are reported annually in the USA. Aerosols formed during storms and hurricanes when *K. brevis* is blooming irritate the respiratory tract, exacerbating allergies and asthma attacks in Florida inhabitants (Watkins et al. 2008). Brevetoxins can also weaken the immune systems of humans and other animals and increase the danger of infections and other secondary diseases.

Diarrhetic Shellfish Poisoning (DSP) Toxins

Diarrhetic shellfish poisoning (DSP-toxicity) is induced by a whole series of microalgal toxins, including okadaic acid, dinophysistoxins, pectenotoxins, and yessotoxins (Fig. 3). All these toxins contain polyether structures and, like many other above-mentioned toxins, can accumulate in shellfish.

Okadaic acid was first isolated from the sponges *Halichondria okadai* and *H. melanodocia* (Tachibana et al. 1981). However, it was later established that it enters sponges from the

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dinoflagellates of the *Prorocentrum* and *Dinophys* genera (Yasumoto et al. 1987). It is well known that microalgae *Prorocentrum* spp. often participate in symbiotic relationships with different benthic marine invertebrates and macrophytic algae. That is why okadaic acid easy penetrates into planktivorous mollusks as well as into other marine animals through the food chain.

Okadaic acid has an ability to inhibit protein serine/threonine phosphatases due to the interaction with the PP1- and PP2A-catalytic domains of these enzymes. Okadaic acid inhibits both human and animal enzymes, as well as protein phosphatases of yeasts and plants equally. The half-maximal inhibitory concentrations (IC_{50}) against the PP2A and PP1 types of protein phosphatases are ~ 0.2–1.0 and 60–500 nM, respectively (Dounay and Forsyth 2002). In addition, okadaic acid is known as powerful tumor promoter.

Closely related to okadaic acid are the dinophysistoxins, which have similar chemical structures: the former is a methyl homologue of okadaic acid, while the latter are stereoisomers of this homologue (Larsen et al. 2007). The inhibitory activity of dinophysitoxin-1 against recombinant and wild PP2A protein phosphatases is the same as that of okadaic acid, while dinophysitoxin-2 activity is two times less (Garibo et al. 2013). Okadaic acid is usually found in European mollusks, while mollusks from Canada and Japan primarily accumulate dinophysistoxins. Dinoflagellate species belonging to these genera pose a major threat to shellfish aquaculture in Europe, Chile, Japan, and New Zealand due to their diarrhetic toxins (Reguera et al. 2012).

Pectenotoxins, in particular, pectenotoxins-1 and -2, usually accompany okadaic acid. They were first identified in sponges and later isolated from the microalga *D. acuminata.* Traditionally, these compounds are classified as another group of diarrhetic toxins (Dominguez et al. 2010). However, when administrated to mice, they do not cause diarrhea but are highly hepatotoxic. Chemically, natural products belonging to this group are polyether macrocyclic lactones. To date, >15 compounds of this type have been identified. The toxins were frequently found in the mussel *Mytilus galloprovincialis* and oysters from the Mediterranean Sea and other geographic areas.

Biological activity of pectenotoxins is connected with their capability to depolymerize actin filaments (Leira et al. 2002). Pectenotoxin-2 displayed a significant cytotoxic effect against tumor cells (Allingham et al. 2007). Moreover, pectenotoxins have stronger apoptotic properties toward tumor cells than toward normal cells of the same tissue, inducing apoptosis by suppressing the nuclear factor κB signaling pathway (Espina and Rubiolo 2008). Yessotoxin and its first described analog were isolated from the digestive glands of the toxic scallop *galloprovincialis* collected in 1986 and 1993 in Japan (Satake et al. 1996). To date, ~ 40 compounds belonging to this third group of diarrhetic shellfish toxins are known. These compounds damage cardiac muscles, and their toxicity ranges between 20 and 500 $\mu g/kg^{-1}$ following intraperitoneal administration to mice (Aune et al. 2008). It is hypothesized that the toxins are produced by the dinoflagellates *Protoceratium reticulatum*, *Lingulodinium polyedrum*, and *Gonyaulax spinifera*. As with pectenotoxins, these toxins do not inhibit protein phosphatases and do not induce diarrhea but have been historically associated with DSP syndrome because they can be detected in microalgae and mollusks along with toxins of the okadaic acid group. Some scientists believe that attributing these compounds to diarrhetic toxins is erroneous (Paz et al. 2008).

Azaspiracid Toxins

Azaspiracid shellfish poisoning was discovered 1995 when it occurred in people in The Netherlands after consumption of mussels from Ireland. Toxins of this group (azaspiracids or spirolides) were initially found in *Mytilus edulis* collected from Irish waters (McMahon and Silke 1996) and later

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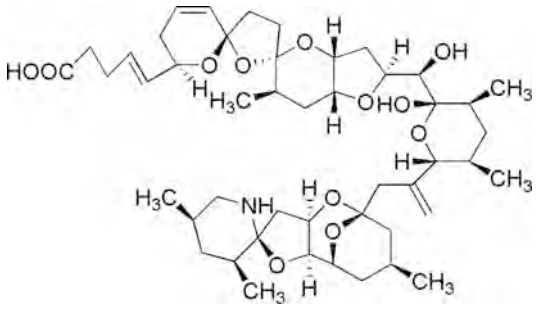

Fig. 4 Structure of azaspirracid-1

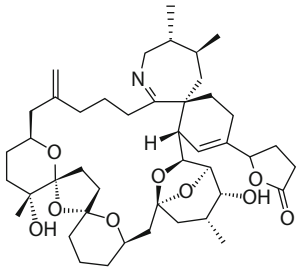

Fig. 5 Pinnatoxin F

discovered in a number of different countries in western and northern Europe, northern Africa, Asia, and South America. As a separate group of toxins, azaspiracids were isolated from contaminated mollusks when one of their spirocycles was found to contain nitrogen instead of oxygen, which is in contrast with other natural polycyclic polyether derivatives having spiro-conjugated pyran and furan rings (James et al. 2003). The structure and particularly stereochemistry of azaspiracid-1 (Fig. 4) were finally established when Nicolaou et al. (2006) accomplished total synthesis of this toxin and obtained several of its degradation products. Now, a whole series of azaspiracids are known, totalling ~ 20 toxins.

Later, it was found that these compounds are produced by a newly identified microalgal species *Azadinium spinosus* gen. et sp *nov.* (Tillmann et al. 2009) and related species of Dinophyceae belonging to the same genus (*Azadinium*). Azaspiracids cause severe and protracted diarrhea in humans, although they have never been included in the DSP group. Recently, azaspiracids were established to be open-state blockers of human Ether-à-go-go-Related Gene (*hERG*) potassium channels and were shown to have high cytotoxicity, teratogenic, and probably carcinogenic activities (Twiner et al. 2012). These physiological effects may contribute to the toxicity experienced following ingestion of these compounds, including injuries to gastrointestinal and lymphoid systems (Twiner et al. 2008). Azaspiracids are highly toxic to humans and animals such as mice. Their LD_{50} is ~ 110–200 μg/kg^{-1} (Ito et al. 2002).

Pinnatoxins

Pinnatoxins – isolated from Japanese, Australian, and New-Zealand mollusks belonging to the *Pinna* genus – have some structural peculiarities resembling those of azaspirolides. Pinnatoxins were first isolated and characterized from Japanese *Pinna muricata* in 1995, following which a

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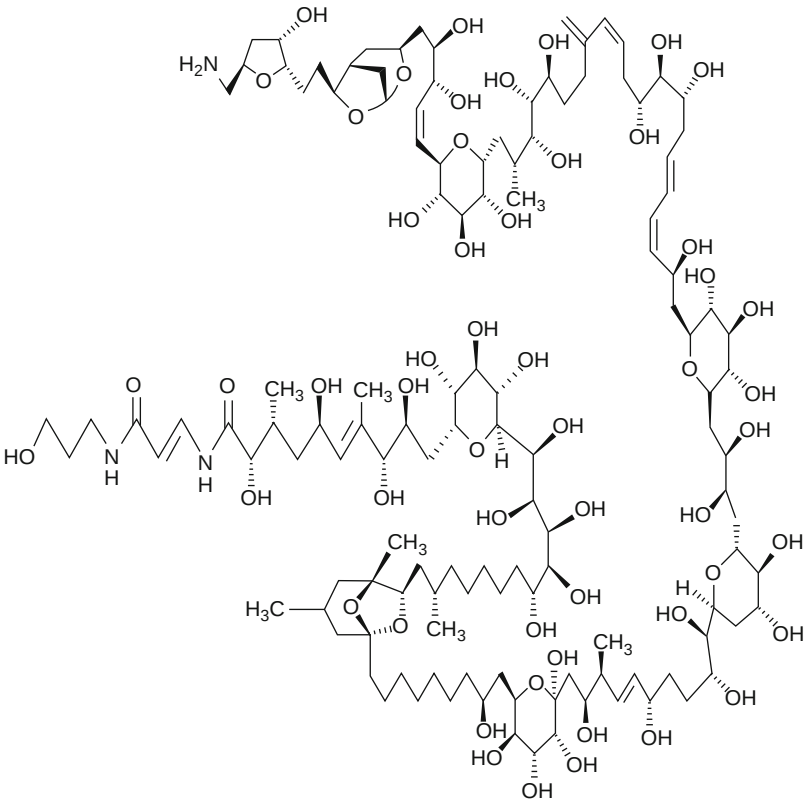

Fig. 6 Structure of palytoxin

series of similar toxins was characterized (Fig. 5) (Uemura et al. 1995). Later, these toxins were found in European mollusks (e.g., mussels). They are referred to as the spiroimine group of toxins and are dangerous to humans. They are produced in nature by dinoflagellates, for instance *Vulcanodinium rugosum*, and then accumulate in shellfish. The LD_{50} of pinnatoxin F is 13 μg/mg^{-1} following intraperitoneal administration to mice. Pinnatoxins purified from the Okinawan bivalve *Pinna muricata* activate calcium channels (Kita and Uemura 2005). After their total synthesis, it was established that pinnatoxins inhibit nicotinic acetylcholine receptors selectively for the human neuronal alpha 7 subunit (Araoz et al. 2011).

Palytoxins

Consumption of marine organisms contaminated by marine toxins can lead to several other types of poisoning. For example, palytoxin, one of the most potent marine toxins to cause poisoning in humans, was first isolated by Moore and Scheuer (1971) from the Hawaiian zoanthid *Palythoa toxica*. Its toxicity is ~ 10,000 times higher than potassium cyanide, and its LD_{50} is ~ 0.30 μg/kg^{-1} following intraperitoneal administration to mice (Munday 2011).

The elucidation of structure of this structurally complex compound – containing 129 carbon atoms and > 60 chiral centers – was carried out by US and Japanese research groups in the 1980s (Fig. 6) (Moore and Bartolini 1981; Cha et al. 1982).

The structural studies on palytoxin and subsequent total synthesis of palytoxinic acid (Armstrong et al. 1989; Kishi 1998) produced very impressive advances in marine bio-organic chemistry in the twentieth century. Its molecular target was shown to be the sodium/potassium/adenosine

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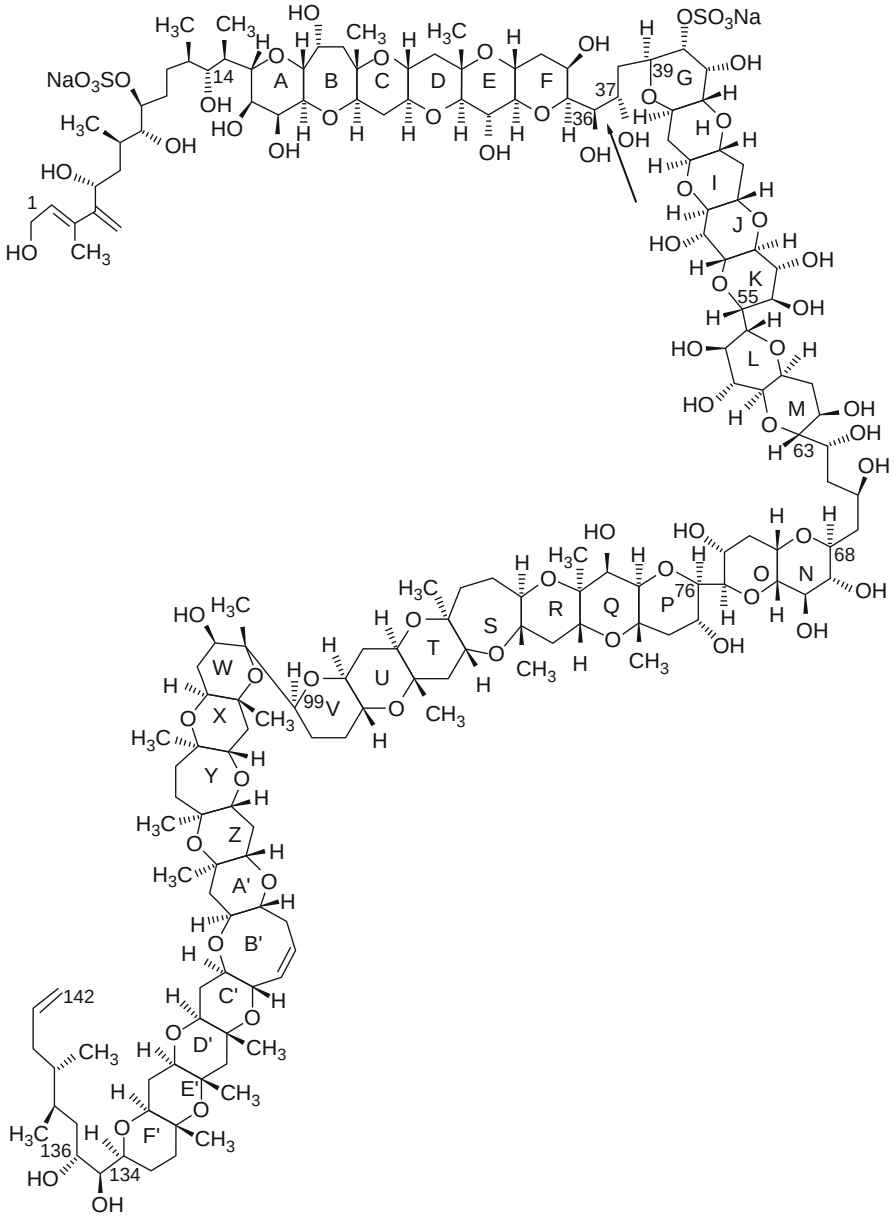

Fig. 7 Structure of maitotoxin

triphosphatase (Na^+/K^+-ATPase) pump), a transmembrane enzyme maintaining the resting potential and the gradient ion concentrations on both sides of biological membranes of nerve and muscle cells (Rossini and Bigiani 2011). The interaction of palytoxin with different cells, including cardiocytes, occurs at approximately the same site as in the case of ouabain, a cardiac glycoside from some higher terrestrial plants. There, the toxin turns the pump into an ion channel, followed by an increase in sodium conduction and influx of calcium ions (Takeuchi et al. 2008). Recently, it was established that palytoxin indeed forms a dimeric supramolecule in aqueous solutions, which interacts with the Na^+/K^+-ATPase pump (Inuzuka et al. 2008).

Palytoxin and closely related ostreotoxins (ostreocines) are probably biosynthesized by the benthic dinoflagellates of the *Ostreopsis* genus, which live on sandy and rocky substrates or macrophytes in tropical, subtropical, and – as recently established – temperate coastal zones. In

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fact, ostreotoxins were isolated from the culture of the microalga *Ostreopsis siamensis*, and their structural similarity to palytoxins was established (Usami et al. 1995). Toxins in this structural group are known to be the cause of food poisoning due to their accumulation in crabs, sea urchins, and saltwater fish (Aligizaki et al. 2011). Occurrence of *Ostreopsis* spp. is presently increasing in the Mediterranean Sea (Italy, Spain, Greece, France), probably in response to climate change. As a result, there is a real danger of severe and numerous human poisonings, because bioaccumulation of palytoxin and related toxins in both filter-feeding bivalve mollusks and sea urchins on the French Mediterranean coast during summer 2008 and 2009 reached very high levels of 200–450 μg/kg of total flesh (Amzil et al. 2012).

Ciguatera Toxicity

Ciguatera toxicity as a phenomenon consists of the fact that edible fishes sporadically may turn toxic in tropical waters where they become contaminated with very potent toxins of microalgal origin. Toxicity results in severe to fatal human poisoning. Although ciguatera toxicity has been known for ~ 200 years, only in the second half of the twentieth century did it begin to manifest in many geographic areas of the world's oceans and was associated with a rapid increase of fishery in tropical seas (Russell and Eugen 1991). More than 500,000 Pacific Islanders suffered from such poisonings since the early 1999s. Thus, in Oceania as well as in some other tropical areas, it is the principal risk factor for people ingesting saltwater fish (Hamilton et al. 2010).

Depending on water solubility, two types of toxins have been distinguished: those insoluble in water (ciguatoxins) and those soluble in water (maitotoxin). Maitotoxin is the most toxic and structurally complex unprecedented marine toxin. It contains 142 carbon and 68 oxygen atoms, excluding those of a sulfate group (Fig. 7). Thirty-two oxygen-containing rings are conjugated into four polycyclic fragments: the first comprises rings A–F, the second rings G–M, the third rings N–V, and fourth rings W–F′. As does palytoxin, maitotoxin contains the longest carbon chain in nature except for biopolymers (Kita and Uemura 2010). The structure of maitotoxin was established by Japanese and US chemists using modern methods of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), as well as syntheses of model compounds (Nonomura et al. 1996; Murata et al. 1996). The molecule was previously degraded using periodate oxidation, and a position of this transformation was identified. Nicolaou wrote of this unique natural compound: "maitotoxin holds a special place in the annals of natural products chemistry. Its fascinating ladder-like polyether molecular structure and diverse spectrum of biological activities elicited keen interest from chemists and biologists. Synthetic studies in the area were developed as part of chemical synthesis programs directed towards the total synthesis of some less complex members of polyether marine biotoxin class, of which maitotoxin is the flagship."

Actually, maitotoxin synthesis fragment after fragment was carried out by the Nicolaou group (Nicolaou et al. 2010, 2011a, b). Maitotoxin toxicity exceeds that of palytoxin by several times and significantly exceeds that of any other nonprotein toxins. Its LD_{50} is ~ 50 ng/kg^{-1} following intraperitoneal administration to mice. Its molecular mechanism of action includes its interaction with calcium-permeable, nonselective ion channels (CaNSC) and its ability to increase calcium influx followed by sodium ion outflow from excitable and nonexcitable cells. In addition, activation of calpain-1 and -2 proteases and membrane permeability disturbance induce oncotic cell death (Frew et al. 2008).

The name of the toxin was derived from the word *maito*, used by Tahiti inhabitants for the surgeon fish *Ctenochaetus striatus,* from which the toxin was isolated. However, the toxin is produced by

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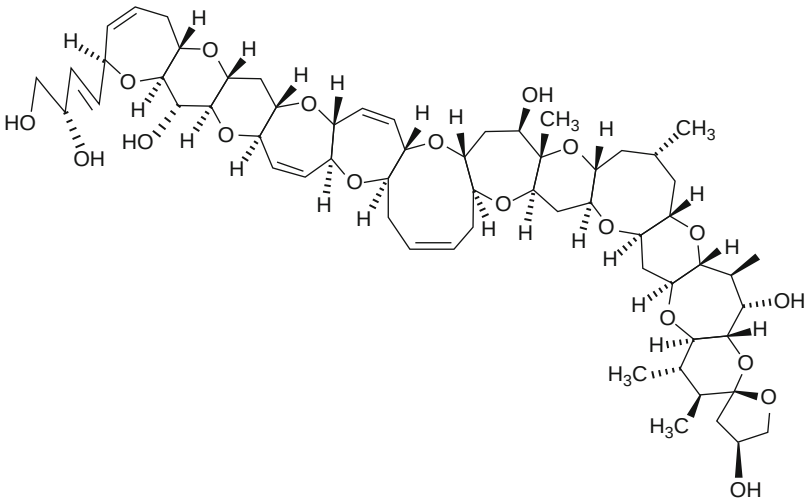

Fig. 8 Structure of ciguatoxin

dinoflagellates belonging to the *Gambierdiscus* genus. The culture of *G. toxicus* was used to obtain maitotoxin in an amount sufficient for its structural elucidation. Benthic ciguatera-causing microalgae of this genus are distributed not only in tropical waters but also in temperate waters, for example, in Japan and South Korea. Toxins from these microalgae contaminate fish via the food chain.

As with maitotoxin, ciguatoxin, the second major toxin inducing ciguatera poisoning, was isolated from contaminated fish (eel *Gymnothorax javanicus*) and cultivated dinoflagellate *G. toxicus* (Lewis et al. 1991). It was also isolated from a variety of dinoflagellates, such as *Coolia monotis*, *Amphidinium carterae*, and some species of the genera *Ostreopsis* and *Thecadinium*.

Ciguatoxin has 13 oxygen-containing conjugated rings, including two spiroconjugated (Fig. 8). Numerous analogous natural compounds (ciguatoxin-3C, gambiertoxin, gambierol, gymnocin A, etc.) accompany ciguatoxin in dinoflagellates and contaminated fish. Some have a smaller number of rings compared with ciguatoxin. All these toxins are more hydrophobic than is maitotoxin and normally contain no more than five or six hydroxyl groups. Natural products belonging to this group are highly toxic, with LD_{50} values in the range of 0.25–2.3 $\mu g/kg^{-1}$ following intraperitoneal administration to mice. Ciguatoxin toxicity itself ($LD_{50} \sim 0.25\ \mu g/kg^{-1}$) is a result of its ability to activate voltage-gated sodium channels and to block potassium channels when administered in large doses (Nicholson and Lewis 2006), thus impairing nerve conduction.

Symptoms of poisoning in humans include gastrointestinal, nervous, and cardiovascular disorders; nausea, headache, dizziness, and – in severe cases, respiratory paralysis and death – have been observed (Daranas et al. 2001). As a specific peculiarity, activation of intense stabbing and burning pain in response to mild cooling following ciguatoxin activation must be mentioned (Vetter et al. 2012).

Summary data of all toxins discussed here are listed in Table 1.

Conclusion and Direction

Prokaryotic (bacteria) and eukaryotic (microalgae) marine microorganisms produce a great variety of very potent toxins. Many of these compounds have unprecedentedly complex structures, and in many cases, their peculiar biological actions remain to be elucidated. These toxins specifically

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Table 1 Brief review of mentioned toxins

Groups of marine toxins	Toxic compounds	Mechanisms of biological action
Produced by microorganisms	Tetrodotoxin and its derivatives	Inhibit the TTX-sensitive channels of neurons
Isolated from marine cyanobacteria	Kalkitoxin, Jamaicamide A	Intersect with the neuronal TTX- sensitive, voltage-dependent sodium channels
Paralytic shellfish poisoning (PSP)	Saxitoxin neosaxitoxin, gonyautoxins, etc.	Inhibit sodium channels
Amnesic shellfish poisoning (ASP)	Domoic acid and its isomers	Activate glutamate receptors (GluRS) of hypothalamic neurons synapse
Neurotoxic shellfish poisoning (NSP)	Brevetoxins	Activate voltage-gated sodium channels
Diarrhetic shellfish poisoning (DSP)	Okadaic acid, dinophysistoxins, pectenotoxins, yessotoxin	Inhibit protein serine/threonine phosphatases. Depolymerize actin filaments. Induce apoptosis through suppression of the nuclear factor κB signaling pathway.
Azaspiracids	Azaspiracid-1	Open-state blockers of hERG potassium channels
Pinnatoxins	Pinnatoxin F	Activate calcium channels and inhibit nicotinic acetylcholine receptors selectively for the human neuronal alpha 7 subunit
Palytoxins	Palytoxin, ostreotoxins (ostreocines)	Turn Na^+/K^+ pump (Na^+/K^+-ATPase), into a shape that allows the passive transport of Na^+ and K^+ ions
Ciguatera	Ciguatoxins	Increase the Ca^{+2} influx followed by Na^+ outflow due to the interaction with Ca^{+2} -permeable nonselective ion channels (CaNSC). Activate of calpain-1 and calpain-2 proteases and disturbance of membrane permeability
	Maitotoxin	Activate voltage-gated sodium channels and block potassium channels, impairing the nerve conduction

TTX tetrodotoxin, *hERG* human ether-à-go-go-related gene

interact with ionic channels and ion-pump systems in biomembranes of target cells and, as a result, usually induce nerve-conduction blockage, cell death, or other dangerous cellular events. These toxins accumulate in marine macroorganisms, such as mollusks and fish, via the food chain, and human consumption of the contaminated marine products causes poisoning. The number such poisonings trend to increase from year to year due to the growth in the seafood industry and the wide distribution and increasingly rapid development of toxic microorganisms under the influence of global warming. Therefore, we expect further discoveries of toxins in marine microorganisms.

Cross-References

► Ciguatoxin and Ciguatera
► Intoxications Caused by the Ingestion of Seafood and
► Saxitoxin and Shellfish, and Other Neurotoxins

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Immunomodulatory Properties of Sea Cucumber Triterpene Glycosides

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Abstract

Sea cucumbers (or holothurians) belonging to the class Holothuroidea (Echinodermata) are marine invertebrate habitually found in the benthic areas and deep seas across the world. Many holothurians are known to be toxic. Triterpene glycosides are the main poison compounds of the sea cucumbers and play a role in the defense of holothuroids as a toxin. The triterpene glycosides are composed of a carbohydrate chain and triterpene aglycone and are widely distributed in sea cucumbers. Most aglycones have 18(20)-lactones and belong to the holostane type. Carbohydrate chains of sea cucumber glycosides have from two to six monosaccharide residues including xylose, quinovose, glucose and 3-*O*-methylglucose and sometimes 3-*O*-methylxylose, 3-*O*-methylquinovose, 3-*O*-methylglucuronic acid and 6-*O*-acetylglucose. They may contain one, two or three sulfate groups. At the micromolar concentrations sea cucumber glycosides show hemolytic, cytotoxic, antifungal and other biological activities caused by membranotropic action. Some sea cucumber glycosides show an immunostimulatory effect at sub-toxic nanomolar concentrations. Incubation of immune cells with the glycosides induces their activation resulting in an increase of immune cell adhesion on an extracellular matrix, enhancement of cell spreading and motility, increase of macrophage lysosomal activity, ROS formation and phagocytic activity. Injection of sub-toxic doses of some glycosides induces an increase in the number of antibody-producing plaque-forming cells in mouse spleens, an increase in the number, size and acidity of lysosomes of peritoneal macrophages, an increase of phagocytic index. It was shown that glycosides moderately induce production of some cytokines, restore the level of some CD-markers of lymphocytes, increase bactericidal activity of leucocytes and induce a significant increase in mouse resistance to lethal doses of some pathogenic microorganisms and radiation. A novel nanoparticulate antigen delivery system, tubular immunostimulating complex, consisting of cholesterol, triterpene glycoside cucumarioside A_2-2, and glycolipid monogalactosyldiacylglycerol from marine macrophytes was developed. This complex influences cytokine mechanisms of immunological regulation and its adjuvant effect varied depending on its composition. Proteomic methods have demonstrated that the mechanism of immunomodulatory action of some sea cucumber glycosides on immune cells includes regulation of the expression of certain proteins involved in formation of the immune response. These glycosides regulate the expression of proteins associated with lysosome maturation, activation and merging, phagocytosis, cytoskeletal reorganization, cell adhesion, motility and proliferation of immune cells. It was found that the most likely membrane targets of cucumarioside A_2-2 can be purinergic receptors of P2X family. These findings suggest that this glycoside can act as an allosteric regulator which able to withdraw purine receptor inactivation by extracellular ATP and provide a recovery of Ca^{2+} conductivity of macrophage membrane.

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Fig. 1 Far-Eastern holothurian *Cucumaria japonica* (Photo by A.V. Ratnikov)

Introduction

Sea cucumbers (or holothurians), belonging to the class Holothuroidea, are marine invertebrate echinoderms and biological cousins of sea stars, sea urchins and sea lilies, habitually found in the benthic areas and deep seas across the world. They are animals with a leathery skin and an elongated body, and many of them are indeed shaped like soft-bodied cucumbers. This class of animals has around 1,100 described extant species (Beirne et al. 2001; Brusca and Brusca 2003).

Some of them are edible and considered a delicacy in many countries around the world. They have high commercial value coupled with increasing global production and trade. Sea cucumbers, informally named as *trepang*, *bêche-de-mer* or *balate*, have long been used for food and folk medicine in the communities of Asia and Middle East. The popular Chinese name for sea cucumber is haishen, which means, roughly, ginseng of the sea (Bordbar et al. 2011). The photograph of popular and commercially harvested in Russia and Japan edible holothurian *Cucumaria japonica* is shown on Fig. 1.

A number of dishes are made with boiled sea cucumbers, and most cultures in East and Southeast Asia countries regard sea cucumbers as a delicacy. But in Japan, sea cucumbers are also eaten raw, as sashimi or sunomoto, and its intestine is also eaten as konowata, which is salted and fermented food. But some authors noted that eating an inadequately cooked sea cucumber can cause an irritation, rash and self-limited gastroenteritis (Holliman 2012), and in some cases the ingestion of undercooked sea cucumber has been known to cause even death (Fell 2012).

Many holothurians, particularly tropical species, are known to be toxic. This toxicity is given as the primary reason for the paucity of predators on holothurians (Bakus 1968; Dyck et al. 2010, 2011). Toxins are elaborated in the body wall and in the skin. Sea cucumber toxins may be released into the sea water continuously, or only when the animal is molested. Some species also possess Cuvierian tubules. These tubules are clusters of fine tubes located at the base of the respiratory tree in the posterior part of the animal that can be ejected toward a predator in response to an aggression. Expelled tubules lengthen into sticky white threads that serve to entangle the predator. Among all these mechanisms, the toxicity of the body wall and the Cuvierian tubules seem to be the most effective against non-specialist predators (Nigrelli 1952; Williamson et al. 1996; Flammang et al. 2002; Dyck et al. 2010). Aborigines of Guam and other regions of Indo-Pacific used some holothurians to poison small lagoons of coral reefs at low tide for killing fish (Frey 1951).

Intoxications from sea cucumbers occur either from ingestion of the fresh (poisoning) or from direct contact with the toxic Cuvierian organs (envenomation). A Cuvierian tubules of most sea

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cucumbers are highly toxic, and skin contact provokes pain and severe inflammatory reactions. The toxic material is also highly irritant to the eyes if transferred there on fingers; claims of "blindness" made in the literature remain unsubstantiated, and are doubtful (Williamson et al. 1996). The toxins exhibits neural blockade in animals. Thus, after the application of holothurian toxins, the mice lost compensation in locomotory movement, and then there came paralysis in which the forelegs were attacked at first, and later followed by the hind ones. Reaction to mechanical stimulus, such as insert by biceps, was totally lost. When the paralyzed mice placed on their back position, they were no more able to do righting reaction. In some animals nose bleeding due to lung blooding was observed. Lethal effect was usually observed within 24 h and intravenous mode of toxin application was most effective (Yamanouchi 1955; Kropp 1982; Zaki 2005).

Toxins are water soluble but at least partially heat resistant. It is hydrolyzed by (gastric) acid into non-toxic products, which may be one of the reasons for the luck of correlations between toxicity studies with different animals and human poisoning from sea cucumbers. Also "edible" species of sea cucumbers probably contain less toxins than other species. It was noted that holothurian poison is most dangerous when it enters into blood circulation (hemolysis), but it is not so harmful when eaten. When eaten raw, the toxins may cause nausea, but boiled and dried animals which are commonly eaten, contain a very low level of toxins in their tissue as a results of the treatment process and are safe for human consumptions (Yamanouchi 1955; Williamson et al. 1996; Descotes 1996).

It was found that the release of the Cuvierian tubules can be accompanied by the discharge of toxic chemicals known as saponins (triterpene glycosides) (Yamanouchi 1955). Triterpene glycosides have long been suggested the main poison compounds of the sea cucumbers and to play a role in the defense of holothuroids as a toxin (Kalinin et al. 2008).

These naturally occurring biologically active compounds, saponins (triterpene glycosides), are mainly produced by plants, but also by some marine animals (typically by holothurians and some sponges). They derive their name from their ability to form stable, soap-like foams in aqueous solutions. Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone which may be triterpenoid or steroid in nature. Therefore, another name of these compounds is steroidal or triterpene glycosides depending on the aglycone moiety structure.

In spite of wide spectrum of biological activities of triterpene and steroidal glycosides described including cytotoxic, membranolytic, antifungal, virucidal, hypoglycaemic activities, molluscicidal, antiprotozoa, antioxidant, glucocorticoid-like effects and even anti-tumor properties (Kalinin et al. 2008), these compounds are of great interest in terms of their effects on the immune system and their applications in vaccines. The greatest progress in study of immunomodulatory properties of glycosides was achieved in the investigation of saponins of plant origin. One of the first plants attracted the attention of researchers was *soap bark tree Quillaja saponaria*, which is a rich source of triterpene glycosides. *Quillaja* saponins have been used for many years as veterinary vaccine adjuvants. The saponin adjuvants most widely used are a type called Quil A, which is a purified *Q. saponaria* saponin fraction. The unique capacity of Quil A and another isolated and purified saponin QS-21 to stimulate both the Th1 immune response and the production of cytotoxic T-lymphocyte against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens as well as for therapeutic cancer vaccines. In effect, there are a series of commercial veterinary vaccines as well as human vaccines formulated with this kind of adjuvant undergoing clinical evaluation. Quil A has been used to prepare an immunostimulating complex, ISCOM, which have been evaluated against a number of viruses, including feline leukemia virus and HIV (Rajput et al. 2007).

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Other plants containing glycosides which were carefully examined by immunologists were ginseng (*Panax notoginseng*), astragalus (*Astragalus membranaceus*, *A. oleifolius*) and number of podded plants (acacia *Acacia victoriae*, soya *Glycine max* (L.) Merr.), and some others. Extensive phytochemical and pharmacological studies on ginseng proved the dammarane-type saponins to be the main bioactive principles. The dammarane-type glycosides of *P. notoginseng* are composed of a protopanaxadiol and protopanaxatriol glycosides. *P. notoginseng* saponin was proved to show a slight hemolytic effect and enhance significantly a specific antibody and cellular response against ovalbumin in mice which were more significant than that of Quil A. The root of a herbaceous plant *Achyranthes bidentata* is a well-known traditional Chinese medicine containing saponins and has been reported as immunostimulatory. *A. bidentata* saponins modulated immune responses significantly enhanced the ovalbumin-specific antibody titers with slight hemolysis in mice. Avicins, a family of triterpenoid saponins from *Acacia victoriae* represent a new class of plant stress metabolites capable of activating stress adaptation and suppressing proinflammatory components of the innate immune system in human cells by redox regulation. It was found that saponins from soybean exhibited high adjuvant activity while the soyasapogenol group exhibited low activity. For more detailed information on plant saponin immunomodulatory properties, a variety of excellent and comprehensive reviews are available (Cheeke 1999; Francis et al. 2002; Sun et al. 2006; Rajput et al. 2007).

For more than 40 years triterpene glycosides from sea cucumbers (holothurians) have attracted the attention of chemists, biochemists, pharmacologists, and biologists-taxonomists. Triterpene glycosides of sea cucumbers demonstrate a wide spectrum of biological effects: antifungal, anti-tumor, hemolytic, cytostatic, ichthiotoxic and some other activities. The mechanism of their toxic membranolytic properties is well known at present and described in details. The application of many preparations from sea cucumbers in traditional oriental medicine is known. The medicinal properties of these preparations are attributed to triterpene glycosides.

At the same time the systematized data concerning the immunomodulatory properties of sea cucumber triterpene glycosides and its effect upon immune system of the animals and human are absent in the literature. The current review summarizes the recent data on immunomodulatory activity of sea cucumber triterpene glycosides and describes some aspects of their molecular mechanism(s) of actions.

Sea Cucumber Triterpene Glycosides

Immunomodulatory Properties

The lanostane triterpene glycosides are characteristic of sea cucumbers (Holothurioidea), and are widely distributed in all the orders of this class. The majority of their aglycones has 18 (20)-lactones and belongs to the holostane type. Carbohydrate chains of sea cucumber glycosides have six monosaccharide residues including xylose, quinovose, glucose and 3-*O*-methylglucose and sometimes 3-*O*-methylxylose, 3-*O*-methylquinovose, 3-*O*-methylglucuronic acid and 6-*O*-acetylglucose. They may contain one, two or three sulfate groups. These compounds possess a wide range of pharmacological properties and are responsible for the organism's environmental defense mechanisms in general (Kalinin et al. 2008). The example of chemical structure of the triterpene glycoside, cucumarioside A_2-2, isolated from sea cucumber *Cucumaria japonica* is shown on Fig. 2.

The ichthyotoxic action of aqueous extracts of sea cucumbers has been studied (Nigrelli 1952; Yamanouchi 1955; Nigrelli and Jakowska 1960). Yamanouchi and Nigrelli showed the presence of ichthyotoxic substances in 30 species of sea cucumbers belonging to four different orders.

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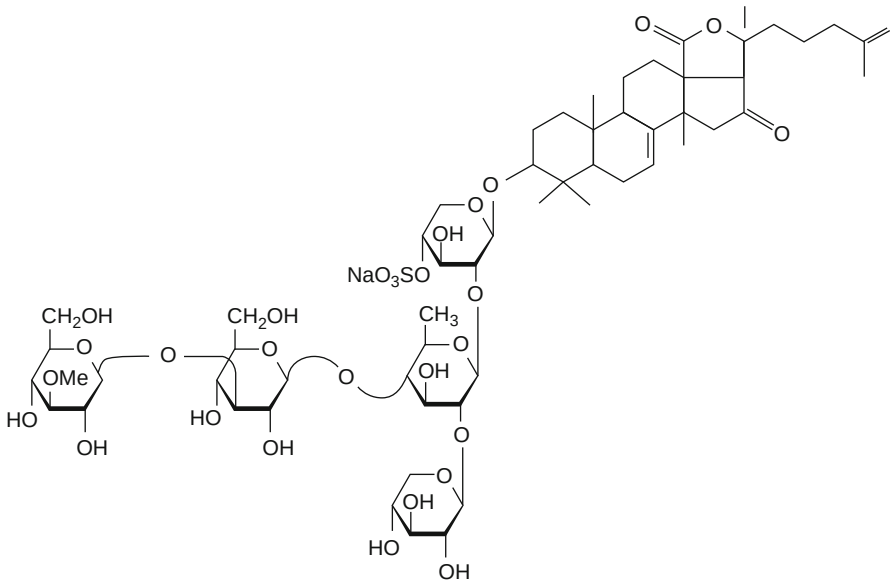

Fig. 2 Chemical structure of cucumarioside A_2-2, the triterpene glycoside isolated from *Cucumaria japonica*

During the last decade there have been several reviews of the published investigations of the cytotoxic activities of triterpene glycosides. These summaries have shown specific correlations between the structure of the triterpenoid saponins and their cytotoxicity and most common biological mechanisms of action (Kalinin et al. 2008; Podolak et al. 2010; Osbourn et al. 2011; Kim and Himaya 2012).

It was established that at the micromolar concentrations sea cucumber glycosides show hemolytic, cytotoxic, antifungal and other biological activities caused by membranotropic action. The basis of membranotropic action of the glycosides is their ability to attach to cell biomembranes and form nonselective ion-conducting complexes with 5(6)-nonsaturated sterol components of those cell membranes, preferably with cholesterol. Such sterol/saponin interaction results in an efflux of some ions, nucleotides and peptides, disrupting ion homeostasis and osmolarity, followed by lysis and cell death. Cytotoxic activity of sea cucumber glycosides against different cell types and cell lines, including human tumor cell lines, has been extensively studied. These studies have shown strong cytolytic effects upon tumor cells in vitro (Kalinin et al. 2008; Podolak et al. 2010; Osbourn et al. 2011; Kim and Himaya 2012).

By contrast, in low sub-toxic concentrations marine triterpene glycosides induce activation and potentiating of cellular functions of immune cells. Stimulatory effects of certain sea cucumber glycosides on the immune cell functions were described for the first time in the 1970s. Applied in low concentrations (0.1–6.0 μg/ml), crude Holothurin, a mixture of triterpene glycosides from tropical holothurians *Actinopyga agassizi*, was shown to stimulate a leukocyte migration, induce an increase in phagocytosis of *Staphylococcus aureus* by human polymorphonuclear leucocyte similarly to serum factors and activate hemopoesis in bone marrow of frog (Nigrelli and Jakowska 1960; Lasley and Nigrelli 1970).

It is known that holothurian triterpene glycosides do not exhibit antibacterial activity itself (Shimada 1969). At the same time it was shown that glycosides have pronounced antifungal effects (Batrakov et al. 1980). Moreover, glycosides from *Cucumariajaponica* were shown to significantly increase the beneficial immune response in animals to bacterial infections elicited by various pathogens. Thus, mixture of cucumariosides was shown to exhibit non-specific antibacterial action

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upon the number of bacteria genus such as *Escherichia*, *Proteus*, *Salmonella* and *Neisseria* after intraperitoneal injection into infected mice. This effect of these glycosides was mediated in-part by the increase in macrophage phagocytic activity, killing and digesting capability which was at least doubled 7–14 days after the *i.p.* injection of the glycosidic mixture (0.03 mg/mouse) (Sedov et al. 1984a, b, 1990).

Glycosides from *C. japonica* were also shown to possess strong adjuvant properties potentiating the effect of some bacterial vaccines and while also demonstrating some antiviral activity, possibly through the activation of T- and B-cell cooperation. Some triterpene glycosides from the Far-Eastern edible holothurians *C. japonica*, are known as active substances of a veterinary immunostimulatory preparations (so-called KD, KM and KM-2) approved for the treatment of mink, pigs, dogs and other animals in Russia. The experimental in vitro research of cucumariosides mixture (KM) action upon functional activity of human blood neutrophils was carried out. It was found that in the range of low concentrations the preparation acted as a modulator of the absorbing capacity stage of phagocytosis and as a stimulator of diminished function of oxidative metabolism (Grishin et al. 1990, 1995; Sedov et al. 1984a, b, 1990; Turischev et al. 1991).

The biological effects of the triterpene glycosides, cucumariosides A_2-2 and A_7-1 from the sea cucumber *C. japonica* and their aglycones were investigated using embryos of the sea urchin *Strongylocentrotus nudus* and the Balb/c line mouse peritoneal macrophages. Cucumariosides were highly cytotoxic in a sea urchin embryo development test with EC_{50} of 0.3 mg/ml and 1.98 mg/ml, respectively. The aglycone was completely lacking of cytotoxicity. In subtoxic concentrations (0.001–0.1 μg/ml) cucumarioside A_2-2 showed more than twofold stimulation of lysosomal activity and induced a rapid short-term increase in cytosolic Ca^{2+} content in mouse macrophages. The maximal stimulatory effect was detected after 1–2 h of cultivation of the cells with this glycoside. Cucumarioside A_7-1 demonstrated considerably weaker effects and even slightly inhibited lysosomal activity, while the aglycone was completely ineffective. At the toxic concentration (1 μg/ml), cucumarioside A_2-2 induced a sharp irreversible increase of intracellular Ca^{2+}concentration. It was suggested that glycosides from *C. japonica*, especially cucumarioside A_2-2, may act as Ca^{2+} agonists due to their membranotropic properties (Agafonova et al. 2003).

Frondoside A, a major triterpene glycoside from North Atlantic commercially harvested sea cucumber *Cucumaria frondosa*, was shown to possess strong immunomodulatory properties in subtoxic doses. Frondoside A stimulated lysosomal activity of mouse macrophages in vivo at a maximal effective stimulatory dose of 0.2 μg per mouse. The effect is maintained over 10 days. This glycoside also showed a 30 % stimulation of lysosomal activity in mouse macrophages in vitro at concentrations of 0.1–0.38 μg/ml. Frondoside A enhanced macrophage phagocytosis of the bacterium *Staphylococcus aureus* in vitro at a maximal effective concentration of 0.001 μg/ml. Frondoside A stimulated reactive oxygen species formation in macrophages in vitro which led to an increase in the number of antibody plaque-forming cells (normally B-cells in spleen) in vivo with a maximal stimulatory effect at a concentration of 0.2 μg per mouse (stimulatory index, 1.86). Frondoside A had a weak effect upon immunoglobulin IgM production after immunization with sheep erythrocytes in mice. Frondoside A did not stimulate Ig production in mice and did not significantly enhance the ovalbumin-stimulated IgM and IgG antibody levels in ovalbumin-immunized mice. Hence frondoside A is an immunostimulant of cell-based immunity including phagocytosis, without a significant effect on amplification of humoral immune activity or adjuvant properties (Aminin et al. 2008a).

Cucumarioside A_2-2 possesses potent immunomodulatory properties. At concentration of 0.02 μg/ml glycoside significantly enhanced macrophage morphology parameters and behavior. The presence of cucumarioside A_2-2 in the incubation medium at different concentrations during

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adhesion experiments increased cell attachment to the Collagen I matrix by 1.5–1.6 times compared to Collagen I alone. Macrophages incubated with cucumarioside A_2-2 had larger geometrical parameters and their shape was more differentiated because of the increase in spreading, elongation, and formation of lamellae and fillopodia compared to the control macrophages. The glycoside induced a statistically significant almost twofold increase in cell area, perimeter and maximal chord, and tendency for an increase of cell shape. Exposure of cells to glycoside leads to a significant increase in cell area variation as well as cell velocity and rotation. Cell motility monitored by cell centroid tracking record was also increased by treatment with cucumarioside A_2-2 (Aminin et al. 2011a).

Six monosulfated triterpene glycosides, frondoside A_1, okhotoside B_1, okhotoside A_1-1, frondoside A, okhotoside A_2-1 and cucumarioside A_2-5, isolated from *Cucumaria okhotensis* stimulate spreading and lysosomal activity of mouse macrophages and ROS-formation in the macrophages. The highest macrophage spreading and stimulation of their lysosomal activity was induced by frondoside A_1, frondoside A and cucumarioside A_2-5. All glycosides similarly stimulate ROS formation in macrophages, but okhotoside B_1 causes minimal stimulation (Aminin et al. 2010a).

Immunomodulatory activity of the triterpene glycosides cucumariosides I_2, H, A_5, A_6, B_2 and B_1 from *Eupentacta fraudatrix* upon mouse peritoneal macrophages was recently studied. Cucumariosides I_2, A_5 and B_2 were shown to increase lysosomal activity of macrophages by 15–17 % at doses of 1–5 μg/ml. Hence lysosomal activity depended on the structure of both the aglycone and the carbohydrate chain and did not have direct correlation with cytotoxicity of the glycosides (Silchenko et al. 2013).

Recently five predominant un-modified sulfated glycosides of *C. japonica* with the same aglycon moiety and different carbohydrate chains (cucumariosides A_2-2, A_3, A_4-2, A_6-2 and A_7-1) and two artificially desulfated derivatives (ds-cucumarioside A_2-2 and ds-cucumarioside A_4-2) were studied using in vivo and in vitro models. Intraperitoneal injection of the glycosides induced macrophage lysosomal activity in a dose-dependent manner (up to 250 % of control). The stimulatory effect was related to the chemical structure of the glycosides and was especially influenced by the number and position of sulfate groups in the carbohydrate moiety of the molecules. Monosulfated cucumariosides A_2-2 and A_4-2 were among the most active compounds, and desulfation of their carbohydrate moiety (DS-A_2-2 and DS-A_4-2) completely abolished this activity. Polysulfated and desulfated cucumariosides lacked stimulatory properties indicating the importance of monosulfation for the immunopotentiating properties of cucumariosides. The number and position of sulfate groups in glycoside molecule were also important to exert maximal stimulation of mouse peritoneal macrophages. Thus, cucumariosides A_2-2 and A_4-2 containing monosulfate group at C-4 position of the xylose residue were significantly more potent in comparison to cucumariosides containing an additional sulfate group at C-6 position of the glucose (A_3) or an additional sulfate at C-6 position of the terminal 3-*O*-Me-glucose (A_6-2). The ability of cucumariosides to stimulate macrophage lysosomal activity was as following: A_2-2 > A_3 > A_4-2 > A_6-2 > A_7-1 > DS-A_2-2 > DS-A_4-2. The effect was seen at picogram doses of cucumariosides with a maximum stimulation around 20 ng/mouse (Aminin et al. 2001).

On the basis of triterpene glycosides isolated from Far Eastern sea cucumber *C. japonica*, a new immunomodulatory lead Cumaside has been created that consists of a complex of monosulfated glycosides (mainly cucumarioside A_2-2) with cholesterol in an approximate molar ratio of 1:2. This complex has been utilized for the prevention and treatment of human immunodeficiency states (Stonik et al. 2005). The haemolytic activity of Cumaside was significantly reduced in comparison with original glycosides due to the glycoside-cholesterol complex formation. The influence of

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Cumaside on mouse macrophages in low doses was accompanied by more than twofold stimulation of lysosomal activity. This preparation was found to increase significantly the animal resistance against bacterial infections elicited by various pathogens such as *Staphylococcus aureus* and *Yersinia* p*seudotuberculosis*. After preliminary incubation during 3 h Cumaside at doses of 0.1 and 1 μg/ml significantly increased human neutrophil and monocyte phagocytosis. Cumaside in small doses rendered a significant influence on parameters of human blood leucocyte bactericidal activities. It stimulated ROS formation, IL6 and TNF-α production in lymphocytes, increased the number of antibody producing cells (plaque-forming cells) and amplified the expression of several cell surface molecules (CD3, CD4, CD8) preliminary cultured with immune depressant compound, hydrocortisone. The entering of Cumaside into culture of healthy donor blood lymphocytes increased the expression of the early activation marker CD25 and did not change the expression of the late activation marker HLA-DR. At the same time the preparation did not affect the delayed-type hypersensitivity, proliferative activity of lymphocytes, cytotoxic activity of NK-cells and cytokine IFNγ and IL12p70 release (Stonik et al. 2005; Aminin et al. 2006).

Recently it was found that Cumaside possess the ability at low dosages to inhibit development of tumors, demonstrates significant antitumor activity in vivo. It delayed Ehrlich mouse carcinoma growth independent of the mode of administration (*i.p*. or oral). Both the ascitic and solid forms of Ehrlich carcinoma were sensitive to Cumaside. The effect was a decrease of ascitic fluid volume, concentration of tumor cells in ascitic fluid and growth rate of the solid form of the tumor. The pronounced antitumor effect was achieved in a prophylactic scheme of lead use alone (several days before tumor inoculation) or at its combined use before and after tumor inoculation. Moreover, a synergetic effect occurs with combined treatment with Cumaside and a cytostatic 5-fluorouracil. One of the author's explanations of Cumaside anticancer effect was related with its clear immuno-modulatory properties and ability to promote proliferation and adhesion of lymphocytes, to activate cell immunity system and the production of series of cytokines including tumor necrosis factor TNF-α (Aminin et al. 2010b).

Antiviral Activity

The high efficacy of cucumariosides was found in prophylactic and curing of tick-borne (vernal) encephalitis. It is necessary to note that the authors explained this activity by hormone-like action of the preparation applied at low doses. The authors noted that a mechanism of antiviral activity of sea cucumber triterpene glycosides may be connected with antiviral protection at the stage of virus-cell interaction. This type of protection was confirmed by experiments on inhibition of the cytopathic effect of vesicular stomatite, poliomyelitis and other viruses in cell culture by cucumariosides in vitro (Grishin et al. 1991). Cucumarioside A_2-2 was shown to be more active than cucumarioside A_4-2 whereas cucumarioside G_1 was not active in the same assay. Holothurinosides A, C and D from *Holothuria forskali* and the desulfated derivative at a concentration 20 μg/ml caused an inhibition of cytopathic effect induced by vesicular stomatitis virus (VSV) in cell culture (20 % inhibition of VSV in baby hamster kidney cell line) (Rodriguez et al. 1991). Two trisulfated tetrasaccharide glycosides, liouvillosides A and B isolated from the Antarctic sea cucumber *Staurocucumis liouvillei* were found to be antiviral against herpes simplex virus type 1 (HSV-1) at concentrations below 10 μg/ml. Liouvillosides A produced a weak inactivation of HSV-1 since at the maximum concentration tested, the residual ineffectivity was 24 % with respect to the control virus sample, whereas after treatment with liouvillosides B in the same experimental conditions, the remaining inactivity was tenfold lower (Maier et al. 2001).

On the other hand the high curing effect was marked for preparation KM-2 with minks affected by virus of Aleutian illness which is the main reason of mink mortality, loss of their productivity and fur

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value. The initial effect of the cucumariosides was related with immunostimulatory activity in vivo and estimated by the dynamics of antibody titer elevation in experimentally infected minks. The comparison of effects of mixture of cucumariosides (preparation KM) and purified cucumarioside A_2-2 (preparation KM-2) had demonstrated the similarity of preparation effectiveness. Therefore, both preparations were recommended for their broad application in veterinary not only for Aleutian illness treatment, but with prophylactic aims to increase in resistance and productivity of healthy and sick animals including pigs and dogs with different infection diseases (Slugin 1982; Grishin et al. 1991, 1995).

Radioprotective Properties

One of the affecting factors of ionizing radiation is its noxious influence on hematopoetic organs and functions of immune systems. During the investigation of physiological activity of sea cucumber glycosides it was found that preparation KM from *C. japonica* exhibited prophylaxis and cure effect on mouse organism lesions after ionizing radiation. Triterpene glycoside injection most considerably influenced on increase in number of karyocytes of femoral bones and number of spleen cells. Evidently, it was correlated with proliferation and migration of pluripotent stem cells, which leaded to the recovery of the function of hematopoetic and immune systems. The rise of animal survival rate up to 47 % was observed on 30th day after irradiation. It was noted that maximal dosage of compound stimulated cell proliferation was approximately 200 times less than that of toxic dose. Individual triterpene glycoside cucumarioside A_2-2 from *C. japonica* and glycoside preparation "trepangin" from Far-Eastern sea cucumber *Apostichopus japonicas* showed radioprotection properties while cucumarioside A_2-2 was significantly more active. Surviving of mice irradiated by γ-rays (^{137}Cs) in absolutely lethal dose (7.7 GR) and administrated by glycoside after 5–10 min after irradiation (1 μg/kg) was about 40 % and after 60 min about 20 %. The restoration of indexes of blood creation was observed (Grishin et al. 1990; Poverenniy 1990).

Radioprotective activity of lead Cumaside has been studied. Female CD-1 strain mice administrated with Cumaside along with a prophylactic scheme were irradiated using a Gamma-therapeutic device with a ^{60}Co source (exposure dose 6.5 Gy, dose rate 1.14 Gy/min) and the average life spans of the mice were determined. The mice administrated with Cumaside and irradiated were killed by pervisceral dislocation on days 4 and 9. Peripheral blood cell composition indexes, blood forming function and cell number in blood-forming organs, and the number of pluripotent blood-forming stem cells were determined and compared with non-treated irradiated mice. The percentage of survivability and average life span were significantly decreased in the irradiated mice, but not in the Cumaside treated groups. Importantly, the number of leukocytes and neutrophils of the blood (bone marrow from hip), and the weight and the cell number of lymphoid organs were increased in the Cumaside treated groups compared to the non-treated group. It was concluded that at low prophylaxis doses, Cumaside possesses moderate radioprotective properties due to its immunomodulatory effects (Aminin et al. 2011b).

Immunostimulating Complexes

The tubular immunostimulating complex (TI-complex) as a new nanoparticulate antigen delivery system was recently developed. The morphology and composition of TI-complexes were principally different from the known vesicular immunostimulating complexes (ISCOMs). An antigen carrier consisting of triterpene glycosides from *C. japonica*, cholesterol, and monogalactosyldiacylglycerol (MGalDG) from marine macrophytes with reproducible properties was developed and high adjuvant activity was obtained. The cucumarioside A_2-2 – cholesterol – MGalDG ratio of 6:2:4 (by weight) was found to provide the most effective formation of TI-complexes and the minimum hemolytic

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activity in vitro. The TI-complex was able to increase the immunogenicity of the protein antigens from *Yersinia pseudotuberculosis* by three to four times (Kostetsky et al. 2011).

Immunization of mice with porin from *Yersinia pseudotuberculosis* (YOmpF) within TI-complexes including cucumarioside A_2-2, comprised of different MGDGs, revealed a dependence of the immunostimulating effect of TI-complexes on the microvicosity of this glycolipid. TI-complexes comprising MGDGs from *Sargassum pallidum* and *Ulva fenestrate* with medium microviscosity induced maximal levels of anti-porin antibodies (four times higher when compared with those induced by pure porin). The adjuvant effect of TI-complexes based on other MGDGs varied by 2.8, 2.3 and 1.3 times for TI-complexes comprised of MGDGs from *Zostera marina*, *Ahnfeltia tobuchiensis*, and *Laminaria japonica*, respectively. MGDGs were also able to influence cytokine mechanisms of immunological regulation. The results obtained suggest lipid "nanofluidics" as a novel strategy for optimizing the immune response to protein antigens within lipid particulate systems (Sanina et al. 2012).

Molecular Mechanisms of Action

In spite of comprehensive knowledge concerning chemical structure and physiological properties of sea cucumber glycosides as well as their practical use in the form of veterinary preparations, the molecular mechanism(s) underlying immunostimulatory properties of the compounds on cellular and sub-cellular levels remains to be elucidated. To address this question and to establish possible structure-activity relationship, the interaction of some sea cucumber triterpene glycosides with different immune cell types was studied.

Some authors associate the high biological and immunomodulatory activity of holothurian triterpene glycosides with their strongly pronounced membranotropic action, notably with changes in biomembrane ion penetration and glycoside impact upon different enzymatic complexes of biomembranes. It was supposed that the changes in cellular ionic homeostasis due to alteration of biomembrane barrier function is the main factor underlined of mechanism of glycoside inhibitory and stimulatory actions. The functional role of membrane sterols in biomembrane ionic penetration also was considered in connection with glycoside stimulatory activity. In the interaction with membranes, sea cucumber triterpene glycosides "pull" cholesterol which has a great affinity to phosphatidylcholine in the native membrane. As a result it significantly influences the physico-chemical properties of membranes: stability (Popov et al. 1982) and microviscosity (Gorshkova et al. 1989a; Aminin et al. 1989) of lipid bilayers, as well as lipid-protein interactions and conformations of membrane proteins (Gorshkova et al. 1989b, 1999, 2000). These properties in many respects determine activities of membrane enzymes and functional status of whole membranes. For instance, activities of membrane Na^+,K^+-ATP-ase and Mg^{2+}-ATP-ase in vitro decreased in the presence sea cucumber triterpene glycosides (Gorshkov et al. 1982). Moreover, the glycosides changed the parameters of active Ca^{2+} transportation in vesicles of sarcoplasmatic reticulum of rabbit skeletal muscles (Rubtsov et al. 1980). The reversibility of rapid short term increase in cytosolic Ca^{2+} was explained in this paper by a direct enhancement of the Na^+-Ca^{2+} exchange induced by saponin and secondary augmentation of the Na^+-Ca^{2+} exchange due to an increased $[Ca^{2+}]_i$ as it was suggested for the plant saponin, digitonin, applied at sub-skinning concentrations (Ishida et al. 1993).

Spleen is a prime organ in which immunostimulation takes place in mammalians. Proteome analysis was used to investigate the elicited effects on mouse splenocytes upon exposure to holothurian triterpene glycosides. Cucumarioside A_2-2 from *Cucumaria japonica*, and frondoside A from *C. frondosa*, respectively, have been used to in-vitro stimulate primary splenocyte cultures. Differential protein expression was monitored by 2D gel analysis and proteins in spots of interest

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were identified by MALDI-ToF-MS and nano LC-ESI Q-ToF MS/MS, respectively. Differential image analysis of gels from control versus gels from stimulated primary splenocyte cultures showed that approximately thirty protein spots were differentially expressed. Prime examples of differentially expressed proteins are NSFL1 cofactor p47 and hnRNP K (both down-regulated in stimulated primary splenocyte cultures), as well as Septin-2, NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, and GRB2-related adaptor protein 2 (up-regulated in stimulated primary splenocyte cultures) which are involved in the processes of lysosome maturation, activation and merging, phagocytosis, cytoskeletal reorganization, cell adhesion, mobility and proliferation of immune cells. Immuno-analytical assays confirmed differential protein expression. Together with results from proliferation and cell adhesion assays, these results shown that particularly cellular proliferation is stimulated by holothurian triterpene glycosides. It was concluded that holothurian triterpene glycosides are thought to express their immuno-stimulatory effects by enhancing the natural cellular defense barrier that is necessary to fight invading pathogens and for which lymphocytes and splenocytes have to be recruited constantly due to the limited lifetimes of B-cells and T-cells in the body (Aminin et al. 2009).

The in vitro interactions between the triterpene glycoside cucumarioside A_2-2 and mouse splenocytes, peritoneal macrophages and its biomembranes were studied. Multiple experimental approaches were employed, including determination of biomembrane microviscosity, membrane potential, Ca^{2+}-signaling and radioligand binding assays. Cucumarioside A_2-2 exhibited strong cytotoxic effect in the micromolar range of concentrations and showed pronounced immunomodulatory activity in the nanomolar concentration range. It was established that cucumarioside A_2-2 effectively interacted with immune cells and reversible increased the cellular biomembrane microviscosity. This interaction led to a dose-dependent reversible shift in cellular membrane potential and temporary biomembrane depolarization; and an sharp and reversible increase in $[Ca^{2+}]_i$ in the cytoplasm. Ca^{2+} influx from the external medium was not inhibited by voltage-sensitive Ca-channel blockers and inhibitor of Na^+/Ca^{2+} exchange. It was suggested that there are at least two binding sites for [^{3}H]-cucumarioside A_2-2 on cellular membranes corresponding to different biomembrane components: a low affinity site with $K_d = 489.6$ nM match to membrane cholesterol that is responsible for the cytotoxic properties, and a high affinity site ($K_d = 3.07$ nM) corresponding to a hypothetical receptor that is responsible for immunostimulation (Pislyagin et al. 2012).

Using confocal microscopy, flow cytometry, Ca^{2+}-imaging and electrophysiology (patch-clamp) it was found that the most likely membrane targets of cucumarioside A_2-2 can be purinergic receptors of P2X family (predominantly P2X1 and P2X4). Structural models of cucumarioside A_2-2–mP2X1 and –mP2X4 complexes generated by *in silico* modeling disclosed that cucumarioside A_2-2 and ATP binding sites are localized at different areas of extracellular receptor domain. Molecular Dynamics simulations results revealed that cucumarioside A_2-2 action on the receptor turn those binding site residues connected through β-strands by H-bonds network. These findings and experimental data suggest that cucumarioside A_2-2 can act as an allosteric regulator which able to withdraw purine receptor inactivation by extracellular ATP and provide a recovery of Ca^{2+} conductivity of macrophage membrane. Such type of glycoside interaction with receptors may trigger an activation of Ca^{2+}-signaling pathway that initiates the amplification of expression of certain intracellular target proteins involved in key stages of immune cell physiology (Aminin et al. 2013; Astashev et al. 2013).

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Toxicological and Pharmacokinetics Study

The toxic properties and influence of immunomodulatory lead Cumaside on the function of different organs during 3-month long-term intragastric administration in experimental rats was studied. It was established that Cumaside did not significantly change animal general condition, body weight dynamics, hemogram, cardiovascular and central nervous systems, functional conditions of liver and kidney and ECG. The preparation did not influence on activity of alkaline phosphatase and some transaminases (aspartate aminotransferase and alanine aminotransferase) in blood serum (Polycarpova et al. 1990; Belousov et al. 2008a, b).

Pathomorphological investigation did not detect any serious negative changes in postmortem specimens of rat brain, cerebellum, hypophysis, lungs, heart, stomach, small and large intestine, liver, kidney, adrenal gland, thyroid body, lymph nodes, spleen, thymus, pancreatic gland and testis after Cumaside treatment. The lethal dosage of Cumaside exceeds 5,000 mg/kg for mice and rats at intragastric administration. Therefore, Cumaside was ranked among low-hazard compounds (Belousov et al. 2010).

Radiolabeled Cumaside was obtained from the tritium-radiolabeled cucumarioside A_2-2, and the pharmacokinetic of the former was studied. The model-independent parameters of the Cumaside pharmacokinetics in various organs and tissues of mice were calculated using an equation of nonlinear regression and the one-compartment model. Fairly rapid Cumaside absorption was observed after a single peroral or intraperitoneal administration. The maximum concentration was reached more rapidly (within 0.02–0.6 h) after intraperitoneal administration. This was a result of immediate and rapid contact of the preparation with blood and organs. After peroral administration, the maximum Cumaside concentration in the organs was reached within 0.3–0.7 h because of rapid initial and gastric absorption. Note that the maximum Cumaside concentrations in the stomach and blood were reached within 0.33 and 0.67 h, respectively. After peroral administration, radiolabeled products were detectable in the blood and organs for a short time: the mean residence time ranged from 0.38–1.45 h for different organs. The time of the preparation excretion from the body depended on the way of Cumaside administration. The value was minimum after peroral administration (0.3–1.4 h). After intraperitoneal administration, the excretion time of the preparation was several times longer. The half-elimination time ranged from 9– 22 h. Finally, authors suggested that Cumaside has proved to be readily absorbable after peroral administration; it does not accumulate, and much less is it retained for a long time, in organs of experimental animals. Peroral Cumaside administration can be recommended for the clinical trial (Aminin et al. 2008b).

The distribution of triterpene glycoside cucumarioside A_2-2 in mouse spleen was determined. For this purpose the stability and dynamics of glycoside content changes over time in Balb/c mouse spleen tissue homogenate as well as the study of the cucumarioside A_2-2 spatial distribution in tissue sections were investigated using radiospectroscopy, MALDI-MS and MALDI-IMS, correspondingly. Cucumarioside A_2-2 was reliably detected by MALDI-MS in the mouse spleen tissue after single *i.p.* injection at a dosage of 5 mg/kg. The glycoside was stable in the spleen and did not undergo metabolic transformation in either tissue homogenates or in the intact organ within 24 h after *i.p.* injection. The glycoside was absorbed fairly rapidly: the glycoside maximum concentration (C_{max}) in tissue homogenate was observed in the first 30 min after injection; the minimum values were registered in 3 h. These results are in agreement with those obtained in the pharmacokinetic study of ^{3}H-cucumarioside A_2-2. It was established by MALDI-IMS that glycoside was mainly located in the tunica serosa part of the spleen and only a small amount was detected within the red and white pulp of the organ. MALDI MS images obtained 15–30 min post dosage clearly reflected high drug concentrations in the regions surrounding the organ followed by its decline in the surface part and a very slight redistribution to the internal part of the spleen (Pislyagin et al. 2013).

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Conclusion and Future Directions

Chronic bacterial, fungal, protozoan, and viral diseases continue to plague humankind. Multiple resistances to antibiotics contribute significantly to the problem, and some are involved in the primary etiology of these diseases. Decreased immunity in large sections of the world's human population also continues to be a problem, and even newer generations of antibiotics can speed the decline in immune status. Therefore, discovery and development of new highly effective immunostimulatory nutraceutical and pharmaceutical agents from terrestrial and marine sources are acutely relevant to the everyday health of populations.

Sea cucumbers (or holothurians) are one class of marine animals which is important as a human food source and traditional medicine, especially in some parts of Asia. It is interesting to note that ancient Chinese medical books mention that unspecified elements in sea cucumber can increase/repair the human immune system, hence improving resistance to diseases, relieve chronic exhaustion syndrome and relieve stress and mental exhaustion. In the last few decades, scientific literature from several countries have reported that, indeed, triterpene glycosides from sea cucumbers do have a wide spectrum of biological effects including cytotoxic, hemolytic, antifungal, ichthiotoxic, and other activities. It has been shown that a majority of these activities are based on the interaction of these compounds with membrane sterols. The triterpene glycosides of sea cucumbers have strong membranotropic action against any cellular and model membranes containing Δ^5-sterols. This interaction with biomembrane Δ^5-sterols results in the formation of pores, changes in membrane ion permeability and viscosity, profound inhibition of some membrane enzymes, especially ATPases that finally lead to cell death. This partly explains the wide spectrum of their biological activities in mammals that includes cancer cell apoptosis or necrosis as a final result.

At the same time, sub-toxic concentrations of sea cucumber triterpene glycosides may result in cellular activation and potentiation of cellular functions. Applied in low concentrations, some glycosides induce an increase of cellular immunity, modulate the immune response in lymphocytes and macrophages and show mitogenic activities, while higher concentrations are found to suppress cellular functions. Immunomodulatory activity of sea cucumber glycosides at very low concentrations has the greatest interest from the pharmacological point of view. It is reasonable to suggest that this immunomodulatory activity is related to the modifying effect on membranes of target cells. Basically, the molecular mechanism(s) of glycoside immunomodulatory actions are currently being investigated in detail for cucumarioside A_2-2 isolated from sea cucumber *Cucumaria japonica*. To date, the results of these investigations provide useful insights into the immunomodulatory mechanism of some sea cucumber glycosides, which can be summarized as follows: (i) at low nanomolar concentrations, glycoside is associated only with high affinity receptors on immune cell biomembranes; and (ii) glycoside interaction and binding with cellular biomembranes triggers a cascade of intracellular reactions leading to the immunostimulatory response of cells. The first step in this cascade is the activation of the Ca^{2+}-signaling pathway via membrane Ca^{2+} channels that initiates an increase in expression of some intracellular target proteins involved in key stages of the immune cell physiology. This leads to increase of cell adhesion, motility and proliferation, ROS formation, lysosomal activity, phagocytosis, elevated synthesis of some cytokines and pathogenic microorganism killing. Ultimately, an activation of cellular immunity resulting in an increase in the immunological resistance of the organism to various opportunistic infections, irradiation and tumor growth is apparent after sea cucumber glycoside *i.p.* administration.

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Cross-References

- ► Classification and Distribution of the Aquatic Venomous and Poisonous Animals
- ► Marine Toxin, Review of
- ► Phylum Echinodermata e Annelida: Sea Urchins, Starfish and Sea Cucumbers, and Marine Worms

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Okadaic Acid and Other Diarrheic Toxins: Toxicological Profile

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Abstract

Okadaic acid (OA) and its derivatives are toxic lipophilic polyethers produced by marine dinoflagellates and accumulated in filter-feeding organisms, where they can undergo biotransformation. The ingestion of marine shellfish contaminated by these toxins causes a gastrointestinal illness in humans known as diarrhetic shellfish poisoning (DSP). OA and its analogues are weak tumor promoters and specific inhibitors of serine/threonine protein phosphatases. Inhibition of these enzymes affects various cellular processes, including the events at the basis of diarrhea and tumor promotion, although other mechanisms of action cannot be excluded. Studies in rodents indicate that these diarrheic toxins are absorbed through the gastrointestinal tract and distributed in the whole body, inducing toxic effects. Their lethal oral doses after acute administration in mice are 2–5 times higher than intraperitoneal ones, with the small intestine and liver as main target organs, while repeated oral OA exposure affects mainly the forestomach, lymphoid organs, pancreas, and, at ultrastructural level, myocardium. The increasing number of toxicological studies on OA, including cytotoxicity, genotoxicity, neurotoxicity, and tumor promotion, suggests that the adverse effects of this compound and/or its analogues could not be limited to acute hazards related to diarrhea induction but can also pose a significant risk after sub(chronic) exposure. Thus, further studies are needed to assess the adverse effects of long-term exposure to low doses of diarrheic toxins. Moreover, periodic monitoring of seafood and harmful algal species in the seawater is recommended to protect public health from foodborne poisonings and to minimize negative economic impacts to the shellfish industry.

Introduction

Okadaic acid (OA) and its analogues, the dinophysistoxins (DTXs), are lipophilic and heat-stable polyethers produced by marine phytoplanktonic dinoflagellates and responsible for the human illness known as diarrhetic shellfish poisoning (DSP). DSP is a food poisoning caused by the ingestion of marine filter-feeding bivalves contaminated by OA and/or DTXs, which symptoms are mainly gastrointestinal distress, diarrhea, nausea, vomiting, abdominal cramps, and sometimes fever that occur 30 min to few hours after contaminated shellfish consumption. DSP is a nonfatal illness and there is a complete recovery within 3 days, without any pharmacological therapy. Anyway, the illness is an important cause of foodborne morbidity worldwide, representing a threat to public health, but also causes serious problems for the shellfish industries and regulators, in relation to the economic losses consequent to the closure of harvesting sites and shellfish marketing during contamination by diarrheic toxins (Yasumoto et al. 1978).

Toxins

The main diarrheic toxins causing DSP are OA and its analogues DTX-1, DTX-2, and DTX-3 (Fig. 1). They are long-chain compounds containing polyether rings, with hydroxyl and carboxyl functions, and methyl groups differing in number or position. DTX-3 is a mixture of 7-*O*-acyl esters of OA, DTX-1,

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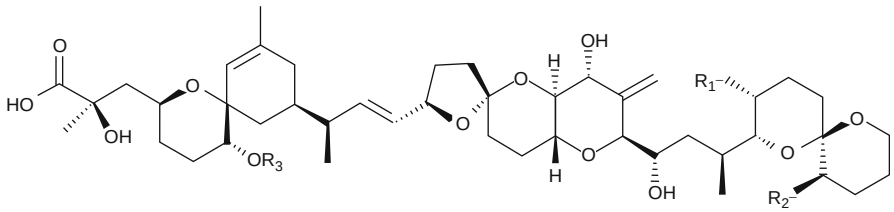

	R_1	R_2	R_3
OA	CH_3	H	H
DTX-1	CH_3	CH_3	H
DTX-2	H	CH_3	H
DTX-3	H or CH_3	H or CH_3	Acyl

Fig. 1 Chemical structure of okadaic acid and dinophysystoxin-1, dinophysystoxin-2, and dinophysystoxin-3 (DTX-1, DTX-2, and DTX-3, respectively)

and/or DTX-2 formed in shellfish fed with toxic dinoflagellates: the C-7 hydroxyl group of the parent compounds is esterified with long-chain fatty acids, forming derivatives collectively known as DTX-3 (Suzuki et al. 1999). Other OA analogues identified in dinoflagellates of the genera *Prorocentrum* and *Dinophysis* are 19-epi-OA and a group of derivatives named OA diol esters, characterized by the conjugation of the OA carboxyl group to different unsaturated C7 to C10 diols. Some diol esters possess a polar sulfated chain (DTX-4, DTX-5a, DTX-5b, and DTX-5c) and represent the water-soluble OA derivatives. DTX-3 as well as the OA diol esters can be subjected to chemical or enzymatic hydrolysis, giving their parent unesterified diarrheic toxins and ultimately sharing with them their mechanism of action and toxicity (Quilliam 2003; Hu et al. 2010).

Producing Organisms

OA was originally identified in the sponges *Halichondria okadai* and *H. melanodocia*. However, similarly to DTX-1 and DTX-2, it is produced by marine dinoflagellates of the genera *Dinophysis*, *Phalacroma*, and *Prorocentrum*. The first documented *Dinophysis* species involved in DSP was *D. fortii*, in Japan and later also in other geographic areas. Other *Dinophysis* species which have been associated with DSP episodes are *D. acuminata*, *D. acuta*, *D. caudata*, *D. miles*, *D. ovum*, and *D. sacculus*, whereas *D. infundibula*, *D. norvegica*, *D. tripos*, and two species of *Phalacroma* (*P. mitra* and *P. rotundatum*) have been found to contain diarrheic toxins, but doubts have been cast on the toxigenic nature of *P. rotundatum*. These species can be present in the marine environment of different parts of the world, but the toxin production may vary considerably among the species and also among their regional and seasonal morphotypes. Diarrheic toxins were identified also in benthic *Prorocentrum* species and in the planktonic species *P. texanum*. However, only the benthic species *P. lima* has been sporadically implicated in shellfish contamination by diarrheic toxins, so far: this species may contribute to the accumulation of diarrheic toxins in shellfish from shallow coastal embayments or in aquaculture sites with high turbulence, where benthic microalgae are easily resuspended in the water column, becoming available to filter-feeder shellfish that can accumulate the toxins (Reguera et al. 2014).

Vectors of Poisoning

OA and its derivatives accumulate mainly in the digestive gland (hepatopancreas) of bivalve mollusks, such as mussels, scallops, oysters, and clams, by filtering seawater containing the toxic phytoplankton. Subsequently, contaminated seafood consumption can cause DSP in humans. In fact, these toxins cannot

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be detected during seafood consumption since they do not influence the taste of the contaminated seafood. Moreover, being heat-stable, diarrheic toxins are not changed by cooking or freezing the contaminated marine organisms.

Epidemiological Data

OA and its analogues are widely distributed all over the world, causing human poisoning in different geographic areas, with most cases in Japan and Europe, and to a lesser extent North and South America, Thailand, Australia, and New Zealand. Anyway, the number of DSP cases is believed to be much higher than the documented ones: the episodes are often not well documented since the relevant symptoms are not always severe, and frequently the poisoned people do not request medical assistance (Valdiglesias et al. 2013). On the other hand, cases of diarrhea, or at least the presence of dinoflagellates producing diarrheic toxins, appear to be increasing, but this perception may also be influenced by the improved knowledge of the disease and the activation of surveillance programs aimed to reduce the number of intoxications.

Toxicokinetics

Experimental Data

"In Vivo" Experimental Data

In vivo studies on toxicokinetics of diarrheic toxins were performed for OA in mice to evaluate mainly the toxin absorption, distribution, and excretion.

After *single intraperitoneal injection* of radiolabeled [^{3}H]OA to mice (28 μCi/0.2 ml saline solution), Fujiki and Suganuma (1993) detected most of the radioactivity in the gastrointestinal content and in the liver: 33 % radioactivity was detected in the gastrointestinal content 3 h after the injection and 5 % after 19 h, while 27 % of the radioactivity was detected in the liver at 3 h and about 16 % was measured 19 h later. The high level of radioactivity in the liver also after 19 h suggested that, after OA intraperitoneal injection to mice, the toxin is subjected to hepatobiliary circulation (Fujiki and Suganuma 1993).

After *intramuscular injection* of [^{3}H]OA to mice (25 μg/kg), radioactivity was detected in the bile and intestinal content 1 h later, and the elimination pattern of the toxin indicated biliary excretion and enterohepatic circulation (Matias and Creppy 1996a).

Studies in mice after *acute oral administration* of OA by gavage show that OA is well absorbed by the gastrointestinal tract, being detected in the whole body, with high levels in the stomach, intestine, feces, and urine (Fujiki and Suganuma 1993; Matias et al. 1999; Ito et al. 2002; Aune et al. 2012). In particular, Fujiki and Suganuma (1993) observed that 3 h after the radiolabeled administration (14 μCi/0.2 ml sesame oil), most of the radioactivity (>77 %) was detectable in the gastrointestinal content, while 19 h later it decreased to 4 %, and 30 % was detected in feces. At these times, only 1 % of the radioactivity was found in the liver. Subsequent studies by Matias et al. (1999) evaluated the toxin distribution in organs and fluids of mice 24 h after single administration by gavage of nonlethal doses of [^{3}H]OA or of the unlabelled toxin (50 or 90 μg/kg). After 24 h, after [^{3}H]OA administration to mice (50 μg/kg), the toxin was detected in all organs and fluids, urine, intestinal content, and feces. The toxin was most concentrated in the urine and intestinal content, followed by the feces, stomach, blood, lungs, skin, and intestine tissues. The toxin distribution was even more pronounced after oral administration of a higher dose (90 μg/kg), which induced diarrhea. The high concentration of [^{3}H]OA in the intestinal tissues and intestinal content 24 h after its administration indicates a slow elimination of the toxin, due to a reabsorption from the intestine and enterohepatic circulation (Matias et al. 1999). Further studies by Ito et al. (2002) evaluated the toxin

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distribution at different times after oral administration of wider oral doses of OA in mice (75–250 μg/kg), using an immunostaining method. After its administration, the toxin was rapidly absorbed from the small intestine, mainly from the jejunum, and reached the liver within 5 min. The toxin was then distributed in the whole body, including the lungs, liver, heart, kidneys, and intestine; it was detectable for 2 weeks in the intestine, liver, and blood vessels. Excretion from the kidney, cecum, and large intestine began even after 5 min of the administration, and the excretion through the intestine continued for 4 weeks. Another immunochemical study revealed the presence of low OA levels in the liver of mice at 24 h and 48 h from its acute oral administration (115 and 230 μg/kg) as well as in the duodenum and ileum (mucosal secretions of the goblet cells), but the toxin was not detected in the colon secretions (Le Hégarat et al. 2006). On the other hand, a recent immunohistochemistry study at 24 h from the acute administration of higher OA doses to mice (500–1,000 μg/kg) detected the toxin in the liver and kidneys of 700 and 1,000 μg/kg treated animals but not at a dose of 500 μg/kg. OA was randomly distributed throughout the liver parenchyma and around the centrilobular areas, whereas the kidneys of mice treated with 700 μg OA/kg showed strong immunoreactivity in the proximal convoluted tubules, and those treated with 1,000 μg/kg showed immunoreactivity distributed in a scattered manner in the proximal tubules (Vieira et al. 2013).

Recently, Wang et al. (2012) quantified OA in the intestinal tissues of mice by liquid chromatography-mass spectrometry (LC-MS) analysis up to 24 h from its oral administration (750 μg/kg), observing that the toxin reached a peak after 3 h and then it decreased by more than 50 % at 24 h. Furthermore, using the same technique, Aune et al. (2012) quantified the toxin distribution in mice organs at 24–30 h from the acute oral administration of slightly lower and higher doses of OA (660–1,140 μg/kg). By far the highest OA levels were found in the gastrointestinal tissues, but low toxin levels were detected also in other organs, such as the liver, kidneys, spleen, and lungs, while very low levels were detected in the blood.

Despite the different exposure levels and the different experimental conditions, these studies demonstrate an OA absorption after its oral administration in mice, with the highest distribution in the gastrointestinal tissues, intestinal content, urine, and feces, where it is detectable for some weeks after exposure.

After oral administration of [^{3}H]OA to pregnant mice at the 11th day of gestation (50 μg/kg), a *transplacental passage* to the fetus was observed, with radioactivity levels higher than those detected in the liver or kidneys of the mother (Matias and Creppy 1996b).

“In Vitro” Experimental Data

Some in vitro studies were carried out on OA, DTX-1, and DTX-2 to evaluate the intestinal absorption and/or metabolism. Using differentiated Caco-2 cells as a model of human intestinal barrier, the absorption of OA, DTX-1, and DTX-2 was recently investigated. Ehlers et al. (2011) showed that an exposure of the cell monolayer to low concentrations of OA (20–200 nM, for 24 h) allows only limited toxin passage from the apical (“luminal”) to the basolateral (“blood”) side of the monolayer. In addition, at noncytotoxic concentrations, an active efflux was observed, which subsequently was demonstrated to involve P-glycoprotein: OA was actively eliminated by P-glycoprotein over the apical cell membrane, and this could represent a defense mechanism against low-dose contaminated shellfish through a reduction of the toxin bioavailability (Ehlers et al. 2014).

Subsequently, Fernández et al. (2014) observed that after incubation of Caco-2 cell monolayers with OA, DTX-1, or DTX-2 (50 and 100 nM, for 3, 6, 12, and 24 h), almost all the OA and DTX-2 remained in the apical side, indicating that these toxins were unable to cross the Caco-2 monolayer. Only DTX-1, disrupting the integrity of the cell monolayer, significantly crossed the intestinal epithelium at concentrations above 100 nM.

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Concerning the in vitro studies on the metabolism, Guo et al. (2010) evidenced that OA is converted to four oxygenated metabolites by two recombinant human cytochrome P450 isoforms: CYP3A4 and, to a lesser extent, CYP3A5. Anyway, three of these metabolites were shown to be only slightly less potent inhibitors of protein phosphatase 2A, with respect to OA. Since this enzyme is the main molecular target for OA, these oxidative biotransformations may not effectively detoxify the toxin (Guo et al. 2010). Nevertheless, a reduction of OA cytotoxicity toward hepatic HepaRG cells was observed only when the CYP3A4 was active and was involved in the biotransformation of OA in two monohydroxylated metabolites. OA oxygenated phase I metabolites were also shown to be formed in vitro after exposure to a fraction from homogenated rat liver, while no formation of glutathione adducts or glucuronidation was observed as phase II reactions (Kittler et al. 2014).

Human Data

There are no quantitative data on diarrheic toxin kinetics in humans. Some evidences indicate that DTX-3 is metabolized to DTX-1 in the human gastrointestinal tract: a poisoning episode in humans due to ingestion of DTX-3-contaminated mussels gave evidence for a toxin biotransformation into DTX-1 since the latter was the only diarrheic toxin detected in the feces of the affected people as well as in the mussel extracts after hydrolysis (García et al. 2005).

Mechanism of Action

OA is a specific inhibitor of serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A), a group of enzymes that dephosphorylate several proteins through a versatile mechanism that regulates different cellular functions, such as metabolism, transport, secretion, muscle contractility, cell division, DNA replication, transcription, RNA splicing, cell cycle progression, differentiation, and oncogenesis. These enzymes represent the most relevant serine/threonine phosphatases in mammalian cells, accounting for over 90 % of all serine/threonine phosphatase activity and being involved in the dephosphorylation of different substrates.

OA binds to a hydrophobic groove near the active site of the protein phosphatases and interacts with basic residues within the active site of these enzymes, inhibiting their activity (Louzao et al. 2005). OA possesses higher affinity for PP2A with respect to PP1 and is two orders of magnitude more potent against PP2A with respect to PP1. Furthermore, it inhibits also other types of serine/threonine protein phosphatases, including PP4, PP5, and to a lesser extent PP2B, PP3, and PP6 (Louzao et al. 2005). Also other OA analogues inhibit some protein phosphatases to a greater or lesser extent. Experimental evidences indicate that the affinity of PP2A for DTX-1 is 1.6-fold higher than that of OA, while the affinity for DTX-2 is twofold lower (Huhn et al. 2009). On the contrary, other studies showed that OA is slightly more potent than DTX-1 as PP2A and PP1 inhibitor. Moreover, using a recombinant and wild PP2A, a recent study determined the inhibition equivalency factors for DTX-1 and DTX-2 with respect to OA, which correspond to 1.1 or 0.9 for DTX-1 and 0.4 or 0.6 for DTX-2, using recombinant and wild PP2A, respectively (Garibo et al. 2013). The differences in PP inhibition might be due to different experimental conditions between the studies, including the enzyme sources and the substrates.

Among other OA analogues, the C19 epimer of OA (19-*epi*-OA) was shown to inhibit PP1 with a significantly lower potency than OA, but it inhibited PP2A similarly to OA, being one of the most selective PP2A inhibitors (Cruz et al. 2007). In contrast, the 7-*O*-acyl esters of OA, DTX-1, or DTX-2 (DTX-3) and the OA diol esters do not inhibit these protein phosphatases, if not hydrolyzed.

Inhibition of protein phosphatases in cells exposed to OA or its analogues leads to increased levels of phosphorylated proteins that affect a diverse array of cellular processes. The diarrhea and the tissue

alterations in the absorptive epithelium of the small intestine induced by the ingestion of these toxins have been attributed to an increased level of the phosphorylated proteins controlling ion secretion in intestinal cells and of cytoskeletal/junctional structures involved in the control of the permeability to solutes. These events have been associated with a consequent passive loss of fluids from the submucosa of the intestinal wall to the intestinal lumen, at the basis of the diarrheic effect of OA and its analogues (Domínguez et al. 2010). In fact, in vitro studies showed that OA affected the actin filaments controlling the integrity and functionality of the tight junctions between epithelial intestinal cells, which are important in the regulation of paracellular permeability in these cells. These effects were observed in rat intestinal cells (IEC-6 cells), where micromolar concentration of OA induced a concentration- and time-dependent cell rounding and retraction, associated with F-actin network reorganization. Within a 30 min exposure, OA induced a marginalization of F-actin and, after 90 min, also a collapse of vimentin filaments around the nucleus, with the formation of a netlike structure of microtubules. No changes in the plasma membrane permeability and transmembrane potential were observed in cells exposed to the toxin as indicators of early cell damage or activation. Other in vitro studies on human intestinal epithelial cells (Caco-2 and T_{84}) showed that exposure to OA decreased the transepithelial electrical resistance in a polarized way, an index of increased paracellular permeability, normally regulated by tight junctions (Tripuraneni et al. 1997; Okada et al. 2000). Thus, the diarrhea induced by OA and its analogues was attributed to a disruption of the barrier function of the intestinal epithelium and increased paracellular permeability, rather than to an effect as secretagogue. This was confirmed also by other studies, which evidenced that the toxin does not stimulate the secretion of Cl^- ions by intestinal cells, but it alters the transport systems involved in nutrient, ionic, and water absorption through small intestinal cells (Louzao et al. 2005).

Protein phosphatase inhibition by OA and its derivatives is also involved in their tumor promotion activity, initially observed in vivo in two-stage carcinogenesis experiments on mouse skin after cutaneous exposure (1 μg, twice a week, for 30 weeks) and rat glandular stomach mucosa after oral exposure (10 μg/rat/day, during weeks 9–55, and 20 μg/rat/day, during weeks 56–72) (Fujiki and Suganuma 1993). This mechanism distinguishes these toxins from the non-12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters, since they do not bind to the phorbol ester receptors on the cell membrane or activate protein kinase C. The tumor-promoting activity of OA and its analogues is related to an increased phosphorylation and then activation of a series of proteins involved in the signal transduction pathways regulating the cell cycle, cell transformation, and promotion of tumor growth, which have not been completely clarified. In particular, it was reported that alteration of cell processes regulating the transitions from G_0 to G_1 and to S-phase of the cell cycle is an early step of the tumor promotion induced by OA. Furthermore, it has been suggested that the tumor promotion by OA is partially mediated by the activation and induction of nuclear transcription factors, such as the activator protein-1 (AP-1) complex (Valdiglesias et al. 2013). Other findings demonstrated that OA prevents apoptosis by suppressing the dephosphorylation of the heterogeneous nuclear ribonucleoproteins A1 and A2, an event that in the context of tumor promotion could indirectly contribute to the survival of initiated cells. In addition, in vitro studies showed that OA induces genotoxic and cytotoxic effects, including DNA strand breaks, DNA adducts, oxidative damage, DNA repair alterations, and micronuclei induction, which might cause genomic instability, but the toxin is not classified as a carcinogen by the International Agency for Research on Cancer (IARC) (Valdiglesias et al. 2013).

Although PP inhibition leads to a dramatic increase in phosphorylation level in a number of proteins, resulting in important cell alterations and toxic effects, it has been supposed that not all the toxic effects observed after exposure to OA or its analogues can be explained by inhibition of these enzymes (Valdiglesias et al. 2013). For this reason, a reevaluation of the mechanism of toxicity of these compounds has been recently suggested (Munday 2013).

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Toxicity

Human Toxicity

Episodes of diarrhetic shellfish poisoning were documented worldwide, and, already in 1990, about 15,000 cases had been recorded, mainly in Europe and Japan. Symptoms in humans include mainly diarrhea, nausea, vomiting, and abdominal pain, which incidences were reported to be 99 %, 80 %, 55 %, and 53 %, respectively. Fever, chills, and headache have also been reported in some incidents. The symptoms start within 30 min to few hours after contaminated seafood consumption, usually from 3 to less than 12 h after shellfish ingestion. As an example, in the first reported episode in Japan, vomiting and diarrhea (detected in 92 % and 79 % of patients, respectively) occurred with a mean frequency of 4 times/day up to 12 times/day in the most severe cases; most individuals had a completed recovery within about 3 days (Yasumoto et al. 1978). Although the poisoning can provoke a high debilitation for some days, no human fatalities due to DSP were recorded so far, and recovery is usually complete within 1–3 days, also without any pharmacological treatment. No information is available for possible long-term effects or repeated exposure.

Other illnesses due to shellfish consumption characterized by diarrhea, such as those caused by bacterial or viral contamination, should be ruled out (Aune and Yndestad 1993). Moreover, it has to be considered that other toxins contaminating edible shellfish, such as azaspiracids, can also provoke diarrhea and symptoms similar to those determined by OA and its analogues. Anyway, since no antidote is currently available for azaspiracid poisoning as for OA and derivatives, differential diagnosis between the two poisonings is not important for the management of the poisoned people.

The majority of outbreak reports provide limited information on the amounts and profile of diarrheic toxins ingested by poisoned people. Moreover, the level of seafood contamination is often uncertain because the analyzed shellfish were harvested at a different time from those actually consumed. In addition, these reports provided no information on the effects of cooking on these toxin levels in shellfish. However, considering that these are heat-stable and lipophilic compounds, it has been reported that loss of fluid during cooking can result in a significant increase in toxin concentration in cooked shellfish flesh, compared to the uncooked shellfish (EFSA 2008).

The first reports related to incidents by DSP in 1976 and 1977 in Japan, involving 164 subjects, indicate that the major detected toxin was DTX-1 (Yasumoto et al. 1978). Analyses of leftovers of mussels from meals of eight persons poisoned in 1977 indicated that dietary exposures corresponded to about 48 μg OA equivalents/person for mild symptoms and 80–280 μg OA equivalents/person for severe symptoms. Other documented episodes of DSP in European countries (Sweden, Norway, Portugal, and the United Kingdom) report that several hundreds of cases were associated with lowest-observed-adverse-effect levels (LOAELs) of about 50 μg OA equivalents/person that for a person of 60 kg body weight corresponds to an LOAEL of 0.8 μg OA equivalents/kg. From this LOAEL, the European Food Safety Authority Scientific Panel on Contaminants in the Food Chain derived an acute reference dose (ARfD) of 0.3 μg OA equivalents/kg, which represents the amount of toxin that can be ingested in a period of 24 h or less without appreciable health risk (EFSA 2008).

Considering the tumor promoter activity and the genotoxic effects of OA and DTXs evidenced by experimental studies, a link between cancer risk and sub(chronic) exposure to low levels of these toxins by seafood consumption had been hypothesized. Anyway, even though few epidemiological studies evidenced a possible correlation between consumption of shellfish and incidence of total and colorectal cancers, available data and potential bias do not allow to determine whether a repeated exposure to OA and/or its analogues played a role in these observations (EFSA 2008). Thus, due to the insufficient information on the chronic effects of these toxins, no tolerable daily intake (TDI) had been determined for

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diarrheic toxins as the daily toxin intake in food that in the light of present knowledge can be consumed every day for a lifetime with no appreciable harmful effects (Munday 2013).

Experimental Toxicity

Single Administration

The main effects of OA and its analogues in rodents include lethality, diarrhea, and alterations at gastrointestinal and hepatic levels, observed both after intraperitoneal (i.p.) and oral administration.

After *single intraperitoneal administration* in adult mice, the median lethal dose (LD_{50}) of OA determined through different studies ranged from 192 to 225 μg/kg, while an LD_{50} value of 352 μg/kg was determined for DTX-2 (Aune et al. 2007). The relative lethal potency of DTX-2 with respect to that of OA had been determined to be 0.6, and the lower potency of DTX-2 was ascribed to the axial 35-methyl group that would determine a lower affinity for PP2A (Aune et al. 2007; Huhn et al. 2009). No LD_{50} data were reported for DTX-1, but lethality in mice was recorded at a dose of 160 μg/kg, and its potency after i.p. injection is considered to be comparable to that of OA, while DTX-3 appears to be less toxic (lethality was recorded at 250–500 μg/kg). Concerning the lethality of other OA derivatives, it had been observed that acylation of the 7-hydroxyl group of OA with a saturated fatty acid to form 7-*O*-palmitoyl-OA, or with a diunsaturated fatty acid to form 7-*O*-linoleoyl-OA, significantly decreased the toxicity of the parent compound since the lethal potency (minimum dose necessary to kill a mouse of 16–20 g in 24 h) of these esters was more than 20 times lower than that of OA. In contrast, the polyunsaturated 7-*O*-docosahexaenoyl-OA was 10 times more toxic than the saturated or diunsaturated esters (Table 1) and about half as toxic as OA. Anyway, it has to be considered that some of the studies on diarrheic toxin lethality do not specify the number of mice per dose, the strain, the gender and the age of mice, and/or the range of the administered doses of toxins (EFSA 2008; Tubaro et al. 2008).

Pathological changes induced by intraperitoneal injection of OA, DTX-1, or DTX-3 to rodents (mice and rats) were recorded mainly at intestinal and hepatic levels. In suckling mice, a study showed that DTX-1 injection (50–500 μg/kg) induced distension of the duodenum and upper part of the small intestine within 15 min and accumulation of mucoid but not bloody fluid. Moreover, the lowest dose of the toxin (50 μg/kg) induced a slight congestion during the first 2 h after injection, whereas higher doses provoked more severe congestion of the villous and submucosal vessels within 1 h. A marked edema in the lamina propria of villi and vacuolization of mucosal epithelial cells were observed in mice administered with a dose of 300 μg/kg. Furthermore, electron microscopy revealed three consecutive stages of injury: edema in the lamina propria of villi due to an increased capillary permeability, degeneration of absorptive epithelium of villi, and desquamation of the degenerated epithelium from the surface of villi. During these stages the crypts appeared only slightly affected (Terao et al. 1986). Also other studies in rodents after i.p. injection of OA or DTX-1 ($\geq$200 and 375 μg/kg) showed similar injuries at the intestinal mucosa within 15 min, mainly at the duodenum and upper jejunum (Terao et al. 1993; Ito and Terao 1994; Tubaro et al. 2003). At sublethal OA doses, these alterations were reversible, and the recovery process, observable already after 2 h, was complete within 24 h (Ito and Terao 1994).

Tissue changes induced by DTX-3 at the intestinal level are less pronounced and are limited to a dilation of the cisternae of the Golgi apparatus and cytoplasmic vesicles in the absorptive epithelial cells (Terao et al. 1993; Ito and Terao 1994).

Intraperitoneal injection of OA, DTX-1, or DTX-3 to mice or rats was shown to induce also alterations in the liver, visible as congestion, vacuolization, and/or necrosis of hepatocytes (Terao et al. 1993; Ito and Terao 1994; Aune et al. 1998; Tubaro et al. 2003).

OA was shown to induce liver injury also after *intravenous injection* to rats, which provoked congestion and dissolution of hepatic bile canalicular actin sheaths (Berven et al. 2001).

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Table 1 Lethal doses of OA and its analogues after single administration in mice

Toxin	Lethal doses (μg/kg)	Animals	Observation time	Reference
	Intraperitoneal injection			
Okadaic acid	$LD_{50} = 192$	Mice	Nor reported	Tachibana et al. – J Am Chem Soc 103: 2469, 1981
	$LD_{50} = 210$	HLA:(SW) BR female mice, 16–22 g	Nor reported	Dickey et al. – Toxicon 28: 371, 1990
	$LD_{50} = 225$	CD-1 female mice, 18–20 g	24 h	Tubaro et al. – Toxicon 41: 783, 2003
	$LD_{50} = 204$	CD-1 female mice, 19–22.5 g	24 h	Aune et al. – Toxicon 49: 1, 2007
DTX-1	LD = 160	Mice	24 h	Yasumoto & Murata – In *Marine Toxins. Origin, Structure, and Molecular Pharmacology*, Hall S & Strichartz G Eds, American Chemical Society, Washington DC 1990, pp 120–32
DTX-2	$LD_{50} = 352$	CD-1 female mice, 19–22.5 g	24 h	Aune et al. – Toxicon 49: 1, 2007
DTX-3	LD = 250–500	Mice	24 h	Yasumoto and Murata – In *Marine Toxins. Origin, Structure, and Molecular Pharmacology*, Hall S & Strichartz G Eds, American Chemical Society, Washington DC 1990, pp 120–32; Ito and Terao – Nat Toxins 2: 371, 1994; Yasumoto et al. – J AOAC Int 78: 574, 1995
7-*O*-palmitoyl-OA	MLD = 100	ddY male mice, 16–20 g	24 h	Yanagi et al. – Agric Biol Chem 53: 525, 1989
7-*O*-linoleoyl-OA	MLD = 100	ddY male mice, 16–20 g	24 h	Yanagi et al. – Agric Biol Chem 53: 525, 1989
7-*O*-DHA-OA	MLD = 10	ddY male mice, 16–20 g	24 h	Yanagi et al. – Agric Biol Chem 53: 525, 1989
	Oral administration			
Okadaic acid	LD = 400	ICR male mice, 4 weeks old	Nor reported	Ito et al. – Toxicon 40: 159, 2002
	$LD_{50} > 1000$ and <2,000	CD-1 female mice, 18–20 g	24 h	Tubaro et al. – Toxicon 41: 783, 2003
	LD = 575	Swiss female mice, 18–20 g	24 h	Le Hégarat et al. – Environ Toxicol 21: 55, 2006
	$LD_{50} = 880$	NMRI female mice, 18–22 g	24–30 h	Aune et al. – Toxicon 60: 895, 2012
DTX-1	LD = 100	ddY male mice, 20 ± 0.5 g	30 h	Ogino et al. – Nat Toxins 5: 255, 1997

LD lethal dose, *LD_{50}* median lethal dose, *MLD* minimum lethal dose, *7-O-DHA-OA* 7-*O*-docosahexaenoyl-okadaic acid

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After acute *oral administration*, OA, DTX-1, and DTX-3 were shown to be less toxic than intraperitoneally. Despite the wide variation in lethality data reported, OA appears to be two to fivefold less toxic by oral administration than by i.p. injection, and lethal oral doses in adult mice were recorded by different studies at 400 and 575 μg/kg (Ito et al. 2002; Le Hégarat et al. 2006). After OA administration by gavage, LD_{50} in mice was initially recorded in the range between 1,000 and 2,000 μg/kg (Tubaro et al. 2003), while recently LD_{50} and LD_{10} (lethal dose for 10 % of treated mice) values were determined at 880 and 780 μg/kg, respectively (Aune et al. 2012). Oral administration of DTX-1 to mice was lethal at 100 μg/kg (Table 1). In rats, oral administration of 1,000 μg OA/kg resulted lethal in 10 % of the treated animals (Berven et al. 2001).

The main clinical sign recorded after oral administration of OA, DTX-1, or DTX-3 was diarrhea. In adult mice, the diarrheic doses of OA recorded in the different studies appear to be variable: e.g., Matias et al. (1999) observed diarrhea after administration of 90 μg OA/kg, while no diarrhea had been observed after administration of higher toxin doses (≥600 μg/kg) by Aune et al. (2012).

To compare the diarrheic potency of OA and DTX-1, the toxins were orally administered to suckling mice, observing that each compound provoked diarrhea already at 0.05 mouse units (MU)/mouse, considering that 1 MU is the minimum toxin dose necessary to kill at least 2/3 mice in 24 h and corresponds to 4 μg for OA and 3.2 μg for DTX-1. Similarly, the diarrheic potency of 7-*O*-acyl-OA esters in suckling mice is comparable to that of OA (Hamano et al. 1986; Yanagi et al. 1989).

In adult mice or rats, oral administration of OA (130–4,000 μg/kg), DTX-1 (750 μg/kg), or DTX-3 (μg/kg) was shown to induce dilation and increased fluid content in the intestinal lumen. The morphological toxicity signs after oral OA, DTX-1, and also DTX-3 administration were similar to those observed after i.p. injection of OA or DTX-1. In mice and rats, a degeneration and desquamation of the absorptive cells from the lamina propria of villi was observed within 1 h after the toxin administration; while the cells in the crypts were unchanged for 24 h, regeneration of the mucosa started within 2 h and was completed after 24–48 h (Terao et al. 1993; Ito and Terao 1994; Ito et al. 2000; Berven et al. 2001; Wang et al. 2012). After OA administration in mice (750 μg/kg), a significant reduction of the protein phosphatase activity was also recorded in the small intestine, but it recovered to normal levels within 6–24 h. In addition, proteomic analysis revealed an altered expression of different proteins involved in macromolecular metabolism, cytoskeleton reorganization, signal transduction, molecular chaperoning, and oxidative stress, which suggests that OA effects in the mouse intestine are very complex and involve multiple biological processes (Wang et al. 2012).

Other studies in rats administered with OA revealed slight structural alterations also at the colon, as indicated by some regenerative changes (fewer goblet cells and distinct nuclei in epithelial cells) noted 24–48 h after the administration (Ito et al. 2000). On the contrary, after oral administration of OA or DTX-1 to mice (2–10 μg/mouse of 6 weeks age), Yuasa et al. (1994) recorded edema or acute inflammation in the squamous mucosa of the esophagus and forestomach as well as erosion of the fundic mucosa, but no significant tissue alterations were observed in other parts of the gastrointestinal tract. Anyway, these toxins increased cell proliferation along the gastrointestinal tract and at skin level, while no effect at the liver and kidney was noted within 36 h. An increased cell proliferation at the intestinal level of mice was observed also by Le Hégarat et al. (2006) at 24 h or 36 h from OA oral administration (115–525 μg/kg), which was associated with increased micronuclei in the gut cells (525 μg/kg). A dose-dependent increase in cell proliferation in the digestive tract was observed also in rats (1–50 μg/rat of 8 weeks of age) orally administered with OA or DTX-1 (Yuasa et al. 1994).

Some studies revealed that single oral administration of diarrheic toxins induces tissue damages also at the gastric, hepatic, and kidney levels. OA administration to mice (1,000 μg/kg) induced lesions at the forestomach of some mice, such as vacuolar degeneration of the epithelium associated with acute submucosal inflammation and less frequently also reactive hyperplasia of the keratinized epithelium

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(Tubaro et al. 2003). DTX-1 (750 μg/kg) and DTX-3 (750 μg/kg) caused degeneration of surface cells of the gastric mucosa of mice and rats (Terao et al. 1993).

Hepatotoxic effects were also observed after oral administration of these toxins, but the findings are sometimes contradictory, probably due to the differences in the experimental conditions. After oral administration of DTX-3 (750 μg/kg) to mice, Terao et al. (1993) recorded a marked presence of fat droplets and foci of necrosis in the midzonal and periportal regions of hepatic lobules, but no liver changes were noted after oral administration of the same dose of OA or DTX-1. Similarly, OA administration to rats (1,000–4,000 μg/kg) did not induce liver injuries (Berven et al. 2001). On the contrary, liver damage was observed histologically (degenerative changes of hepatocytes) and conformed by a marked increase in plasma transaminase levels, after oral administration of 1,000 and 2,000 μg/kg of OA to mice (Tubaro et al. 2003). Moreover, Vieira et al. (2013) has recently reported hepatic lesions after oral administration of OA to mice (750 μg/kg), visible as random multifocal aggregates of necrotic hepatocytes, with dilation and congestion of sinusoids, while neighboring hepatocytes showed swelling, lipid vacuoles, and either pleomorphic or pyknotic nuclei. Scant polymorphonuclear inflammatory infiltrates as well as an altered gene expression of hepatic antioxidant and detoxifying enzymes were also recorded. In addition, tissue changes were noted in the kidneys as vacuolation of the cytoplasm of tubular epithelial cells and nuclear changes pointing out necrosis of the tubular epithelium (Vieira et al. 2013).

Le Hégarat et al. (2006) observed that oral administration of OA to mice (115–230 μg/kg) induced also apoptotic breaks in DNA in the ileum, liver, and kidneys.

Recently, a study *after single oral coadministration* of OA and azaspiracid-1, an algal toxin affecting the gastrointestinal tract and causing diarrhea in humans, had been carried out in mice. Coadministration of OA (780 or 880 μg/kg, the LD_{10} and LD_{50} of OA in the same study) and azaspiracid-1 (570 μg/kg, corresponding to its LD_{10} in the same study) did not result in an additive or synergistic effect for lethality or pathological effects, which were restricted to the gastrointestinal tract. However, after the toxin coadministration, the absorption of OA was slightly reduced (Aune et al. 2012).

After *intravenous injection* of OA to rats, Berven et al. (2001) observed that doses ranging from 50 to 500 μg/kg induced low effects at the intestinal level but caused liver damages. Lethality was recorded within few hours at doses $\geq$0.2 μg/kg; necropsy and histological analysis showed a marked congestion in the liver with a dissolution of hepatic bile canalicular actin sheaths, not associated with altered microtubular or keratin network. Moreover, no apoptotic DNA fragmentation was noted in hepatocytes.

Diarrheic toxin *injection in ligated intestinal loops* of rats and mice was also performed to evaluate their ability to induce intestinal hypersecretion, at the basis of their diarrheic effect. As an example, injection of OA in rat intestinal loops (0.5–5 μg/loop) was shown to induce a rapid fluid accumulation, which peaked within 2 h. Swelling of the epithelial cells at the top of villi and their detachment from the basal membrane was observed within 15 min after OA injection and after 90 min most of the villi enterocytes shed into the lumen, while large parts of the flattened villi were covered by goblet cells, which were not altered by the toxin (Edebo et al. 1988).

After *intracerebroventricular injection* in rats, OA and its analogues have been studied for their effects on the central nervous system. Injection of nanograms of OA into the brain of rats causes neuronal cell death and memory impairment, as reviewed by Kamat et al. (2013) and Munday (2013). OA or DTX-1 (300 ng) caused hyperexcitability in rats, and epileptiform electroencephalogram (EEG) discharges were observed until 4–6 h. In contrast, DTX-1 (300 ng) induced EEG discharges with a longer latency and lower frequency than those recorded after OA injection, but it did not cause neurodegenerative effects (Arias et al. 2002). The neuronal damage induced by OA was accompanied by hyperphosphorylation of tau protein and increased formation of neurofibrillary tangles and deposits of β-amyloid, even though these effects had not been evidenced by some studies (Kamat et al. 2013; Munday 2013).

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Repeated Administration

OA, daily administered by gavage to mice (185 μg/kg/day, for 7 days), did not induce diarrhea, while light microscopy evidenced epithelial hyperplasia of the forestomach and slight focal subacute inflammation of its submucosa only in 1/5 mice. Ultrastructural analysis of the heart revealed only occasional mitochondrial assemblage and dilated sarcomeres in cardiomyocytes (Sosa et al. 2013). In the same experimental conditions, increasing OA dose to 1,000 μg/kg/day, the toxin provoked diarrhea within 30 min after each daily administration, together with reduced food consumption and body weight and the death of 2/5 mice at the 5th day of treatment. Toxic effects were noted mainly in the forestomach as ulcerated and/or hyperplasic mucosa and acute/subacute inflammation of the submucosa. Anyway, considering that the forestomach is typical of rodents and its lesions are probably "species-specific," OA effects at this level are not predictive for toxicity in humans. OA induced injuries also at the hepatic level, evidenced as atrophic signs of hepatocytes and by the increased plasma levels of transaminases. The effects on the liver appeared of lower entity than those recorded after single oral OA administration (Tubaro et al. 2003), probably due to an activation of regenerative mechanisms and/or detoxifying hepatic processes activated during the repeated exposure to the toxin. Other tissue changes were observed in the lymphoid organs, pancreas, and fatty tissues as atrophic signs, tentatively consequent to the body weight loss of mice. Electron microscopy analysis showed also a package of rounded mitochondria and fiber alterations in cardiomyocytes, not accompanied by DNA break indices of apoptotic changes. Contrary to observations after single oral OA administration, no degenerative changes were noted in the small intestine, probably due to the activation of resistance to OA at this level and/or to activation of regenerative processes, such as those noted after single oral exposure to the toxin (Tubaro et al. 2004).

The effects of *repeated oral co-exposure* of OA and yessotoxin, a dinoflagellate polyether often detected in edible shellfish together with OA, were studied in mice after daily administration, for 7 days. Oral exposure to a combination of fixed doses of OA and yessotoxin (185 and 1,000 μg/kg/day, respectively) did not induce lethal effects, signs of toxicity, diarrhea, or hematological changes. Light microscopy revealed changes at the gastric level (multifocal subacute inflammation, erosions, and epithelial hyperplasia) in 2/5 mice coadministered with both the toxins, while ultrastructural analysis of the heart showed some cardiomyocytes with "loose packing" of myofibrils and aggregated rounded mitochondria. Moreover, the combined oral doses of the two toxins did not exert cumulative or additive toxic effects in mice, in comparison to the single toxins (Sosa et al. 2013).

Repeated oral administrations and topical skin application of OA or DTX-1 were performed to evaluate the tumor-promoting activity of these toxins in rats and mice. The observed effects will be described in the section dedicated to tumor promotion.

In other studies, OA was administered to rats by a *chronic intracerebroventricular infusion* for up to 4 months (70 ng/day), observing severe memory impairment, changes in gray and white matter areas typical of Alzheimer's disease, and apoptotic cell death (Kamat et al. 2013).

Mutagenicity and Genotoxicity

OA was not mutagenic in *Salmonella typhimurium* in the absence or in presence of metabolic activation, but its mutagenic effects were evidenced in different eukaryotic cells in vitro (Aune and Yndestad 1993; Valdiglesias et al. 2013). No genotoxicity data are available for DTX-2 and DTX-3. Genotoxic effects of OA include different alterations of DNA, such as micronuclei formation, sister chromatid exchanges, minisatellite mutations, 8-hydroxy-deoxyguanine adduct formation, and DNA strand breaks, but the mechanism of genotoxicity has not been completely clarified. Some studies carried out in different cell types evidenced its aneugenic potential, whereas some authors support double-strand breaks as mechanism of genotoxicity. Other studies indicate that OA must be metabolically activated to exert its genotoxic effects, while other studies showed a direct toxin genotoxicity. It can be hypothesized that these different

results might be related to a highly dependent genotoxic effect, cell type, and experimental conditions. The genotoxicity of OA is not only related to a direct damage of the genetic material, but it can be due also to indirect effects, such as the alteration of the repair of DNA damage induced by other genotoxic compounds (Valdiglesias et al. 2013).

The in vivo significance of the genotoxic in vitro effects is unclear and has not been investigated. Moreover, despite its genotoxic effects, OA is not classified by the International Agency for Research on Cancer (IARC) as a potential carcinogen.

Tumor Promotion

The tumor promotion effects of OA and DTX-1 had been evidenced by two-stage carcinogenesis studies in mouse skin (Fujiki and Suganuma 1993) and rat glandular stomach (Suganuma et al. 1992). The tumor promotion of OA in the glandular stomach of rats was studied after initiation with MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) and administration of the toxin in drinking water (10 μg/rat/day, during weeks 9–55 of the experiment, and 20 μg/rat/day, during weeks 56–72). OA administration significantly increased the incidence of neoplastic formations (adenomatous hyperplasias and adenocarcinomas) in the glandular stomach (Suganuma et al. 1992), after the increased dose of OA. At skin level, 1 week after initiation with a single cutaneous application of DMBA (7,12-dimethylbenz[α]anthracene), OA or DTX-1 (1 μg) was applied twice a week, for 30 weeks. A high percentage of tumor-bearing mice and a high average number of tumors (carcinomas and papillomas) per mouse were recorded (Fujiki and Suganuma 1993).

Treatment

The treatment of the diarrheic poisoning in humans is symptomatic and supportive for the short-term diarrhea and loss of fluids and electrolytes. In general, hospitalization is not necessary since the loss of fluid and electrolytes can be replaced orally. Other diarrheic illnesses associated with shellfish consumption such as bacterial or viral contamination should be ruled out on the basis of anamnesis of the patients (Aune and Yndestad 1993).

Conclusion and Future Directions

OA and DTXs are polyether marine toxins that can contaminate edible filter-feeding shellfish and induce diarrhetic shellfish poisoning in humans. These compounds are inhibitors of protein phosphatases, a mechanism at the basis of their diarrheic effect as well as of their tumor promotion activity. The current European regulation (Regulation (EC) No. 853/2004, 29 April 2004) on the level of diarrheic toxins in shellfish for human consumption focuses on prevention of the gastrointestinal symptoms. The regulation establishes a maximum admitted level of 160 μg of OA equivalent per kg shellfish edible parts (European Commission 2004), whereas the European Food Safety Authority proposed to decrease this level to 45 μg OA equivalents/kg (EFSA 2008). The risk of acute effects in humans due to ingestion of seafood contaminated by diarrheic toxins is managed by monitoring shellfish for toxicity: frequency of sampling and analysis of edible shellfish should ensure that the toxin content does not rise to dangerous levels in temporal or spatial gaps between sampling times or locations. Moreover, monitoring the potentially toxic dinoflagellate species in the water column and the toxin content in algal cells allows to detect a toxic bloom earlier than shellfish contamination, protecting public health and minimizing negative economic impacts to the shellfish industry.

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Various methods have been developed for diarrheic toxin analysis. The mouse bioassay has been the reference and most commonly used analysis method to detect these lipophilic toxins in shellfish. However, this technique suffers from low accuracy, specificity, and ethical problems due to the animal experimentation. Thus, chemical, functional, and immunological methods have been developed to assess the presence of diarrheic toxins in seafood. Immunoassays include enzyme-linked assays (ELISA kits are commercially available) or radioactivity-based immunoassays and lateral flow immunochromatography (LFIC) tests, which use visual end points and are now commercialized. Protein phosphatase 2A inhibition assays, based on the mechanism of OA action, are sensitive in vitro functional assays currently commercialized as kits. Chemical methods of analysis include high-performance liquid chromatography (HPLC), capillary electrophoresis, and liquid chromatography-mass spectrometry (LC-MS) techniques. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been validated and approved by the European regulation as the new official control method for lipophilic toxins in shellfish (Sassolas et al. 2013). Alternative or complementary methods can be used when properly validated, but there are inherent advantages in using LC-MS/MS to monitor OA and its analogues since other classes of regulated toxins can be detected at once.

Cross-References

► Azaspiracid Toxins: Toxicological Profile
► Microcystins: Toxicological Profile

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Marine and Freshwater Toxins
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Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Short Review

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Abstract

Polypeptide and low molecular weight toxins are widely found in marine invertebrates and vertebrates. These dangerous toxins cause painful and sometimes lethal stings. Due to their properties, some are of interest for application in medicine and as excellent biochemical tools in nerve-conduction studies and the discovery of new molecular targets for pharmacology.

Introduction

Some marine invertebrate species, even some as large as phylum, are known for their toxicity. As a rule, they contain toxin-producing cells or glands where, in the majority of cases, toxic polypeptide compounds are produced and encapsulated. In addition, some invertebrates have the ability to accumulate toxins in their intestines and skin from a diet contaminated by microbial toxins, as mentioned in the another review in this Handbook (Stonik and Stonik). Finally, invertebrates sometimes use low molecular weight deterrents and natural toxic products from other invertebrate species upon which they feed.

Toxins of Marine Invertebrates

Cnidaria

The Cnidarian phylum – which combines the corresponding Hydrozoa, Scyphozoa, Cubozoa, and Anthozoa classes with the most ancient metazoans – includes many organisms belonging to various venomous animals. As with other cnidarians, animals of the subphylum Medusozoa (first three classes) have specialized cell nematocysts in tentacles and other body parts. Organelles nematocysts within these cells contain toxic proteins and low molecular weight compounds in rigid proteinaceous capsules, with a long, spiny tubule for each nematocyst. When a nematocyst is stimulated by its sensory receptor (cnidocil), it discharges quickly; toxins from nematocysts of jellyfish tentacles infiltrate into tissues of prey, predators, and sometimes humans.

Jellyfish venom (medusas) is usually a mixture of proteins that are mainly cytolytic and cardiotoxic components. Cytolytic cnidarian proteins form pores in biomembranes of target cells, and in the majority of jellyfish, protein toxins are high molecular weight compounds (20,000–100,000 Da). They are often labile, and structure investigation is therefore difficult.

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It is known that LD_{50} values (intraperitoneal administration in mice) of the most potent toxins from jellyfish range between 30 and 300 $\mu g/kg^{-1}$ (Brinkman and Burnell 2009; Nagai et al. 2000).

Many people on beaches in Australia, the Philippines, the Maldive Islands, and other countries get stung by tentacles of the cubomedusan *Chironex fleckeri,* the venom of which contains several polypeptide toxins that induce very potent action on human skin and central nervous system (CNS). These toxins cause painful stings and sometimes endanger human life. Symptoms of envenomation include severe pain, acute inflammation, dermonecrosis, hyper- or hypotension, shock, cardiac dysfunction, etc. Exasperation of these symptoms may proceed extremely rapidly, and in severe cases, death can occur within minutes as a consequence of pulmonary and cardiac failure.

Thus far, 61 proteins have been identified in the nematocysts of *C. fleckeri*, including toxins and proteins important for nematocyte development. The most frequently identified toxins are related to other potent cnidarian toxins and are associated with cytolytic, dermonecrotic, and lethal properties of *C. fleckeri* venom. Many of these toxins have been posttranslationally modified by glycosylation (Brinkman et al. 2012). Toxins-1 (CfTX-1) and −2 (CfTX-2), containing 436 and 445 amino acid residues, respectively, share a significant homology with other known toxins from jellyfishes, such as toxins from *Chiropsalmus quadrigatus*, *Carybdea rastoni,* and *Carybdea alata.* Compounds from Cubomedusae are lethal and demonstrate hemolytic properties and contain transmembrane-spanning fragments that may be responsible for the pore-forming mechanism of their action (Brinkman and Burnell 2007, 2009).

Another group of dangerous jellyfish is the small carybdeid jellyfish, such as the Australian *Carukia barnesi,* which cause minor stings resulting in muscle pains, especially in the lower back, as well as vomiting, agitation, vasoconstriction, and hypertension, followed in cases of severe human envenomation by acute heart failure (Irukandji syndrome). Molecular mechanism of action of this toxin includes neuronal sodium channel modulation, leading to release of catecholamines. Heart disfunction is associated with pore formation in myocardial cellular membranes due to troponin leakage (Tibballs et al. 2012).

Physalia physalis (Portuguese man-of-war), a jellyfish-like colonial animal belonging to the family Physaliidae from the class Hydrozoa, is a dangerous cnidarian with tentacles that deliver powerful stings. A colony consists of many minute individual polyps (zooids) of four different types attached to each other by connective tissue. One type of polyps (dactylozooids) forms tentacles ∼10–20 m in length but that sometimes can be up to 30–50 m. Another type of polyp, the pneumatophore, is a gas-filled member of the colony known as a sail, which swims on the sea surface. Two other polyp types are used for feeding and reproduction. The animal is responsible for many thousands of human stings annually in Australia and tropical countries of the Pacific, Indian, and Atlantic oceans. In warm seasons, these animals are also distributed in subtropical and even temperate waters, including the northern Atlantic. Stings cause severe pain and often lead to allergic reactions and, rarely, fever, shock, heart and lung problems, and death. Nematocysts of *P. physalis* contain a mixture of enzymes, cytolytic polypeptides, and a neurotoxin almost as powerful as cobra venom.

Sea anemones (Anthozoa, Actiniaria) are also well known as toxic invertebrates. Numerous groups of sea anemone polypeptide toxins, namely neurotoxins, cytolytic polypeptides, enzymes, and other proteins were isolated from different sea anemones (Honma and Shiomi 2006; Norton 2009; Frazao et al. 2012). Among their neurotoxins are ∼40 polypeptides with molecular weights ranging between 3.5 and 6.5 kDa with three disulfide bonds. Typically, they are 46–49 amino acid residues long. As with other neurotoxins from different sources, these neurotoxins are capable of binding voltage-gated sodium channels. Type 1 neurotoxins interact with extracellular sites of the sodium channel, delaying their inactivation at signal transduction (Norton 2009; Wanke et al. 2009) Sometimes they strongly stimulate mammalian cardiac muscle contraction (Yasunobu et al. 1985).

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Another subgroup (type II) contains shorter amino acid sequences (27–32 amino acid residues). Their biological action is also characterized by deceleration of sodium-channel inactivation. Sea anemones produce a variety of potent modulators of voltage-dependent sodium channels that are able to paralyze and kill prey and are used by their producers for hunting. LD_{50} values of neurotoxins range between 1 and 100 μg/kg (in mice).

Sea anemone neurotoxins that interact with potassium ion channels have also been identified. These polypeptides contain 35–37 amino acid units (type I), either 58–59 residues (type II) or 41–42 amino acids (type III), and all have three disulfide bonds. Finally, toxins with 28 amino acid units and two disulfide bonds (type IV) have also been identified. Peptides in group I demonstrate pore-blocking effects on Kv-1 channels. Long (58–59 amino acids) toxins possess both potassium channel inhibitory and enzymatic activities. Medium size (42–43 amino acids) sea anemone toxins affecting potassium channels modulate the gating function of these channels and alter their voltage-dependent properties. Some toxins of this type may be considered as prospects and useful biochemical tools for the diagnosis and treatment of autoimmune diseases (Lazdunski et al. 1998; Diochot and Lazdunski 2009).

Recently, toxins specifically interacting with neuronal voltage-insensitive cationic channels activated by extracellular protons [acid-sensing ion channels (ASICs)] were also identified in sea anemones: for example, the toxin APET × 2 from the sea anemone *Anthopleura elegantissima* (Diochot et al. 2004). Administration of APET × 2 into the gastrocnemius muscle blocked the development of acid-induced and inflammatory pain and hypersensitivity in rats (Spencer et al. 2010).

Specific tertiary structures and conformational flexibility of sea anemone toxins play an important role in their receptor-binding ability. These and other cnidarians contain toxins not only in nematocysts but also in some other cells. Toxins are used mainly for prey deterrence and prevention against predator attacks.

Another class of sea anemone toxins is the cytolysins, which disturb membrane permeability in target cells followed by pore formation and cell lysis (Honma and Shiomi 2006; Norton 2009; Frazao et al. 2012). Some cytolysins from sea anemones combine to the comparatively low molecular weight peptide toxins (~5–6 kDa), whereas another group belonging to type II consists of actinoporin polypeptides with a molecular weight ~20 kDa. Sphingomyelin proved to be an actinoporin receptor. In a multistep process, pore formation includes recognition of sphingomyelin in membrane, binding to the membrane with participation of the N-terminal region of toxin and oligomerization of three or four toxin molecules to form transmembrane pores by their amphipathic alpha helices (Kristian et al. 2009).

As an example of their structural study following the isolation of new actinoporins from the sea anemone *Heteractis crispa* (= *Radianthus macrodactylus*), determination of their primary structures, exact molecular masses, and sequencing genes encoding these polypeptides may be mentioned (Monostyrnaya et al. 2010; Tkacheva et al. 2011). Actinoporin RTX-A from the same sea anemone shows extremely potent cytotoxic activities against JB6 P^+ C l41 cells and HeLa, THP-1, MDA-MB-231, and SNU-C4 human tumor cell lines (IC_{50} = 0.57, 2.26, 1.11, 30.0, and 4.66 nM, respectively). It is of special interest that the toxin inhibits malignant transformation of mouse JB6 P^+ C l41 cells stimulated by epidermal growth factor (EGF) in soft agar, with the inhibition of a number of cell colonies ($INCC_{50}$) at a dose as extremely small as 0.034 nM (Fedorov et al. 2010).

Cytolysins of types III and IV have molecular masses ~30–40 and 80 kDa, respectively. They are represented to date only by cytotoxins from the genus *Urticina* and from *Metridium senile,* respectively. In many cases, cholesterol proved to be another receptor following the action of some cytolysins in addition to sphingomyelin.

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Toxins with Kunitz-type protease inhibitory activity and phospholipase A_2 activity were also discovered in sea anemones. First, analgesic peptide compounds, inhibitors of vanilloid receptor 1 (TRPV-1), was recently isolated from *Heteractis crispa* (Andreev et al. 2008). Polyfunctionality is probably a characteristic property of many sea anemone toxins. Recently, bifunctional polypeptides, such as APEKT × 1 from *Anthopleura elegantissima*, with Kunitz-type protease and potassium-channel-inhibiting properties, were found (Peigneur et al. 2011).

On the whole, sea anemone toxic polypeptide mixtures are very complicated. Indeed, a mixture from 156 peptide compounds was described in a single-species venom (Rodriguez et al. 2012). Thus, the diversity of polypeptide toxin is extremely great in sea anemones. In addition, numerous non-proteinaceous substances, such as purines, quaternary ammonium compounds, betaines, etc., were found along with peptide toxins in sea anemones.

Porifera

Sponges are marine invertebrates belonging to the phylum Porifera, which is characterized by very impressive diversity of secondary metabolites. Many sponge species are indeed symbiotic systems, composed of host cells and bacterial or microalgal symbionts. Although these unmovable animals should contain a variety of different deterrent compounds to prevent against predators, highly toxic compounds have rarely been found in sponges. As an example, the isolation of okadaic acid from the sponge *Halichodria okadai* might be referred to. Similarly, other highly toxic metabolites of microalgal and cyanobacterial origins were also found in sponges.

Probably, some sponge polymers play a chemopreventive role in sponges. Suberitine, the blue toxic 7–8 kDa protein with the potent hemolytic activity was isolated from the sponge *Suberites domuncula* by Carrielo and Zanetti (1979). This protein is capable of a noncovalent interaction with retinal, formed in the sponge in result of enzymatic transformation of carotenoids from symbiotic sponge-associated microorganisms at the action of host-encoded, light-inducible dioxygenase. As a result, hemolytic activity is reduced.

Pyridinium sponge toxin preparations, such as halitoxin, from several marine sponges belonging to the genus *Haliclona*; and amphitoxin, from the Jamaican sponge *Amphimedon compressa*, contain pyridinium rings connected to each other by hydrocarbon chains (Fig. 1). They have the ability to form transient pores/lesions in cell membranes and to cause a collapse in membrane potential. These polymers are toxic to fish and mice (Tucker et al. 2003; Thompson et al. 2010).

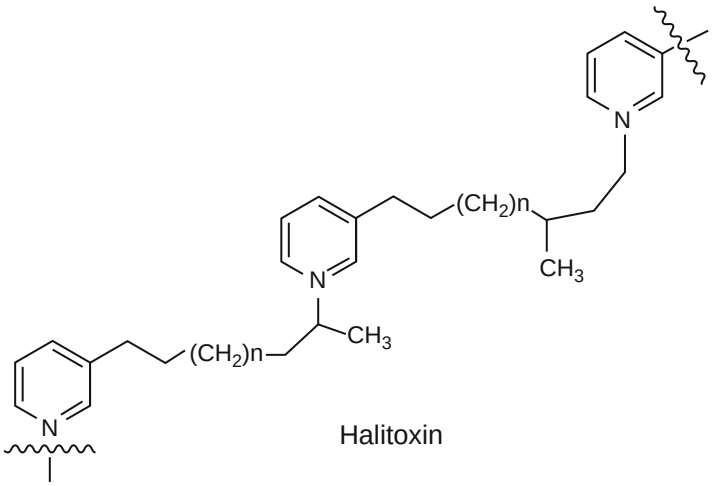

Fig. 1 Structure of halitoxin

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Mollusca

Peptide toxins from mollusks (sea snails) belonging to the family Conidae (class Gastropoda), have been attracting attention for many years due to their structure and properties. This family comprises predatory animals that feed on fish, marine worms, and other mollusks. Some species specialized in fish hunting, for example *Conus geographicus*, are dangerous for people collecting mollusks on coral reefs (Bingham et al. 2012): 70 % of human sting cases are fatal. Several dozens of people have been victimized by this and other *Conus* venoms (Hao et al. 2012).

All mollusks of this family have poison glands connected by a duct with a harpoon used for predation. More than 400 species of these mollusks have thus far been identified, but it is believed that there are as many as 700 species of this family.

Conopeptides with several disulfide bonds are conventionally referred to as conotoxins. Conotoxins are low molecular weight peptides, comprise only 12–25 amino acid units, and have from four to ten cysteine residues and two to five disulfide bonds. Usually, conotoxins contain posttranslationally modified amino acids, such as hydroxyproline, glycosylated serine and threonine, 6-bromotryptophan, and others. D-amino acids were also found in amino acid compositions of conotoxins (Terlau and Olivera 2004).

It is known that each species contains a characteristic set of peptide components (at least 100 different conopeptides) in its venom gland, although the same compound in different species is rarely found. Therefore, there is a great diversity of conopeptides and conotoxins in these mollusks, producing ~100,000 distinct conopeptides, a majority of which have not yet been identified (Olivera et al. 1990; Han et al. 2008).

Several superfamilies of conotoxins have been identified on the basis of number of cysteine residues and their distribution along the polypeptide chain. These superfamilies have names such as A, I, M, O, P, S, T. For instance, representatives of the superfamily M contain six cysteine residues, which are distributed in accordance with the scheme CC-C-C-CC, where the «C» is a cysteine residue, and «-» means that one or several residues of other amino acids are located between cysteine residues. The O superfamily comprises conotoxins with the C-C-CC-C-C motif. In each superfamily, groups of conotoxins are designated as families using Greek letters: For example, the O superfamily is divided into the δ, μ, κ, and ω families, which differ from each other in their ability to bind different molecular targets: Conotoxins of the δ and μ families interact with sodium channels; those of the α family with acetylcholine receptors, and those of the κ and ω families with potassium and calcium channels, respectively (Han et al. 2008; Terlau and Olivera 2004).

It is particularly important that individual conotoxins exhibit a high specificity in their interaction with ionic channels or membrane receptors. They not only recognize one or another type of molecular target but can also “select” one specific subtype or isoforms among them. This makes conotoxins useful tools for studying neurophysiology or molecular mechanisms connected with the conduction of nerve impulses.

More than two decades of studies on cone-snail venom peptides has led to several compounds that have reached human clinical trials, mainly for the treatment of pain. Remarkably, none of the conopeptides mediate analgesia through opioid receptors, as do many known analgesics, but interact with other molecular targets (Han et al. 2008).

As result of long-term studies, the US Food and Drug Administration (FDA) approved a synthetic equivalent of the naturally occurring conotoxin, MVIIA, as the pharmaceutical drug ziconotide (Prialt^{R}, Elan Pharmaceuticals, Inc.). This compound was first obtained from the Pacific mollusk *Conus magus* (Ramilio et al. 1992; Chung et al. 1995). It is a linear peptide consisting of 25 amino acid residues and having three disulfide bonds (Fig. 2). Due to its spatial structure, ziconotide specifically blocks the function of neuron-specific voltage-sensible calcium channels (NVSCC) and

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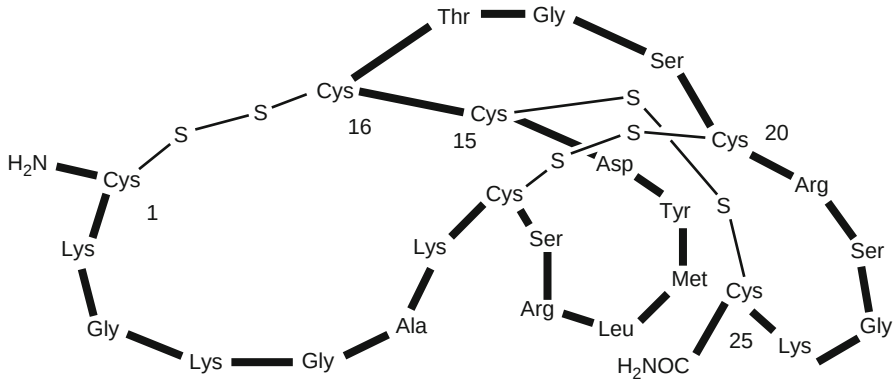

Fig. 2 Ziconotide

pain signal transmission. It is the first "topically" active analgesic for managing chronic intractable pain (Klotz 2006). It is ~1,000 times more effective than morphine but is not addictive and has no hallucinogenic effect (Kaas et al. 2012a; Williams et al. 2008).

Some conopeptides studied to date in animal models exhibited antinociceptive, antiepileptic, neuroprotective, or cardioprotective activities. Screening results also suggest potential applications of conotoxins in cancer and psychiatric disorders. Additional important applications of conotoxin research are the discovery of new therapeutic targets and novel binding sites on already validated molecular targets. In spite of the structural and functional diversity of conotoxins being sufficiently investigated, *Conus* venoms continue to be excellent sources for discovery of new, highly active compounds and, potentially, new therapeutics.

The database ConoServer (http://www.conoserver.org) specializing in sequences and structures of conotoxins expressed by marine cone snails provides not only structural data but also information on the ability of these toxins to bind target receptors, channels, and transporters (Kaas et al. 2012b).

Echinodermata

The phylum Echinodermata (echinodrems) consists of five now-living classes: Echinoidea (sea urchins), Holothurioidea (sea cucumbers or holothurians), Asteroidea (starfish), Crinoidea (sea lilies), and Ophiuroidea (brittle stars). Holothurioidea and Asteroidea are known by the glycosidic nature of their toxins. Holothurians are represented for the most part by soft-bodied, slow-moving animals. However, they are successful survivors that belong to one of the most numerous benthic animals, in particular at great depths. Some holothurians belonging to the order Aspidochirotida and have specialized glands (the Cuvierian organ) attached to the cloak. The Cuvierian organ is a system of sticky tubules containing large quantities of triterpene glycosides, which are highly toxic to fish. When in danger, these animals expel their tubules through the anus. Toxic glycosides cause hemolysis in gill capillaries and repel predatory fish. In marine aquaria, this brings about immediate death to the predators. Although many holothurians do not have Cuvierian organs, they do contain hemolytic glycosides in their body walls.

More than 200 structure variants of triterpene glycosides have been identified in different species of sea cucumbers, and the majority of them were discovered by Russian scientists (Fig. 3). Many glycosides contain lanostane aglycones with 18(20)-lactone fragment (the holostane series of these toxins). However, compounds without this fragment and those containing shortened side chains were also isolated. Triterpene glycosides were found in the majority of the ~60 chemically studied

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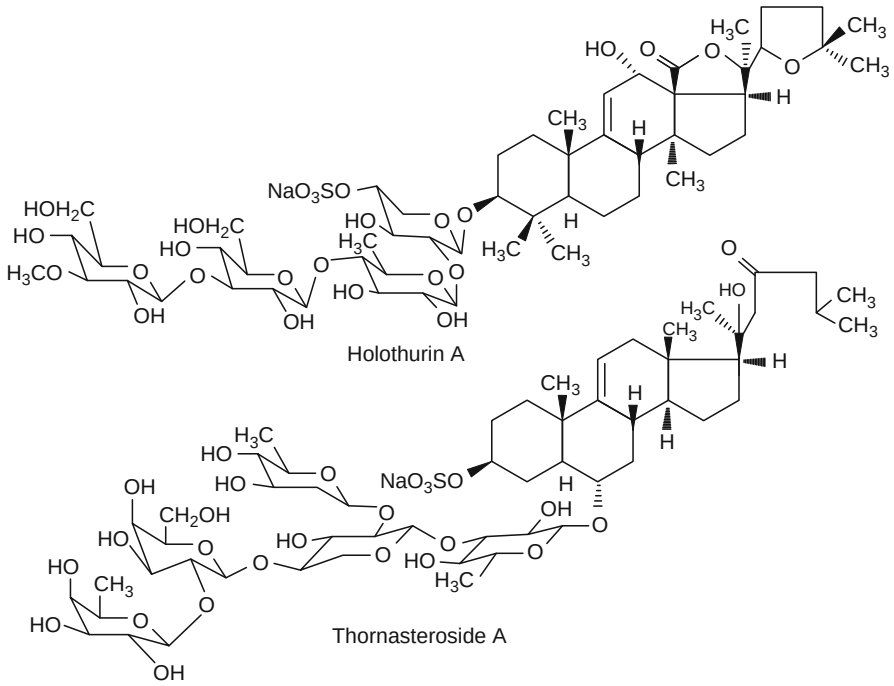

Fig. 3 Some glycoside toxins from echinoderms

sea cucumber species. Their carbohydrate moieties include from two to six monosaccharide units and frequently are sulfated (up to three sulfate groups per molecule) (Stonik et al. 1999; Kalinin et al. 2005).

Holothurin A from *Holothuria vagabunda* was one of the first marine ichthyotoxins studied. Its LD_{50} for mice is 750 $\mu g/kg^{-1}$ when administrated intravenously but only 400 mg/kg^{-1} on peroral application. Strong membranolytic action and hemolytic activity of holothurins and related compounds is a result of the formation of short-living ion-conduction channels and larger pores in target-cell membranes as consequence of the formation of complexes of sea cucumber glycosides with membrane sterols (Kalinin et al. 2008).

Thus, triterpene glycosides from sea cucumbers are very potent hemolysins and membranolytics and are much more active in comparison with triterpene saponins from higher terrestrial plants. On the other hand, these toxins – which also demonstrate antifungal and antitumor properties – are less toxic following peroral administration. Inhabitants of some Pacific coral islands long ago began using holothurians to stun fish in small lagoons, squeezing their glycoside-containing intestines into the seawater. Some sea cucumber species – trepangs – are edible and used in commercial fishery. After removal of the majority of toxins and appropriate cooking, these animals are considered a delicacy and a healthy food.

Representatives of another echinoderm class, Asteroidea, Echinodermata, use a similar strategy of chemical defense against predators. They produce cytolytic toxins primarily in their outer tissues, which are referred to as asterosaponins (Kicha et al. 2001). In their chemical nature, they are also glycosides, being sterol oligoglycosides rather than triterpene glycosides (Stonik 2001; Ivanchina et al. 2011). Asterosaponins – for instance, thornasteroside A from the starfish *Acanthaster planci* (known also as crown of thorns) – are weaker hemolytic agents in comparison with sea cucumber triterpene glycosides, but they are sufficiently toxic against many other marine invertebrates and fish.

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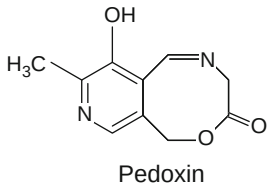

Fig. 4 Structure of pedoxin

Toxic polypeptides are also found in some echinoderms, mainly in their spines, and pedicellariae – pincers-like formations located between spines, especially in starfish and sea urchins. Two lethal protein factors – plancitoxins I and II, both with LD_{50} values of 140 μg/kg^{-1} (intravenous administration in mice) – were isolated from spines of the starfish *Acanthaster planci*. Plancitoxins consist of two subunits, α- (10 kDa) and β- (27 kDa), bridged by a disulfide bond. These toxins show some homology with mammalian deoxyribonucleases II; one of them, plancitoxin I, also exhibited DNA-degrading activity (Shiomi et al. 2004).

Several species of echinoids (Echinoidea) are dangerous to humans, causing toxicosis by stings from either pedicellariae or spines. For example, some species of sea urchins contain potent peptide toxins in their globiferous pedicellariae, particularly sea urchins belonging to the genus *Toxopneustes*. Peditoxin, cytochrome-b-like apoprotein from pedicellariae of *T. pileolus* (∼10 kDa), is itself nontoxic, but the purified holoprotein is extremely toxic (LD_{50} 70 μg/kg^{-1}), causing anaphylaxis-like shock and death in experimental animals. Small amounts of the prosthetic group added to the holoprotein enhanced its toxicity. The prosthetic group of this apoprotein proved to be 3-hydroxy-2-methyl-5-methoxy-4-pyridineformyl-glyciliden ester (pedoxin) (Fig. 4). A high concentration of glycine was found in the blood of humans following stings from pedicellariae of this sea urchin (Ruwabara 1984).

The venoms of pedicellariae of these animals contain also lectins. Two D-galactose-specific lectins [SUL-I (∼32 kDa) and SUL-II (∼29 kDa)] and a heparin-binding lectin (TGL-I, ∼23 kDa) were isolated and purified from the pedicellariae of *T. pileolus* and *T gratila*. The function of pedicellarial lectins from *T. pileolus* and *T. gratilla* might be defense against a foreign body. Furthermore, a novel hemolytic lectin (∼29 kDa) was isolated from the coelomic fluid of *T. gratilla* (Nakagawa et al. 1999, 2003). Recently, one more lectin – SUL-IA (∼32 kDa) – from pedicellarial venom was shown to possess immunomodulatory properties (Edo et al. 2012).

Toxins of Marine Vertebrates

To date, approximately 1,000 species of venomous fish have been described, including such groups as lionfish, scorpionfish, etc. For example, the marine family Scorpaenidae displays a variety of sharp dorsal and pectoral spines coated with venomous mucus or attached to the base of poison glands. However, venoms of the majority of fish genera are less toxic compared with those of the *Synanceia* spp.

The stonefish *Synanceia horrida* is one of the most venomous fish in the ocean and is found in the coastal waters of the Indo-Pacific and the Caribbean Sea. Its venom causes excruciating pain, described as the worst pain known. The multifunctional toxic agent, stonustoxin, a component of the venom, is secreted from poison glands at the base of the needle-like dorsal fin. The toxin comprises two polypeptide subunits (α-, 71 kDa; and β-, 79 kDa) with five disulfide bonds in this heterodimer (Ghadessy et al. 1996). This lethal toxin shows hemolytic activity due to its ability to

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Fig. 5 Comparison of amino acid sequences of sea snake *Praescutata viperina* and land snake *Naja naja atra* neurotoxins

form pores in the cellular membrane, elicits the potent hypotensive via the nitric oxide pathway, and activates potassium channels. The LD_{50} of stonustoxin is 17 μg/kg following intravenous injection in mice.

Sea snakes inhabit warm tropical coastal waters of the Pacific and Indian oceans and are a serious public health problem in these regions. Some of these snakes (subfamily Hydrophiinae) spend their lives in the sea only. Others, such as *Laticauda semifasciata* (subfamily Laticaudinae) spend part of their lives among rocks on shore. Sea snake venoms are more toxic than venoms from corresponding land snakes, but the amount of venom in their glands is small: from 0.6 to 18.0 mg per sea snake. Sea snake toxins are similar to the corresponding toxins from terrestrial snakes of the genus *Elapidae*. The toxins are differentiated into two types: short-chain toxins containing 59–63 amino acid residues with four disulfide bridges, and long-chain toxins containing 68–72 amino acids, with four or five disulfide bridges. The most potent and abundant sea snake toxins belong to short-chain group and are known as three-finger neurotoxins because they bind to two α-subunits of an $\alpha_2\beta\gamma$ acetylcholine receptor located on the postsynaptic membrane, blocking nerve signals transmitted by acetylcholine. Tryptophan and tyrosine residues are of critical significance to the toxicity of these neurotoxins (Tu 2011).

Comparison of sea snake neurotoxins with cobra neurotoxins showed many similarities, especially in positions of the four disulfide bonds. However, some difference exists in amino acid compositions, and their three-dimensional structures are quite discriminated, as shown using computer modeling (Fig. 5) (Komori et al. 2009).

Beside postsynaptic neurotoxins, sea snake venoms contain presynaptic neurotoxins, toxins of haemorrhagic action, cardiotoxins, enzymes, and other polypeptide constituents. The symptoms of sea snake envenomization differ and depend on the snake species. However, motor impairments are observed in a short time, which expedites the hunting of these animals for fish.

Conclusion and Direction

Toxic compounds produced by marine organisms include low molecular weight secondary metabolites, mainly of glycoside or alkaloid nature, and peptides and proteins. These compounds vary greatly not only in their chemical structures but also in their capabilities to interact with target macromolecules, such as ionic channels, receptors, and enzymes. High specificity of many marine toxins allows them to be regarded as promising biochemical tools and prospective models for creating new drugs. Some toxins are used – or studied to be used – in medicine. It is of special interest that toxins interacting with the same molecular targets differ from each other in their biological activities due to reaction with certain sites on these targets and their different affinities to various tissues and organs.

The most dangerous toxins are produced by marine microorganisms, such as bacteria, cyanobacteria, and microalgae, and in nematocysts or venom glands of some marine animals.

Microbial toxins may contaminate sea food and cause lethal human poisonings. Toxins of some marine animals causing stings represent a serious problem for those who fish, swimmers, and inhabitants of many countries, in particular, those in tropical and subtropical geographic areas.

Marine toxins have two primary biological functions: predation and defense. These functions play important roles in interspecies interactions in marine biological communities.

Studies on marine toxins produced a significant impact on the development of modern experimental methods in marine bio-organic chemistry, such as separation techniques for complicated mixtures of natural products, using 2D nuclear magnetic resonance (NMR) and tandem mass spectroscopy (MS/MS) techniques, etc. Many compounds, especially microbial toxins, impose a challenge to the finest groups of organic chemists working in organic synthesis. As a result, outstanding synthesis techniques have been carried out and new synthetic reactions and approaches developed.

Cross-References

- ▶ Classification and Distribution of the Aquatic Venomous and Poisonous Animals
- ▶ Clinical and Therapeutic Aspects of Envenomations Caused by Sponges and Jellyfish
- ▶ Equinatoxins: A Review
- ▶ Immunomodulatory Properties of Sea Cucumber Triterpene Glycosides
- ▶ Intoxications Caused by the Ingestion of Seafood and Fish
- ▶ Other Marine and Freshwater Toxins
- ▶ Phylum Echinodermata e Annelida: Sea Urchins, Starfish and Sea Cucumbers, and Marine Worms
- ▶ Phylum Porifera and Cnidaria

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Toxinology
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Phylum Porifera and Cnidaria

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Abstract

The main features to recognize members of Porifera (sponges) and Cnidaria (corals, sea anemones, jellyfish) are presented. Some toxinological information is highlighted focusing on dangerous species like sea anemones and box jellyfish.

Introduction

The two phyla presented in this chapter are among the most ancient animal lineages that have appeared on Earth. After the paleontological record, they have appeared at least during the Ediacaran, over 600 million years ago, and molecular estimates indicate they could have appeared about one billion years ago, during the Cryogenian. Recent inferences on animal evolution also place them among the basal lineages of the animals, together with the phyla Ctenophora and Placozoa (Marques and Collins 2004; Erpenbeck and Wörheide 2007; Collins 2009; Hooper et al. 2011). However, different from ctenophores and placozoans, the Porifera (sponges) and Cnidaria (corals, sea anemones, jellyfish, hydroids) are well known to be highly toxic (Williamson et al. 1996). Therefore this chapter will be covering the dawn of the animal toxin, making them key-groups to understand the evolution of animal toxinology.

Here it is presented the basic features of the sponges and cnidarians in order to help to distinguish these peculiar animals in a context of their toxic nature, but the reader should refer to Zoology textbooks to get more general and biological characters of them (Brusca and Brusca 2003; Ruppert et al. 2003). Bibliography at the end of the chapter includes qualified literature for key information about the several groups of sponges and cnidarians (Bergquist 1978; Bouillon et al. 2006; Daly et al. 2007). The focus here are on the essential information needed by a Toxinology audience, providing the general morphological characters that define each phylum and their subgroups, a classification framework for both phyla, as wells as some ecological and toxinological information important to understand their natural history.

Phylum Porifera

This phylum comprises the so-called sponges. Members of the phylum have a variety of body forms (Fig. 1). Sponges have in common a distinctive type of cell, named choanocytes, which are flagellated and promote circulation of water through a unique system of canals (Maldonado 2004).

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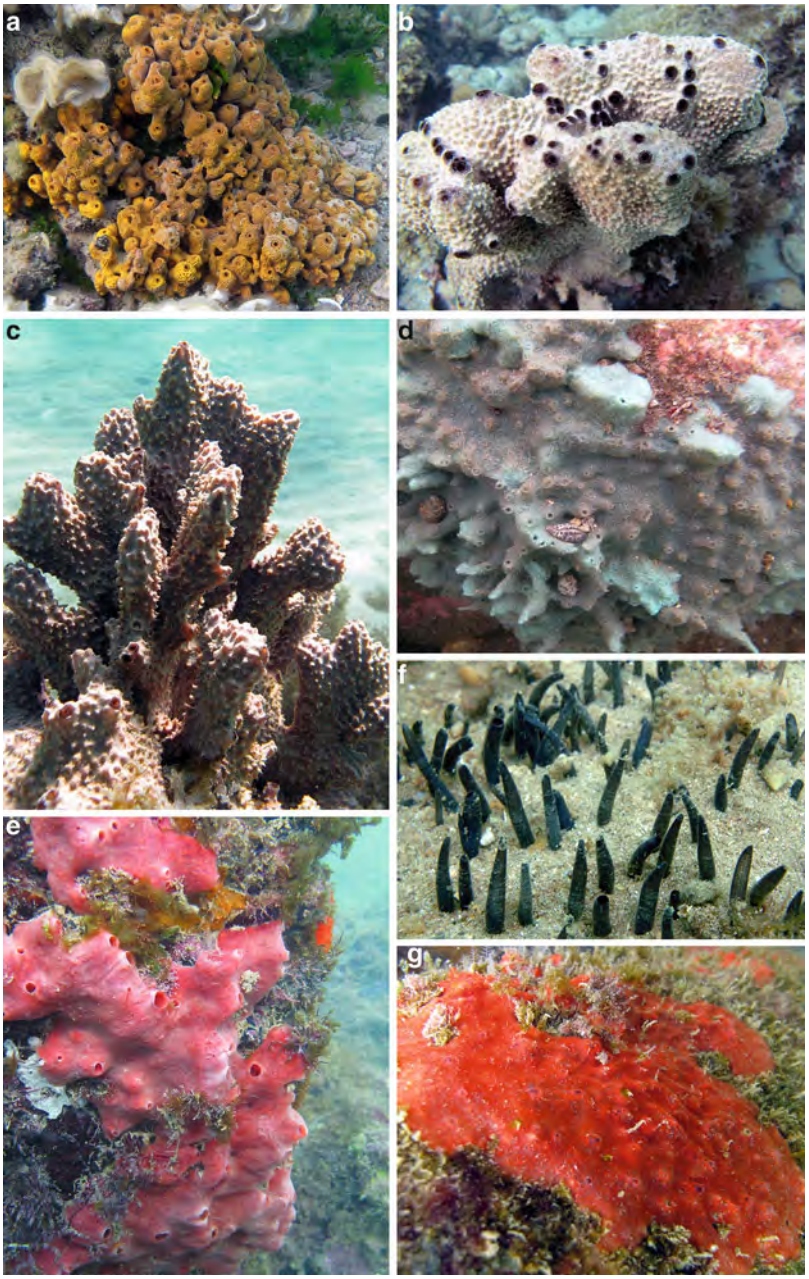

Fig. 1 Different sponges illustrating diversity of the group. (**a**) *Aplysina aerophoba* Piran, Slovenia. (**b**) *Ircina felix* Natal, Brazil. (**c**) *Dysidea robusta* Natal, Brazil. (**d**) *Amphimedon viridis* Guarujá, Brazil. (**e**) *Desmapsamma anchorata* Salvador, Brazil (Photo courtesy of Dr Eduardo Hajdu). (**f**) *Polymastia janeirensis* Guarujá, Brazil. (**g**) *Mycale microsigmatosa* Guarujá, Brazil

Poriferans are benthic sessile animals and can be found living in the marine or fresh water environments, occurring in all depths and latitudes. The body of a sponge is organized in a parazoan pattern and this means that they lack true embryological germ layering. There are no true tissues or organs, and although being a multicellular metazoan sponges function much like unicellular organisms in terms of their physiological aspects (Bergquist 1978).

The classification of the phylum Porifera in higher hierarchical categories is among dispute in recent times; mostly due to several findings based on molecular studies (Erpenbeck and Wörheide

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2007; Hooper et al. 2011). There are about 8,700 described living species (data from late 2012) with a higher diversity in pristine tropical habitats. Traditionally the phylum is divided into three main classes based on the composition of the spicules and their main shape (number of living species in brackets): Calcarea [684 species], Hexactinellida [689 species], and Demospongiae [7,356 species].

Body Organization

The corporal pattern of sponges is intimately related to the cell types, because there are no organized tissues in their body. For understanding the poriferans it is important to have in mind two points: the aquiferous system and the totipotent nature of the cells.

The body of a sponge is composed of two layers: the external is called ectosome and the internal is called choanosome. The canals and all external surfaces are covered by the pinacoderm, composed by pinacocytes and presenting small holes called dermal pores or ostia. The water is pumped through these apertures to the water channels due to the flagella beating of the choanocytes, which are organized in choanocyte chambers that occupy most of the choanosome. The skeleton and various types of cells are embedded in an extracellular matrix (mesohyl) mostly composed of collagen fibers. It may vary in thickness depending on the species but is extremely important for the sponges because several physiological processes occur there (digestion, skeletogenesis, transport, gamete production).

A sponge is completely dependent of the water flow promoted by the flagella of the choanocytes through the aquiferous system. The larger the area of the choanocyte chambers the more food is captured by the animal. To increase the area for filtration it is possible to find different body arrangements in the poriferans, which are mainly folding of the surface. There are classically three types of organization (which have no phylogenetic meaning): asconoid, a single central cavity covered by choanocytes, with no folding; syconoid with some folds, and leuconoid with discrete flagellated chambers. The asconoid condition is found only in small calcareous species (up to 10 cm in height) presenting a vase-shaped form, which are clearly radially symmetrical. The central cavity of the vase-shaped body is called atrium or spongocoel, which is connected to the outside by a single aperture called osculum. In some species the entrance of water occurs via specialized cells of the pinacoderm named porocytes (tube-like). The water flow in such sponges is: ostium (aperture of the porocyte) → atrium (spongocoel) → osculum. The syconoid condition is achieved by simple folding of the body wall forming lateral projections, and the choanocytes are restricted to the inner side of such projections (choanocyte chambers). The aperture through the water comes into the choanocyte chambers is called prosopyle and the exit is the apopyle. Such sponges also can be radially symmetrical and the water flow is: pore (ostium) → prosopyle → choanocyte chamber → apopyle → atrium → osculum. The leuconoid condition is due to further folding of the body and formation of choanocyte chambers of different shapes. The organization is basically the same as in the syconoid sponges, but in leuconoid forms the atrium is reduced and a well developed system of inhalant and exhalant canals can be observed; due to that there are also some more oscula; these sponges are asymmetrical. In leuconoid sponges the water flow is: dermal pore → inhalant canal → prosopyle → choanocyte chamber → apopyle → exhalant canal → osculum. There are at least two more types of arrangements of the aquiferous system (sylleibid and solenoid), but there might be some intermediate forms (Cavalcanti et al. 2011). In general, the organization of hexactinellid sponges is different from the other two groups, and this led some researchers to propose a different phylum for them (Symplasma).

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Cellular Organization

Because there are no organized tissues with specialized functions, different cell lineages play important roles in the life of a sponge. As mentioned before the pinacocytes are the cell type responsible for the layering of external surfaces and in some internal areas (inhalant and exhalant canals). Although the pinacoderm function as an epithelial tissue, it cannot be defined as such due to the apparent absence of a well-developed basal membrane in most species. The shape of the pinacocytes can vary depending on the area they occur, and they can engulf small particles. The porocytes are tube-like cells of the pinacoderm forming ostia (water entrance) and can have some contractile capacity that helps in regulating the diameter of the aperture. Choanocytes are one of the most characteristic cell type in sponges: due to the presence of a beating flagellum they create a negative pressure that drives water into the aquiferous system. The flagellum is surrounded by a collar of cytoplasmic microvilli in which food particles are collected and engulfed through phagocytosis or pinocytosis. Several different types of cells are found in the mesohyl, mostly ameboid. Some of these are able to secrete collagen (collencytes, lophocytes and spongocytes); others produce calcareous or siliceous spicules (sclerocytes) that form the skeleton. Some cells have a limited contractile capacity, the actinocytes. Among the ameboid cells of the mesohyl there are the archaeocytes that are highly mobile and can differentiate into any other cell type. They play an important role in digestion, transport of nutrients and excretory products. Sponges have a great capacity of regeneration, being able to form a functional individual from single cell suspension, and this is a prominent line of research.

Skeleton

Mainly two cell types produce the skeleton of sponges: spongocytes secrete collagen fibers and sclerocytes form the spicules. The collagen, which is a feature present in all metazoans, can appear as fibrils or organized in a matrix called spongin. The spicules (silica or calcium) are produced in an organized way by the sclerocytes. Depending on the type of the spicule more than one cell can be involved in the production. These mineral skeletal structures are classified according to their relative size (as microscleres and megascleres), shape, number of axis and ornamentation and have an important role in the systematics of sponges. The organization of the skeleton may involve distinct types of spicules embedded or connected by collagen fibers or a matrix in different degrees and can include inorganic accumulated material such as sand grains. Some lack spicules and its skeleton is composed only by dispersed collagen fibrils or organized in fibers, a group that includes those species used as bath sponges.

Physiological Aspects

Digestion in sponges occurs intracellularly, mainly on choanocytes and archaeocytes due to phagocytosis and pinocytosis. The openings of the body (ostia) will affect the size of particles that gets into the aquiferous system, normally smaller than 50 μm. Depending on the size of a food particle it will be captured on the way to the choanoderm surface and the mobility of the mesohyl cells will ensure the adequate distribution of nutrients to all body areas. Dissolved organic matter (DOM) is also incorporated by sponges. Excretion products are released by the cells into the matrix and then diffuse to the water system, although the formation of fecal pellets was observed in some species. Sponges are filter-feeding animals, but there is a family (Cladorhizidae) in which the members are carnivores and predators. Most of these species are deep-water forms, and they capture prey with tentacle-like projections and hook-shaped spicules. Simple diffusion takes also place for gas exchange and freshwater sponges present contractile vacuoles for osmoregulation. There are

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neither nerve cells nor sense organs in sponges, but they can respond to environmental stimuli by body contraction, closing ostia and oscula, stopping or inverting the water flow.

Life Cycle and Reproduction

Due to the totipotency of the archaeocytes, sponges have a high regeneration capacity through several asexual reproducing processes. Pieces or fragments can regenerate new individuals, and this is widely used by "farmers" in bath sponge cultures (Pronzato et al. 2008). Freshwater species (and a few marine species) produce small spherical dormant structures named gemmules. These structures are composed by aggregated archaeocytes enclosed by a thick collagenous layer and siliceous spicules, which confers the capacity to withstand lower temperatures and/or desiccation. When conditions are favorable a small aperture opens (micropyle) and the archaeocytes migrate to the exterior and begin to form a new sponge individual. Regarding sexual reproduction, sponges are in the majority hermaphroditic animals but producing eggs and sperm in different periods of time. Eggs are formed by differentiation of choanocytes and archaeocytes while sperm formation seems to be derived primarily from choanocytes. Gametes are released in the water and fertilization is external, but in some species it is internal (inside the aquiferous system). There are roughly three types of larval forms: coeloblastula, parenchymula, and amphiblastula; which are ciliated outside. When these larvae are released they seek for an adequate substrate for settling, and the outer layer looses the flagella and flagellated choanocytes start to differentiate internally. There are young forms (just after settling and choanocyte formation) called rhagon and olynthus depending on the group.

Many other animals use sponges as a substrate or hiding place like crustaceans, ophiuroids, annelid worms and fishes. But some cnidarians (hydrozoans and scyphozoans), mollusks and crustaceans take advantage of the water current to have a continuous supply of food. It has been found that the sponges harbor a diverse and important microbiota, which can be species-specific and compose more than half of the dry weight of the animal. Freshwater forms have association with symbiont green algae, zoochlorellae. Some sponges are capable of bioerosion, excavating calcareous substrates (corals, mollusks shells) and promoting a cycling of the available Calcium Carbonate in the sea. The process involves mechanical and chemical action.

Historical Aspects

In the past, due to the benthic sessile habit and the asymmetrical growth, the sponges were considered among the Zoophyta, later together with cnidarians they were treated as Coelenterates and Radiates until the elevation as a phylum in 1826 by Grant.

Sponges are the earliest lineage that diverged in the animal tree of life. The lack of organized tissues and the physiology based in distinct cell types, especially the flagellated choanocytes, suggests the view of a flagellated protist origin, most probably among the Choanoflagellata (Maldonado 2004).

Classification

Class Calcarea. Sponges with spicules composed of Calcium Carbonate. The group is divided into two subclasses (Calcinea and Calcaronea) based on the type of larvae and the position of the nucleus in the choanocytes. Examples are the genera: *Chlatrina*, *Leucascus*, *Sycon*, *Leucetta*, *Amphoriscus*.

Class Hexactinellida. Sponges with spicules composed of silica usually six-rayed, and a syncytium of somatic cells. The group is divided into two subclasses (Amphidiscophora and Hexasterophora) based on the type of substrate that it is attached, and fusion of spicules in a network or not. Examples are the genera: *Hexactinella*, *Hyalonema*, *Dactylocalyx*, *Euplectella*, *Lophocalyx*.

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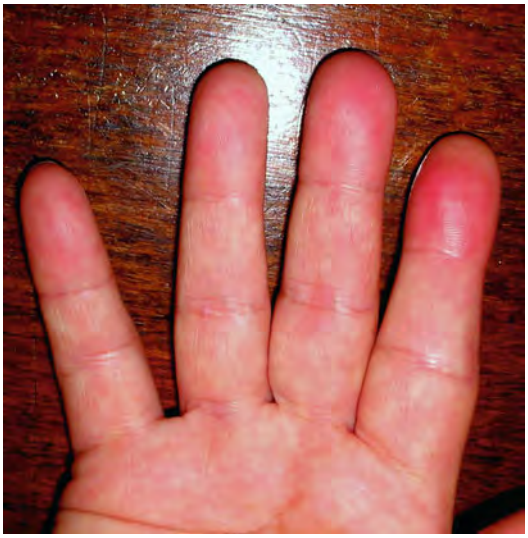

Fig. 2 Erythema and edema in the finger tips of a sponge collector. Contact with animal occurred 12 h before image was taken (Photo courtesy of Dr Vidal Haddad Jr)

Class Demospongiae. Sponges with the skeleton composed by spicules of silica (usually monaxonic or tetraxonic); by collagen fibers, or by combinations of both in various degrees. The group is divided into many orders that are not yet clearly resolved in terms of phylogenetic relationships. Examples are the genera: *Amphimedon*, *Cliona*, *Thetya*, *Mycale*, *Tedania*, *Geodia*.

Toxinological Aspects

Sponges are sessile benthic animals that cannot move around to avoid predators; thus these animals developed a wide spectrum of chemical substances (see Blunt et al. 2013, and previous reviews of this series). Several of these substances are being studied focusing on their pharmacological potential and it is known that several metabolites with interesting properties are in fact produced by the associated microbiota (Thomas et al. 2010). Research in biochemical compounds derived from sponges revealed a widespread occurrence of antimicrobial agents; but antitumor, cytotoxic and neurotoxic extracts are common. The spicules can provoke some skin irritation but the chemical compounds are most important to cause dermatitis and also allergic reactions (Fig. 2). Species of the genera *Tedania*, *Haliclona*, *Neofibularia* among others were reported causing accidents.

Phylum Cnidaria

This phylum includes a huge variety of beautiful and deadly animals (Fig. 3), such as the feared jellyfish, the Portuguese man-o-war and its highly complex organized relatives, the colorful and varied stony corals, sea anemones, gorgonians, the ubiquitous, small and highly abundant hydroids, the delicate and gorgeous stalked jellyfish. Ecologically, cnidarians may act as "producers" like the corals and their symbiotic algae in the coral reef ecosystem, or be related to massive biomass production such as in jellyfish blooms, or even occupy high levels in trophic chains like the box-jellyfish. Despite the huge biological diversity of the members of the phylum, there is a single feature that unites all cnidarians: the presence of cnidae. The cnida (Fig. 4) is a secretion product of the Golgi apparatus of a single cell (called cnidocyte), which is used for different

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Fig. 3 Different cnidarians showing diversity of body forms in the group. (**a**) Scyphozoan jellyfish *Rhizostoma pulmo* Piran, Slovenia. (**b**) Anthozoan hard coral *Mussismilia hispida* São Sebastião, Brazil (Photo courtesy of Dr Sergio N. Stampar). (**c**) Hydrozoan hydroid *Hydrocoryne iemanja* Brazil. (**d**) Anthozoan sea anemone *Anemonia sulcata* Piran, Slovenia. (**e**) Staurozoan stalked jellyfish *Haliclystus antarcticus* King George Island, Antarctica. (**f**) Hydrozoan siphonophore *Physalia physalis* Guarujá, Brazil

functions: capturing prey, defense, fixation/adhesion, building tubes, etc. They are the most ancient specialized structure to inoculate toxins in animal evolution (Özbek et al. 2009).

The majority of cnidarians are marine animals, with only a few species living in freshwater, like the well-known and model organism *Hydra*. They can be found in all places from the intertidal zone down to abyssal depths, from tropical areas to polar regions, and colonizing the benthos (sea bed)

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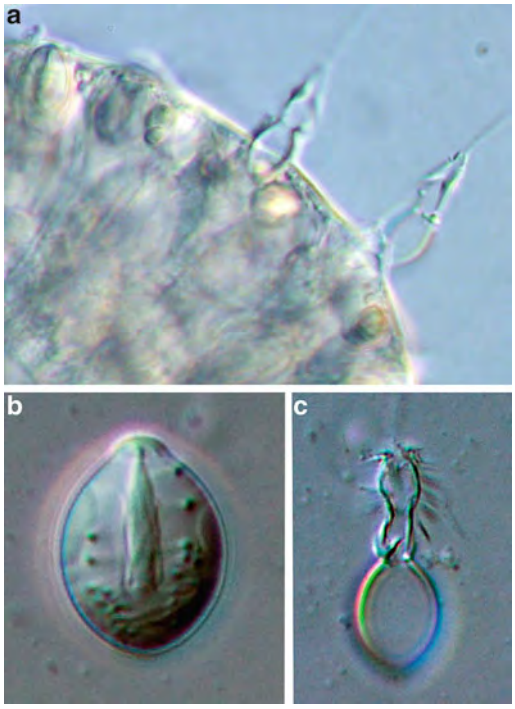

Fig. 4 Nematocyst of the hydroid *Hydrocoryne iemanja*. (**a**) Tip of medusa tentacle, magnification 400×. (**b**) Undischarged stenotele nematocyst, magnification 1000×. (**c**) Discharged stenotele nematocyst, magnification 600×

and the plankton (water column) and, although cnidarian species are not considered to be part of the nekton, some of them are very good swimmers. They exhibit an astonishing array of life histories, including most of the embryological pathways of development (Byrum and Martindale 2004). Their developmental programs are varied – many times the same genome may be found in completely different forms, either in the plankton or in the benthos, reproducing sexually or asexually, etc. These different expressions of forms exhibiting quite different ecologies evidently reflect into the morphological diversity of the group. A major feature of many cnidarians is the presence of two major phases in their so-called metagenetic life cycles, in which different stages are expressed along their life span: polyp and medusa. They have essentially the same body architecture, cytology and histology, but the polyps generally live on hard substrate and the medusae generally have a free-swimming habit.

The classification of the phylum Cnidaria in higher hierarchical categories was highly improved recently, based both on morphology, life history and DNA characters (Bridge et al. 1995; Won et al. 2001; Daly et al. 2003; Marques and Collins 2004; Collins 2009). The cnidarians comprise around 11,300 described species (Daly et al. 2007) that can be divided into two main groups based on the life cycle pattern: those with polyp and medusae in their life cycle and those with only the polyp stage. The classes Cubozoa (36 species), Hydrozoa (∼3,500 species), Scyphozoa (∼200 species), and Staurozoa (∼50 species) compose the subphylum Medusozoa (sometimes called Tesserazoa or Metagenetica) – cnidarians with metagenetic life cycles. The subphylum Anthozoaria (sometimes called Ametagenetica), with its single class Anthozoa (7,500 species), presents cnidarians with only the polyp stage in the life cycle. Although classification systems are liable to change and updating the recent advances in phylogeny and classifications for a given group is mandatory, an updated view of classification of the cnidarians is presented here.

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Body Organization

The general body plan of the cnidarians is simple. They have only two tissue layers, the epidermis (derived from the ectoderm) and the gastrodermis (derived from the endoderm), and animals with only these two epithelia are called diploblastic, considered to be an intermediary grade of the evolution the animals. The epidermis and gastrodermis are separated by the mesoglea – a layer of variable thickness mostly composed of collagen fibers, occasionally with some scattered cells, but not related to the mesoderm of the triploblastic animals. The general shape looks like a sac: one end opens and connects with the outside world through the mouth (defining the oral end) and the other end (defined as aboral end) is closed, like a *cul-de-sac*. The oral-aboral axis establishes the body symmetry of the cnidarians: it is elongated in the polypoid forms (cylindrical or columnar in general shape), and not so elongated in the medusoid forms (defined as bell, umbrella, dish or cubic-shape). Considering only the external morphology, the majority of the cnidarians are radially symmetrical, with a mouth located on a small elevation of the oral region and encircled by tentacles; the opposite (aboral) end forms the basal or pedal disc and works as the attaching point to the substrate. The internal space is named gastrovascular cavity because it works for digestion and distribution of nutrients, O_2, etc. to different body parts. This cavity was also known as coelenteron and it was used in the past to define the Coelenterata, joining cnidarians and ctenophores into a single phylum, what is currently not accepted.

Polyps of cnidarians have a huge morphological diversity derived from the many varieties of asexual reproduction, ultimately enabling a colonial (or modular) organization. The basic pattern (or module) is characterized by a simple gastrovascular cavity in small forms. However, in larger animals, such as sea anemones, this cavity may be subdivided by mesenteries which vary in number and arrangement depending on the taxa. External symmetry is essentially radial, but there might be biradial or tetraradial forms when the internal symmetry is observed. In anthozoans, most of the polyps are solitary forms fixed to the substrate by their pedal discs; but in forms living on soft bottom this disc can become rounded (called physa in sea anemones) and used for burrowing and anchoring the animal to the sediment. The mouth can be on a somewhat elevated part of the oral disc (oral cone for scyphozoans; hypostome for hydrozoans) or on a wide oral disc like in sea anemones. In Anthozoa the mouth is slit-shaped and opens into an epithelio-muscular actinopharynx of ectodermal origin that connects to the gastrovascular cavity. This actinopharynx might possess one or two ciliated grooves called siphonoglyphs that helps to move water in and out of the cavity. The presence and the number of such siphonoglyphs define the anthozoan symmetry. Anthozoans with a single siphonoglyph have a single plan of symmetry internally, and are considered to be “radio-bilateral”, in which the side defined by the ciliated groove is named sulcal (or dorsal) and the opposite is named asulcal (or ventral). Anthozoans with two siphonoglyphs have two plans of symmetry and, therefore, are called “biradial”. In scyphozoan polyps (scyphistomae) the gastrovascular cavity possesses four mesenteries, and therefore these animals have a “tetramerous” symmetry, likewise the staurozoan polyps (stauropolyps). Finally, hydrozoan and cubozoan polyps (hydropolyps and cubopolyps, respectively) have no internal features – therefore they have infinite plans of symmetry and are considered to be “radial”.

The number of mesenteries varies greatly among the groups, from no mesenteries in cubopolyps and hydropolyps to hundreds in some sea anemones. Anthozoan octocorallian polyps have a fixed number of eight mesenteries, but anthozoan hexacorallians have a varied number, depending on the group, what makes this a relevant character for taxonomy. In anthozoans these mesenteries are named “complete” or “perfect” if they reach the pharynx, otherwise they are named “incomplete” and “imperfect”; but all mesenteries have a free region aborally in relation to the pharynx, and the rim of that, named mesenteric filament, have cilia, glandular cells and cnidae.

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Colonial or modular cnidarians are formed by asexual reproduction. In Anthozoa the zooids of the same colony are usually connected by their gastrovascular cavities, but in some octocorals there are canals called solenia interconnecting the internal spaces of polyps. The hydrozoan colonies have diverse organization and varied shapes, from reptant to erect colonies. The reptant colonies are formed by the growth of the stolon (or hydrorhiza) over the substrate, occasionally budding off polyps or stems. The erect colonies may also have a stolonal growth, but their shape is characterized by the development of the main supporting axis of the colony in the form of a hydrocaulus, resulting in stems and branches. Erect colonies may have a monopodial growth in which the primary polyp elongates itself at the end of the hydrocaulus forming a stem, from which secondary and tertiary polyps/hydrocaulus buds off, forming the lateral branches that may be arranged alternate/opposite/randomly, at a single/multiple plane(s), etc. Alternatively, it is also possible to observe sympodial growth in which the primary polyp stops its growth at a certain point and the colony develops by the budding of new polyps. Colonial organization is highly developed in hydroids presenting an exoskeleton called perisarc. This exoskeleton protects underneath living tissues, collectively named coenosarc. The perisarc has different names related to its regional specialization, like hydrotheca (perisarc surrounding the hydranth), gonotheca (surrounding the gonozooid), nematotheca (surrounding the nematophores/nematozooids), etc. The presence of the perisarc defines the two main groups of hydrozoans, collectively called hydroids (a non-monophyletic taxon): thecates (those with hydrotheca) and athecates (without hydrotheca). The polymorphism of the polyps of Hydrozoa is usual in thecates and athecates, and the specialized modules play different roles in the colony: gastrozooids or hydranths are in charge for capturing and digesting prey, dactylozooids or nematozooids for defense, gonozooids or gonangia for sexual reproduction, etc. Evidently, gastrozooids, gonozooids, etc. have particular morphologies in each different group, and these peculiarities are majorly used in the taxonomy of the hydroids. The gonozooids have a tissue axis called blastostyle from where the gonophores bud off. Gonophores are the structures bearing the gonadal elements, and may be a medusa or medusoid (ultimately released to the water column) or a fixed gonophore (ultimately retained by the gonozooid). The hydroids have several levels of reduction of the fixed gonophores.

However, the highest developed polymorphism can be observed in the Siphonophorae (hydrozoans like the Portuguese man-o-war) and in the Pennatulacea (anthozoans like sea-pen and sea-pansies). The siphonophores form colonies of highly differentiated polymorphic zooids with special functions, like for swimming (nectophores), floating (pneumatophores), defense/attack (dactylozooids), feeding (gastrozooids), and sexual reproduction (gonozooids). The zooids are organized in highly complex groups named cormidia that may be protected by bracteae. In the pennatulaceans the colony develops from the primary polyp that differentiates into a stalk and a frond (raquis). The stalk is in charge to attach the colony to the substrate, and the raquis develops secondary zooids, that can be autozooids (feeding) or siphonozooids (promoting water circulation). The raquis can be elongate and cylindrical or flat and wide depending on the taxon. Sessile colonial cnidarians in general have their morphology (size and shape) modulated by the habitat hydrodynamics caused by the water flow.

The medusa stage does not form colonies. Medusae are free-swimming stages, although there are a few benthic and sessile forms. The medusae usually have a thicker mesoglea than the polyps, and the body is in the form of a bell, umbrella or somewhat flattened like a dish. The aboral surface is called exumbrella and the oral surface is called subumbrella. The subumbrella has a tubular projection in the central part named manubrium, with the mouth at its end. The gastrovascular cavity occupies the center and, it can be divided marginally by septae or canals (radial and circular ones). The cubomedusae and hydromedusae usually have four radial canals or extensions of the

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gastrovascular cavity, and in hydromedusae there is also a marginal circular (or ring) canal connecting them. The scyphomedusae have the central part of the cavity divided into four gastric pouches. The internal anatomy of cubomedusae, hydromedusae and scyphomedusae reflect their tetramerous symmetry. The hydromedusae also possess a thin membrane at the umbrella margin, the velum; analogous to a membrane at the margin of the cubomedusae called velarium, in which canals of the gastrovascular cavity are present.

Cellular Organization

The two epithelia of the cnidarians have a limited portfolio of cells. The epithelio-muscular cell has its most apical portion with typical epithelial lining functions but its most basal portion has contractive capacity. Intracellular fibers of neighboring cells can connect to each other forming areas capable of true muscle contractions. Besides those cells, the epidermis has also cnidocytes, glandular, sensory, and interstitial cells. The undifferentiated interstitial cells can develop into any cell type, thus explaining the high regeneration and morphogenetic capacity of the cnidarians. The cnidarians are the first metazoans to present sensory structures, and also have an elaborated repertoire of types of cell junctions (gap junctions, septate junctions, and desmosomes).

The cnidocytes are the cells that bear the cnidae, organelles secreted by the Golgi apparatus. Cnidae are capsules with a coiled thread immersed in a mixture of toxins. They may be triggered through different types of stimuli (chemical, electrical, and/or mechanical) over the prey or predator serving for attack or defense (Özbek et al. 2009). In medusozoans (except by Staurozoa) the cnidae also possess a cilium, the cnidocil, that works as a mechanical trigger for the discharge. By triggering, the operculum (present in the Hydrozoa and Scyphozoa) opens and the thread disentangles penetrating the tissues of the prey/aggressor and injecting the toxin of the capsule. In anthozoans there is no cnidocil and the operculum is tripartite. The reactions of prey/predator are highly differentiated and depend on the type of cnida and chemical composition of the toxin. Some studies report the discharge of cnidae (a kind of exocitosis) as the fastest living mechanism reaching 2 ms with an acceleration of 40,000 g. What could promote such mechanism? There are three hypotheses to explain the discharge of the cnidae: (1) osmotic – a fast entrance of water; (2) tension – tension generated by the formation of the cnida; and (3) contractile – in which contractile units press the capsule. The developing cnidae are formed by the cnidoblasts, cells differentiated from interstitial cells located in the epidermis and gastrodermis, depending on the taxon. The evolutionary origin of the cnidae is speculative – there is a hypothesis that suggests the organelle is a kind of symbiosis with specific unicellular organisms (protists) at the origin of the cnidarians. There are three major types of cnidae: (1) nematocysts possess a double capsule with toxins composed by phenols and proteins, threads with spines and with an apical pore; (2) spirocysts have a single capsule and the inside fluids are composed of mucous and glycoproteins (more adhesive) and there is no apical pore; (3) ptychocysts have a folded thread, no spines, and no apical pore, being exclusively adhesive. Nematocysts, the most common and ubiquitous form of cnida, have a diversified morphology of the thread and capsule, being of taxonomical importance (Mariscal 1974). They appear very early in the life cycle, being already present in the planula (the first larval type of cnidarians), and are present all over the living tissues (although they are not present in the gastrodermis of hydrozoans).

The body of the cnidarians is internally lined by gastrodermis, which is responsible for the digestion that occurs both in the gastrovascular cavity and inside the cells. The gastrodermis can be more developed in some areas forming mesenteries or septae that divides the cavity into smaller parts, thus increasing the digestive area and providing further support to the soft body.

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Skeleton

Like many other marine invertebrates, cnidarians developed a hydrostatic skeleton that works basically with the entrance of water into the gastrovascular cavity, and with the closing of body aperture (mouth). The tension of the water kept in the gastrovascular space is provided by the epithelio-muscular cells arranged longitudinally and circularly, making it possible to support the body structure. When the mouth opens and the water comes out, the animal loses rigidity and its size is reduced. This dynamic process is essential for physiological demands, like excretion, respiration, circulation, etc.

Skeletons are more evident in colonial cnidarians, but they also exist in solitary ones. In all cnidarians the skeleton is exclusively produced by the epidermal cells and is external. In hydroids, a usually thin perisarc layer made of chitin protects the hydranths, hydrocaulus, stolon and reproductive structures. This type of exoskeleton also protects scyphopolyps, in which they are named periderm. The octocorals developed other types of skeleton in the form of free sclerites and/or a horny axis (solid or with spaces) composed of proteinaceous fibers (gorgonin), within the group it is possible to follow the complexity increase of the skeleton. In stony corals and some hydroids (informally named hydrocorals) the exoskeleton is impregnated by the secretion of Calcium creating a massive structure that, by overgrowth and accumulation, originates the famous coral reefs known from tropical regions.

Skeletal structures are widely used to identify species and define higher taxa. In stony corals the individual skeleton of each polyp is called corallite, with an external wall (theca) and the floor (plate). A holding axis arises form the basal plate there, the collumela, as well as internal septae that hold the soft body of the polyp. Corals grow slowly, but the symbiosis with photosynthetic dinoflagellates (generally referred as zooxanthellae) speeds up calcareous deposition. During colony growth the corallites are sealed by tabulae that work as support for continuous and overgrowth developing of the several linings of polyps. The two non-related hydrocoral families (Milleporidae and Stylasteridae) have the skeleton with pores from which gastrozooids and dactylozooids emerge.

Motion

Motion of cnidarians occurs by different manners depending on the life cycle stage and, habitat. Larval forms such as planulae usually displace by ciliary beating, although some planulae without cilia creep and crawl over the substrate (e.g., staurozoans). In polyps, the action of epithelio-muscular cells promotes contractions reducing the height and increasing the diameter of the body, making some species able to move over the substrate. The ability to stretch the body column is an important mechanism to capture prey and is also related to the hydrostatic skeleton, body musculature, tentacles and oral disc. In sea anemones the body wall musculature of the column is more developed, and they also have longitudinal bundles on the sides of the mesenteries, called retractor muscles. In some species a set of ring musculature of the column forms a sphincter, usually located between the column and the oral disc. Also, a circular fold, forming a collar, can contract and protect the oral disc and the mouth. The ceriantharians, however, do not have a sphincter nor retractor muscles in the mesenteries, and the epidermal longitudinal musculature is continuous and allow a fast retraction inside the tube, partially dug on soft substrate.

Interstitial species have a different organization of the muscle fibers, allowing vermiform movements inside the substrate. Epifaunal non-sessile individuals, like *Hydra*, use body contractions and tentacle adhesion to tumble over the substrate – to a certain extent this may be also observed in staurozoans. Stauromedusae motion can be compared to polyps because they have an adhesive pedal disc besides sticky tentacles and rhopalioids. On the other hand, some sea anemones are able to swim by detaching from the substrate, using alternate contractions of the body column and shaking

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tentacles; other species can attach to the surface tension film and thus move around. This drift strategy is evidently also common in neustonic/pleustonic colonies, such as the Portuguese man-o-war (*Physalia physalis*), Blue Button Jellyfish (*Porpita porpita*) and the by-the-wind sailor (*Velella velella*).

In medusoid forms, the epithelio-muscular cells are organized in specific areas of the body, on the epidermis or just underneath that. These cells are generally organized in rings and near the margin of the bell, forming a coronal or ring musculature, but in some species these cells can have radial arrangements. The contraction of these specialized structures together with the antagonistic action of the gelatinous mesoglea promotes the swimming of the medusae. When they are relaxed (i.e., muscles not contracting and with mesoglea not tensioned) the subumbrellar cavity is filled with water. When the ring muscles contract the subumbrellar space is reduced and water is expelled; thus the animal displaces towards the aboral region– depending on the morphology and biology of the animal, this water flow may work as a jet (the so called jet-propulsion of jellyfish) or a row (a rowing movement). The radial muscles can help to return to the relaxed condition, determined by the shape of the dense mesoglea. The presence of a velum (in the hydromedusae) and a velarium (in the cubomedusae) promotes a more efficient and stronger water exhaling flow by reducing the aperture of the bell. This strategy of using tissue membranes is not present in the scyphomedusae, but they are also highly efficient in swimming. Different medusae may have distinct swimming modes: while some swim continuously towards the water surface and then stop moving sinking slowly in the water column, others have the ability to change direction or are attracted to light sources (Costello et al. 2008). The presence of environmental discontinuities like changes in temperature and salinity may concentrate medusae in some areas. A few jellyfish species have benthic habits, and these animals move using their tentacles or body structures like adhesive warts. The swimming strategies are intimately related to food capture and can vary a lot depending on the group.

Physiological Aspects

The nervous system of cnidarians has many physiological aspects similar to bilateral animals, as well as ctenophores. Nerve cells are arranged in a net-like pattern, densely concentrated at the oral region, tentacles and pedal disc – thus forming a non-centralized and diffuse system. There are two nerve nets underneath the two epithelial layers (epidermis and gastrodermis), between those layers and the mesoglea. The subepidermal net is more developed than the gastrodermal one. The majority of the nerve cells are non-polarized, and electric impulse spreads to all directions.

Together with ctenophores (with uncertain affinities), cnidarians are the most basal animal group presenting sense cells; organized as pigment spots, statocysts, motion receptors, and sense pits. There is no central processing area like a brain or cerebral ganglion, thus the information has to be processed equally in the nerve net all over the body of the animal. In polyps the motion receptors are distributed over the body surface, but more concentrated in the tentacles in order to help in perception of prey and predators. In hydromedusae the nerve cells are arranged in two circular rings near the margin and innervate tentacles, muscles, and sense organs. Ocelli, when present, are located in areas with pigmented and photoreceptor cells forming spots or pits. Statocysts are also located in a pit on the body wall or in closed vesicles containing a statolith and sensory cilia. Bell movement tilts the body and cilia of the statocyst are stimulated but the statolith, inhibiting muscle contraction on that side of the body. Many cnidarians live in illuminated areas to favor the development of zooxanthellae in their tissues. In cubomedusae and scyphomedusae the sense cells are grouped in a unique sensory structure, the rhopalia. These structures are located in clefts between the marginal lappets of the scyphomedusae or in niches on the exumbrella of the cubomedusae. Characteristically the rhopalium of cubomedusae is highly developed, including

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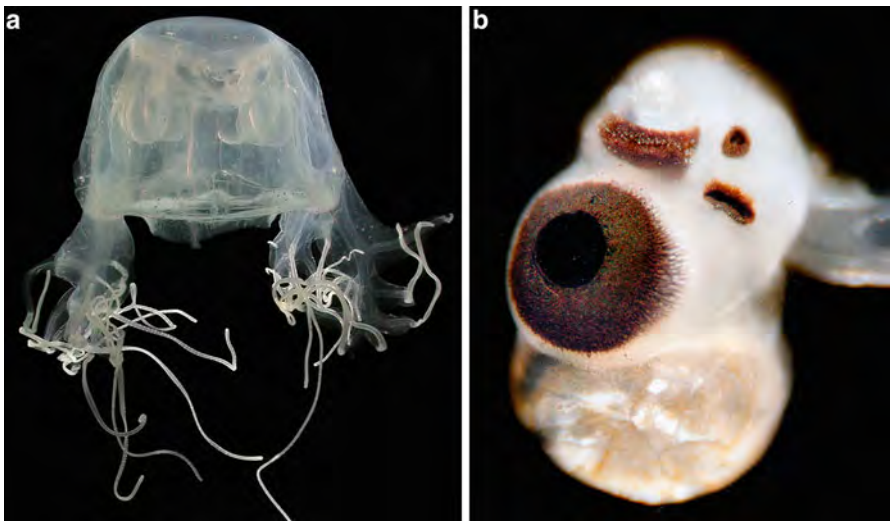

Fig. 5 (**a**) The box jellyfish *Chiropsalmus quadrumanus* (Cubozoa) from the Brazilian coast (Photo courtesy of Dr Alvaro E. Migotto, http://cifonauta.cebimar.usp.br). (**b**) Rhopalium of the same species in higher magnification

photoreceptor structures forming complex eyes with cornea, lens and retina, plus a statocyst and ocelli (Fig. 5). Thus, cubomedusae may chase their prey actively, moving away from obstacles, and may even use environmental landmarks to guide their displacement.

Many cnidarians are bioluminescent, especially deep water jellyfish, but also some polyps can emit light. Bioluminescence is used to attracting prey, reproductive partners, and to avoid predators. The conservative process is the reaction luciferin-luciferase, but there are some other proteins involved (aequorin) and bioprospection of these compounds is an important field of research.

Food capture occurs with the tentacles surrounding the mouth of the polyp. The cnidae of the epidermis of these tentacles are stimulated and then discharged. Cnidae work as small harpoons injecting toxins and holding the prey. In some species the tentacles wrap the prey and direct them to the mouth, in others the mouth and hypostome extend towards and "swallow" the prey. In the medusae the swimming activity creates water flows that direct the prey into distinct parts of the animal (tentacles, oral arms, umbrella) and subsequently transferred to the mouth.

Digestion starts inside the gastrovascular cavity right after ingestion of prey. Gastrodermal cells secret digestive enzymes starting an extracellular process. Ciliary beating and body contractions help to move and mix the content. Smaller particles are engulfed through phagocytosis and pinocytosis by the gastrodermal cells starting the intracellular digestion. Nutrients are distributed all over the body by contractions and ciliary movement. In anthozoans the mesenteric filaments present a free margin with a tri-lobed end: the median lobe (cnidoglandular lobe) kills the prey with the cnidae and produces digestive enzymes for digestion; the lateral lobes are ciliated and help moving food inside the cavity. In a group of sea anemones this median lobe is much developed and full of cnidae, called acontium, and helps killing the prey (internally) or as a defense when exteriorized through the mouth or small side apertures named cinclids. Species with reduced tentacles, like some corallimorpharians and sea anemones, use contractions of the oral disc to capture prey. Many cnidarians may also use mucous to capture small organisms. Food rests (like prey carapaces and undigested pieces) are expelled through the mouth by body contraction and ciliary beating.

The cnidarians are essentially carnivorous, but several species may be associated with unicellular symbionts that photosynthesize (zoochlorellae in freshwater and zooxanthellae in sea water

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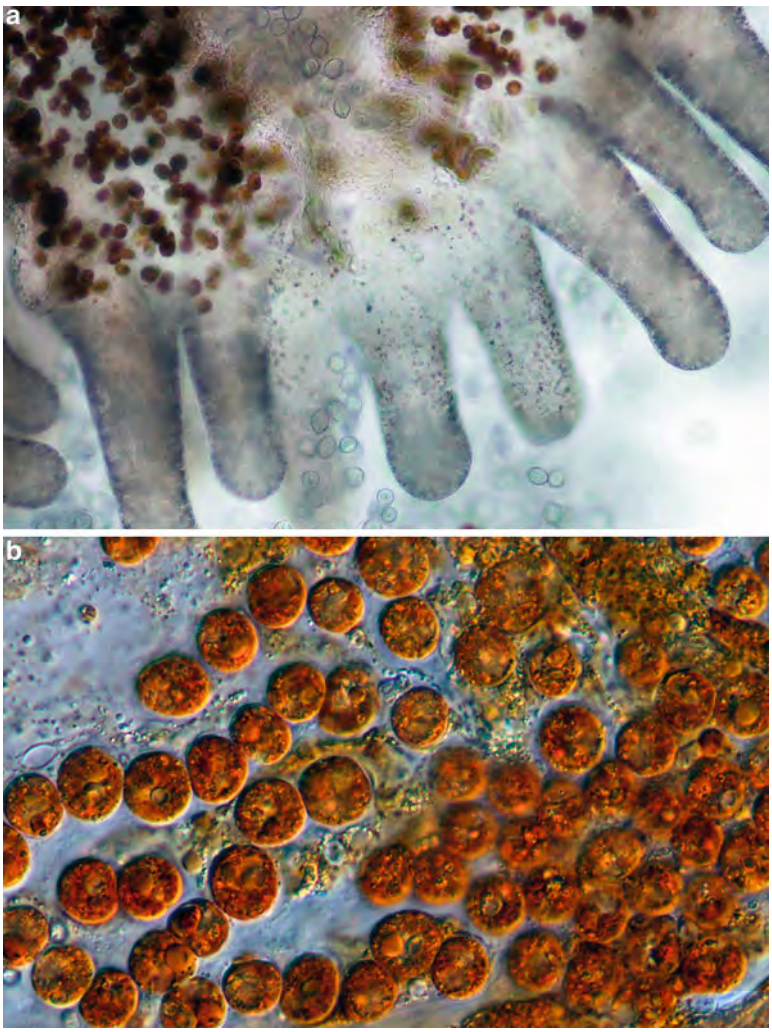

Fig. 6 Zooxanthellae symbiotic of the scyphozoan jellyfish *Cassiopea*. (**a**) Tip of medusa oral arm showing zooxanthellae and digitata, magnification 100×. (**b**) Squash preparation of zooxanthellae, magnification 1000×

animals). This kind of association is well known for stony corals that use the additional nutrients produced by the zooxanthellae to enhance the secretion of calcium carbonate. Besides corals, some species of sea anemones, hydroids, scyphozoans, and one species of cubopolyp also present symbiosis with zooxanthellae. The symbionts are usually located in the gastrodermis, and are responsible for most of the coloration of these cnidarians (Fig. 6). Zooxanthellae provide mainly sugar and amino acids for the hosts and these provide back nitrogen and phosphorus for their metabolism. This association is so important for some corals that many species can only build coral reefs when the symbionts are in good health, and this is the reason they are restricted to warm, clear and shallow waters. Adverse conditions may cause corals bleaching, a process in which the corals loose their zooxanthellae (Fig. 7). Such reduction in the concentration of the symbionts can be caused by many environmental factors, which culminate with the expulsion or consumption of the zooxanthellae by the coral tissues, frequently causing the death of the coral as well.

Gas exchange occurs by diffusion through the whole body surface that is in contact with the environment. The proportional increase of the exchangeable area by the presence of the septa in the gastrovascular cavity is very important to promote a more efficient diffusion. Occasionally, some species may change to anaerobic respiration under certain specific conditions.

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Fig. 7 Bleached small colony of the stony coral *Mussismilia hispida*

Cnidarians do not have a single and specialized organized circulatory system, and this function is provided by the gastrovascular cavity, besides digestive and exchanging functions. Jellyfish have a more developed canal system promoting a better distribution of the nutrients and a way to move out the wastes.

Excretes produced by cellular metabolism (mainly ammonia) are released by diffusion through the body wall or eventually released in the cavity and from there to the external environment. The majority of the cnidarians is marine, thus body concentration is the same or similar to the marine environment, making osmoregulation negligible. In freshwater species, like *Hydra*, there is a periodical discharge of fluids through the gastrovascular cavity that is kept as hyposmotic.

Life Cycle and Reproduction

Cnidarians usually have separate sexes (i.e., they are gonochoristic). There are only a few hermaphroditic species that can be simultaneous or sequential (Tardent 1985). Male and female usually release gametes directly to the water and fertilization is by chance (Campbell 1974). However, several species developed different strategies to maximize gamete fertilization, for instance, synchronizing the gamete release to short periods of time; having aggregation behavior of the mates in large populations to reproduce; developing internal fertilization, sometimes even grouping gametes in packages or spermatophores; and even evolving mating behavior in some species. Cnidarians are certainly the most basal clade of animals to have mating behavior with courtship and indirect transference of spermatophores.

There is no general pattern of embryonic development for the whole group of cnidarians – the different groups have several different types of cleavage, sometimes more than one in the same group. The early cleavages have a radial and holoblastic pattern, but later there are many distinct pathways of development. The gastrulation process, forming the epidermis and gastrodermis, is extremely variable in the group and some species even have a combination of patterns during the embryogenesis (Byrum and Martindale 2004). At the end of gastrulation the result may be a hollow (i.e., coelogastrula) or a solid (stereogastrula) gastrula, demanding or not feeding for its development. The presence of this first cavity in the larvae (planulae) is important to understand its mechanisms for dispersal and settlement/recruitment. The free-swimming or crawling planulae

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seek for a suitable substrate to settle and metamorphose to the initial polyp of a given species. The substrate may be specific (i.e., the planulae may have a strict association with a given substrata that triggers the metamorphosis) or the planulae may be generalist concerning the type of substrata. Ecologically this is quite important because specialized planulae cause the range distribution of their species constrained to the range distribution of their substrata; conversely, generalist planulae may have a broad geographical distribution, and are more prone to be dispersed. This latter case is the phenomena involving invasive species – they usually have a generalist behavior concerning substrata making it possible to colonize different regions of the world.

Asexual reproduction is widespread in the phylum Cnidaria. The main process of asexual reproduction in the polyp stage of hydrozoans is by budding, increasing the polyp population and/or forming colonies. Medusae may also bud by forming new individuals from the body wall (either from the exumbrella or from the manubrium), but this is more rare than in polyps. Some species may present other types of asexual reproduction like fission, either longitudinal or transversal to the oral-aboral axis of the polyps. The presence of large numbers of interstitial (undifferentiated) cells provides the cnidarians a higher regeneration capacity. This may be periodically adopted by certain species – many hydrozoan species periodically dedifferentiate their polyps and use interstitial cells to reorganize their tissues again, like in a rejuvenation process.

As mentioned before, in some hydrozoans the medusa stage is degenerated to a certain degree or even suppressed (like in *Hydra*) and the polyps assume the sexual reproduction. Combining the many possibilities result in a variety of different life cycles. Therefore, in some species the polyps may reproduce both asexually by budding and sexually by producing gametes. There is also a direct life cycle without a polyp stage, in which the planula differentiates directly into another larva called actinula and from this to a medusa. In all hydromedusae the gametes develop in a specific area of the epidermis near the radial canals or the manubrium. Differently, the gametes develop in the gastrodermis in all other groups of cnidarians (Tardent 1985).

In scyphozoans with metagenesis the polyps undergo strobilation, basically a process of asexual reproduction in which the polyp body is apically cut into transversal successive discs, and each one of these discs differentiates into a small larval jellyfish called ephyra. Developing polyps (named strobilae) can produce different numbers of ephyrae and then are considered to have a polydisc (when producing many ephyrae) or monodisc (a single ephyra). After releasing all ephyrae the scyphistoma (scyphozoan polyp) usually regenerates and, after a period of growth and reserve accumulation, it strobilates again. After reaching sexual maturity, scyphomedusae either release gametes in the water or the fertilization occur internally – brooding of planulae can be observed in the latter process. Only two scyphozoan species have holopelagic life cycles (i.e., without a polyp stage during their life cycle), *Pelagia noctiluca* and *Periphylla periphylla*. Curiously in *Pelagia* the planula larva itself differentiate into an ephyra before reach the adult medusa stage. In some members of the order Coronatae a reduction of the medusa stage is also observed, in a convergent process with the Hydrozoa.

In cubozoans the polyps are reduced, but they have mechanisms of budding, increasing the number of individuals (i.e., cubopolyps). Medusa formation occurs by metamorphosis, in which a single polyp transforms into a single medusa. However, in a few species it was observed the existence of remnant polypoid tissue after the metamorphosis is complete, and these remnants can regenerate a new small polyp. For species in which the sexual reproduction is known, they tend to group and some may exhibit mating behavior, including courtship and the male medusae transfer sperm packages, sometimes in the form of spermatophores, to the females, or may release them in the water – in any case, fertilization is internal.

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For staurozoans it is known that some species have a polyp (named stauropolyp), which differentiates into a stauromedusa, and this latter phase produces and releases gametes. The planula larva of this group is creeping and has no cilia.

For anthozoans, the unique polyp stage is evidently the sexual phase, and the gametes are in the mesenteries. They have distinct mechanisms of asexual reproduction like longitudinal and transversal fission, budding, and pedal laceration, but transversal fission and budding from tentacles are rare. Hermaphroditism is commoner in anthozoans than in medusozoans, although both exist depending on the species. Many species brood their planulae inside the gastrovascular cavity, and these planulae are released only on late stages of development, when they are ready to settle. Stony corals have a synchronous and simultaneous gamete release – such mechanism of mass spawning is interpreted as a mechanism to maximize the likelihood of fertilization as well as reduce predation over the eggs. As already mentioned, anthozoans do not present a medusa stage, so the dispersive stage of this group is the planula larva. Many larvae have short lives, but in Ceriantharia the planktotrophic cerinula larva can live for longer periods in the pelagial. Coral larvae can also live for weeks or months in the plankton, thus favoring dispersal. Also, many species use different asexual modes of reproduction while others develop coloniality, including some groups with modular polymorphism.

Few cnidarian species are parasites. The only intracellular parasite of the group is *Polypodium*, an enigmatic parasite of eggs of sturgeons. There is some doubt if the myxozoans, previously classified as protists, should be considered as highly derived cnidarian fish parasites. Extracellular parasites are commoner, including sea anemones and narcomedusae parasitizing other species of medusae. Mutualism between cnidarians and other animals is also common. Many hydroids are associated with crustaceans, gastropods, bivalves, worms and even other hydroids. Some sea anemones are symbiotic of hermit crabs, in some cases even producing a somewhat rigid covering for the crustacean (carcinoecium). Such associations provide easier access to food source and mobility to the cnidarian, and the crustacean usually benefits from the cnidae as a further source of defense. But there are some animals that feed on cnidarians but do not digest the cnidae, subsequently using them for defense (cleptocnidae), like flatworms (*Microstoma*), comb jellies (*Haeckelia*) and many nudibranch species. Several fish species also benefit from the stinging ability of the cnidarians as a defense, like the clownfishes (*Amphiprion*) and sea anemones, and shepherd fish (*Nomeus*) and the Portuguese man-of-war. But some associations do not represent advantages for the cnidarians, like with some hyperid amphipods, majid crabs, and palaeomonid shrimps, that use to feed on the cnidarian.

Understanding the different levels of metagenesis expression is essential to have a picture of the evolution and ecology of the cnidarians. The study of the biology of the species reveals that there are many instances for medusa or polyp reduction, and the interpretation of such observations (life cycle patterns) together with environmental characteristics can be important tools to understand the diversification, abundance and evolutionary pathways of cnidarians.

Historical Aspects

Cnidarians are known since the ancient Greek times (Aristotle, ∼380–320 BC) when they were classified as polyps (*knide*) and medusae (*akalephe*). Later on the group was treated by Linnaeus (1758) as *Zoophyta*, a category between animals and plants that included animals with radial symmetry or totally asymmetric like sponges, bryozoans, and flatworms. Cuvier (1812) used the name *Radiata* to group medusoid cnidarians and echinoderms. Only in 1847 Leuckart divided radiate animals into Echinodermata and *Coelenterata* (which included sponges, cnidarians, and ctenophores). Finally, the name *Cnidaria* was proposed by Verrill in 1865 for the polypoid forms,

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and the status of phylum was proposed in 1888 by Hatscheck, by splitting that from the former Coelenterata.

The precise chronological origin of the cnidarians is uncertain because there are several fossils that are not clearly defined as cnidarians, but present some similarities with polyps or jellyfish. Based on the most recent studies the oldest cnidarian fossils would have occurred during the Ediacaran period (~630–540 million years ago).

Cnidarians are a key group in the traditional hypotheses for the origins of the animals, like the colonial theory (flagellate colonial protists forming a hollow ancestral organism – the blastaea), and the syncytial theory (ancestral cnidarians similar to turbellarian flatworms). However these are nowadays considered as alternative scenarios independent of the general phylogenetic pattern of the origin of the metazoans. But to understand the evolutionary origin of cnidarians, it is important to recognize which body pattern was presented in the ancestral: polypoid or medusoid. These two possibilities were intensely discussed in the literature by the middle of the last century. The idea of a medusoid cnidarian ancestor has few morphological arguments. Currently the idea of a polypoid ancestor is widespread and supported by morphological data as well several different molecular markers.

Classification

Subphylum Anthozoaria, Class Anthozoa. Members of this group are sea anemones, corals, sea fans, and gorgonians. They are solitary or colonial, but exclusively marine. All members studied present a circular mitochondrial DNA, and never present a medusoid stage in the life cycle. They have cnidae in both epithelia (epidermis and gastrodermis); eight hollow tentacles or multiple of six tentacles, with cavities contiguous to the wide gastrovascular space (divided by longitudinal mesenteries with a free rim aborally forming the mesenteric filament). An actinopharynx is present and may have 0, 1, or 2 opposite ciliated grooves (siphonoglyphs). Germ cells differentiate and develop in the gastrodermis.

Subclass Octocorallia. Members of this group are called octocorals and they are colonial forms, their polyps have 8 pinnate tentacles around the mouth opening, eight complete or perfect mesenteries, a single siphonoglyph, and free or fused calcareous skeleton sclerites. Order Alcyonacea includes soft corals (without a support skeletal axis) and gorgonaceans (with a support skeletal axis composed of a horny substance – gorgonin – or calcareous); Order Helioporacea (blue corals) characteristically present a calcareous skeleton composed by aragonite crystals; Order Pennatulacea (pennatulaceans) include polymorphic colonies with the primary polyp working as an anchoring structure (peduncle) from which a wide supporting raquis with secondary polymorphic polyps emerge (autozooids and siphonozooids), and there is also calcareous sclerites. Examples are the genera *Carijoa*, *Muricea*, *Leptogorgia*, *Renilla*, *Alcyonum*.

Subclass Hexacorallia. Members of this group are stony corals, sea anemones, corallimorpharians, black corals, and ceriantharians. They can be either solitary or colonial, usually have a hexamerous symmetry and characteristically present spirocysts. The monophyletic status of the group is dubious because of their wide morphological variability. Order Actiniaria (sea anemones) never form colonies or calcareous skeleton, the column has specialized structures (warts, papillae, vesicles), and typically bear two siphonoglyphs. Order Antipatharia (black corals) have a horny spiny axial skeleton, polyps with six tentacles, six complete mesenteries and up to six secondary mesenteries. Order Ceriantharia (tube anemones) have marginal and oral crown of tentacles, live inside the substrate within a tube produced by mucous secretion and discharged cnidae (ptychocysts). Order Corallimorpharia (corallimorpharians) can be solitary or clonal, without a calcareous skeleton, without siphonoglyphs or a ciliated part of the mesenteric filament; they are

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usually confounded with sea anemones but with many similarities to stony corals. Order Scleractinia (stony corals) is mostly composed by colonial forms, do not have siphonoglyphs or ciliated parts of the mesenteric filaments, and they produce a calcareous skeleton with septae. Order Zoanthidea (zoanthids) have two circles of marginal tentacles, polyps with a single siphonoglyph, colonial forms have a stolon-like structure with gastrodermal canals, and most species incorporate sand grains in the body wall. Examples are the genera: *Bunodosoma*, *Anemonia*, *Anthopleura*, *Antipathes*, *Ceriantheomorphe*, *Pachycerianthus*, *Discosoma*, *Corynactis*, *Siderastrea*, *Mussismilia*, *Acropora*, *Fungia*, *Palythoa*, *Zoanthus*.

Subphylum Medusozoa. Cnidarians with life cycle alternating from polyp to medusa. Solitary or colonial forms, almost exclusively marine with a few species in freshwater. Characteristically presenting linear mitochondrial DNA, sometimes segmented like in Cubozoa. Divided into four classes.

Class Cubozoa. Cubomedusae, sea wasps, box jellyfish; the name is derived from the cubic shape of the bell; 36 marine species, some tolerating estuarine waters. Characteristically with four rhopalia with complex eyes, a velarium of subumbrellar origin, pedalia holding tentacles, gonads developing in the gastrodermis and metagenetic life cycle with solitary polyps that metamorphose into medusae. Carybdeida are the medusae with a single tentacle per pedalium and without gastric saccules. Chirodropida are the medusae with ramified pedalia holding several tentacles and gastric saccules on the subumbrella. Examples are the genera: *Alatina*, *Carybdea*, *Chironex*, *Chiropsalmus*, *Chiropsella*, *Copula*, *Tamoya*, *Tripedalia*.

Class Hydrozoa. Hydromedusae and hydroids, ca. 3,500 species, mostly marine, some estuarine and a few living in freshwater. Characteristically they have a gastrovascular cavity without mesenteries, cnidae only in the epidermis, colonial forms with variable degrees of polymorphism, gametes stored in the epidermis, medusae originating from lateral buds and with a velum. The group can be divided into two subclasses.

Subclass Hydroidolina. When present, members of this group have statocysts of ectodermal origin; colonial forms are polymorphic. “Anthoathecates” (a non-monophyletic taxon) are represented by hydroids without a skeleton around the hydranth, medusae without statocysts and gonads restricted to the manubrium. Order Leptothecata is represented by hydroids with skeleton around the hydranth (theca) and gonophores (gonotheca), medusae with gonads along radial canals. Order Siphonophorae is represented by polymorphic polypoid colonies with zooids for swimming and floating, with holopelagic life cycle. Examples are the genera: *Aglaophenia*, *Apolemia*, *Clytia*, *Corymorpha*, *Eudendrium*, *Obelia*, *Pennaria*, *Physalia*, *Sertularia*.

Subclass Trachylina. When present, members of this group have statocysts of ecto-endodermal origin. Order Limnomedusae have minute polyps without theca (when present), medusae with gonads over radial canals. Order Narcomedusae are jellyfish with lobate margin and tentacles from the exumbrella. Order Trachymedusae is mostly composed by species with holopelagic life cycle, medusae with gonads usually on radial canals, but one group of trachymedusans have interstitial habit with direct life cycle from a stage similar to an actinula larva. Examples are the genera: *Aglaura*, *Cunina*, *Gonionemus*, *Liriope*, *Olindias*, *Solmaris*.

Class Scyphozoa. “True” jellyfish, scyphomedusae, around 200 species, exclusively marine, some living in estuarine waters. The medusoid stage is the most conspicuous of the life cycle, but they also do have polyps. Characteristically the medusae have the margin with clefts and projections (marginal lappets), usually eight rhopalia in the clefts, gonads differentiating in the gastrodermis. Polyps with four mesenteries with septal funnels with muscular strains. Medusae are formed by transverse apical fissions (strobilation) originating ephyrae. Order Coronatae with medusae presenting a coronal furrow on the exumbrella and polyps with a periderm tube (exoskeleton).

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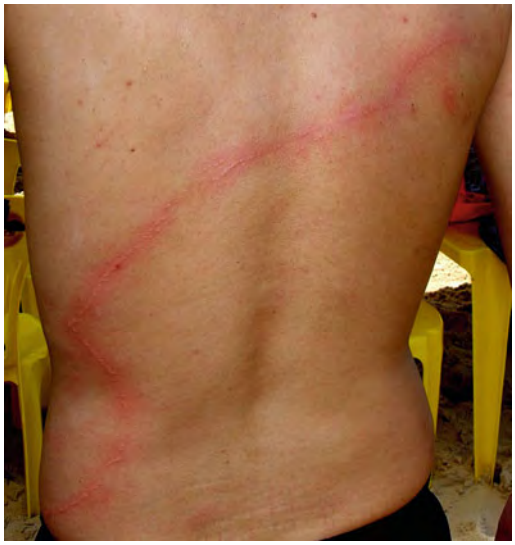

Fig. 8 Recent acute sting caused on the back of a bather by a Portuguese man-of-war (*Physalia physalis*). Photograph taken 15 min after contact (Photo courtesy of Dr Vidal Haddad Jr)

Order Semaeostomeae with medusae presenting four long oral arms, gastrovascular cavity divided by septae or canals, usually tentacles on umbrella margin. Order Rhizostomeae with medusae presenting eight oral arms with minute mouth openings, central mouth and tentacles absent, gastrovascular cavity divided by a network of canals. Examples are the genera: *Aurelia*, *Chrysaora*, *Cyanea*, *Linuche*, *Lychnorhiza*, *Nausithoe*, *Periphylla*, *Rhizostoma*, *Rhopilema*.

Class Staurozoa. Sessile medusae, stauromedusae, with ca. 50 marine species. Members of the class have capitate tentacles in eight groups, metamorphosis of polyp into stauromedusa, absence of strobilation and ephyrae, creeping planula larva without cilia. Taxonomy of the groups needs thorough revision due to several inconsistencies of the present classification. Examples are species of the genera: *Craterolophus*, *Haliclystus*, *Kishinouyea*, *Lucernaria*, *Manania*, *Stenoscyphus*.

Toxinological Aspects

The cnidae of the cnidarians is a stinging structure potentially harmful to man. Although cnidae are microscopic structures, some of them can inflict painful reactions and even cause death (Mariottini and Pane 2014). Envenomation caused by cnidarians can occur occasionally during professional (fishing) or recreational (bathing, diving) activities.

There are reports of accidents involving all groups of cnidarians and all life cycle stages, polyp, medusa and even the planula larvae. Polypoid forms like sea anemones, plume-like hydroids, soft corals, fire corals or hydrocorals, and siphonophores can sting due to the presence of different types of cnidae (and also different types of venom) in their tentacles, warts, or any specialized structure of the body column. Medusoid forms like scyphomedusae, cubomedusae and hydromedusae can also sting, but mostly due to the presence of the cnidae on their tentacles and mouthparts.

Envenomation by cnidarians may be quite variable, depending on two variables: the person and the cnidarian. Concerning the person, the severity of the envenomation may be related to the age of the person, the extension of the area of the body affected, health condition of the person, and potential allergic reaction. Concerning the cnidarian, it is related to the species involved, dimensions of the animal, condition (healthy or moribund), and types of cnidae. There might be local reactions (inflammation, dermatitis, pain) (Fig. 8), long-term reactions (keloids, pigmentation, scars) (Fig. 9), systemic reactions (pulmonary edema, blurred vision, vomiting, convulsions), and fatal reactions

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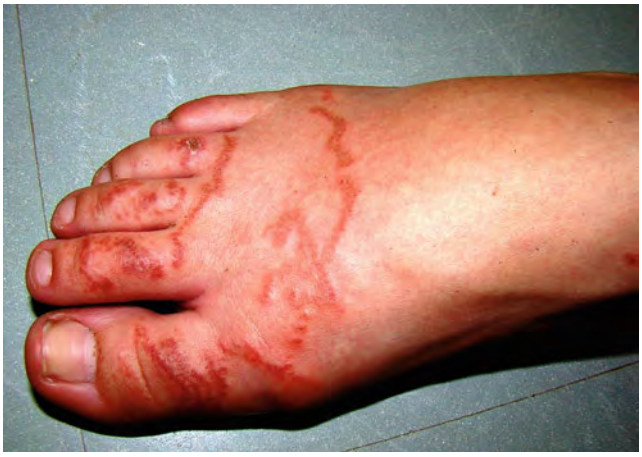

Fig. 9 Late sting (1 week after contact) caused on the foot of a bather compatible with Portuguese man-of-war or cubomedusae. Note linear markings with superficial necrosis (Photo courtesy of Dr Vidal Haddad Jr)

caused by cardiac and respiratory arrest, renal failure, and anaphylaxis. A list of the most dangerous cnidarians includes several species of jellyfish: the cubozoan *Chironex fleckeri*; the hydrozoan *Gonionemus vertens*; the scyphomedusae *Pelagia noctiluca* and *Cyanea capillata*. But there are also some dangerous polyps: sea anemones of the genera *Alicia*, *Actinia*, *Urticina* and *Anemonia*; and the hydrozoans *Physalia physalis* (a polypoid colony), *Aglaophenia*, *Macrorhynchia*.

Cnidarians, as well as sponges, are long known as a source of potential natural bioactive compounds that, combined with pharmacological techniques, may be the basis to develop new drugs or biomedical/biotechnological materials/processes. Several molecules, including anticancer and antioxidant compounds, were isolated from cnidarians (Zaharenko et al. 2008). Prostaglandins, local anesthetics, and vasoconstrictors have already been developed based on isolated bioactive substances from anthozoans, for instance, the most investigated group.

Many cnidarians were studied focusing on the hemolytic action of their venoms on vertebrates, and they present different levels of activity: the scyphomedusae *Cyanea*, *Pelagia*, and *Rhizostoma*; the cubomedusae *Carybdea* and *Chironex*; the octocorals *Litophyton* and *Sarcophyton*; the anemones *Metridium* and *Urticina*; and the hydromedusae *Crossota* and *Pandea*. Octocorals (*Clavularia*, *Xenia*), hexacorals (*Tubastrea*, *Palythoa*, *Actinia*, *Anemonia*), scyphozoans (*Chrysaora*, *Cyanea*), cubozoans (*Chiropsalmus*, *Chironex*) and hydrozoans (*Physalia*, *Aequorea*) were studied concerning the antiproliferative and cytotoxic activities (neurotoxic, hepatotoxic, and cardiotoxic), and also have effects over cell division. In summary, according to several studies, the action of the cnidarian venoms cause pore formation in membranes or oxidative stress.

Conclusions and Future Directions

Although the morphology of sponges and cnidarians is well known there are still classification issues to be solved. Novel methodologies on molecular techniques will certainly provide further advances into taxonomy combined with detailed morphological approaches. Sponges have been a source of a myriad of bioactive compounds and much more can be discovered. Cnidarians possess intracellular organelles that inject toxins (cnidae); those structures and toxins are underexplored in terms of pharmacological aspects.

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Cross-References

► Clinical and Therapeutic Aspects of Envenomations Caused by Sponges and Jellyfish
► Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Review

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Marine and Freshwater Toxins
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Clinical and Therapeutic Aspects of Envenomations Caused by Sponges and Jellyfish

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Abstract

The injuries caused by aquatic animals have increased in recent decades due **to** the increase of human beings in these environments, especially for recreational activities. The highest percentages are associated with invertebrates such as sea urchins, jellyfish, and **Portuguese man-of-war** (echinoderms and cnidarians), but there are injuries caused by numerous other aquatic animals, including marine and freshwater sponges. In this review, the clinical, therapeutic, and preventive aspects of injuries and envenomation caused by sponges and cnidarians around the world **are** discussed.

Introduction

Due to the growing interface between humans and aquatic animals, the interest in this theme has increased in recent years. In marine and freshwater environments, there are many potentially dangerous animals that come into contact with humans due to the large influx of bathers to beaches, the increase in the commercial and sportive and underwater fishing, and other activities such as scuba diving (Haddad Jr 1999, 2000, 2003, 2008).

Aquatic animals capable of causing accidents in humans, grouped according to the frequency and severity of accidents, are sponges, cnidarians, worms, mollusks, echinoderms, crustaceans, fish, and reptiles (Haddad Jr 1999, 2000, 2003, 2008). **Injuries** involving invertebrate aquatic animals (especially marine animals) **reach** their peak during the summer, when the population of some coastal towns increases exponentially. Bathers constitute more than 90 % of the victims, and the incidence of this type of accidents **is 0.1 % or 1 in 1,000 patients in emergency** rooms (Haddad Jr 1999, 2000, 2003, 2008). **Among the victims, approximately 25 % are bathers who have contact with jellyfish and Portuguese man-of-war** (Haddad Jr 1999, 2000, 2003, 2008).

Phylum Porifera (Marine and Freshwater Sponges)

Marine sponges are simple multicellular animals that present a body in a tube constituted by a **spongin** and a "skeleton" consisting of calcium carbonate and silica. The sponges also present an irritating slime on their surface, effect increased by body spikes (Haddad Jr 1999, 2000, 2003, 2008). The main marine sponges associated with lesions in humans are of the genus *Neofibularia* sp. **and the species** *Tedania ignis* (the fire sponge) and *Microciona prolifera*, the red sponge (Fig. 1).

The dermatitis caused by marine sponges presents as a sudden eczematous eruption, constituted by erythema, edema, papules, plaques, vesicles, and, more rarely, blisters (Fig. 2). The itch is

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Fig. 1 Fragment of a *red* marine sponge on a beach in West Atlantic Ocean (Photograph courtesy of Vidal Haddad Junior)

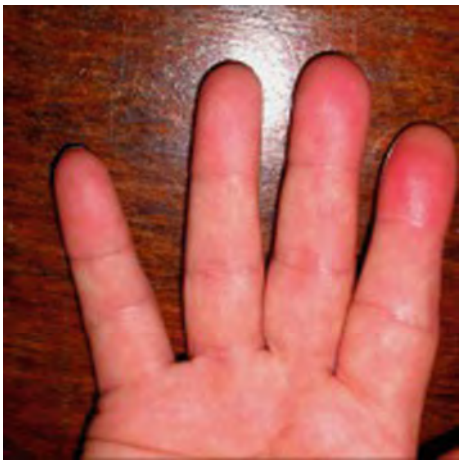

Fig. 2 Erythema and edema on the fingers of a marine biologist who collected sponges for research (Photograph courtesy of Vidal Haddad Junior)

intense. The process is common in the hands and the effects are only local (Fig. 2). The rare complications are the erythema multiforme, severe conjunctivitis, local hyperpigmentation, and anaphylactic reactions (Haddad Jr et al. 1999, 2000, 2003, 2008). It is recommended to apply an adhesive tape for removal of the micro-spines and the use of corticosteroid creams and cold compresses to relieve the inflammation (Haddad Jr et al. 1999, 2000, 2003, 2008).

Sponges also exist in freshwater environments and some species can cause skin disorders similar to those caused by marine sponges (Volkmer-Ribeiro et al. 2006). Belonging to the class Demospongiae, they are popularly called *cauxi* in the Amazon region and *pó-de-mico* (monkey powder) in Savannah-like areas (cerrados), being found in the ponds of these ecosystems (Fig. 3). The lesions caused by freshwater sponges are disseminated, manifesting as pruritic erythematous papules with central vesicles and exulcerations crusts, and secondary infections (Haddad Jr 2008). All the skin and mucous membranes can come into contact with the spicules of sponges (Fig. 4). The treatment used **for injuries caused** by marine sponges is also effective. If the manifestations are severe, it is important to **use** systemic corticosteroids, 30–40 mg of prednisone per day for about a week, with gradual withdrawal. **A study associated human eye injuries (uveitis and leukomas) in**

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Fig. 3 Freshwater sponge. Note that the sponge is attached to a branch of subaquatic vegetation (Photograph courtesy of Vidal Haddad Junior)

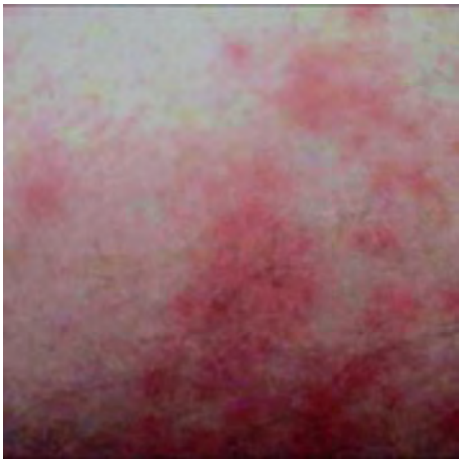

Fig. 4 Diffuse erythematous lesions caused by bathing in the pond with spicules of freshwater sponges in suspension (Photograph courtesy of Vidal Haddad Junior)

riverside people with the presence of spicules of two freshwater sponges (*Drulia uruguayensis* and *D. ctenosclera*). The findings were confirmed by histopathology exam of the lesions, demonstrating the etiology of ophthalmic process (Volkmer-Ribeiro et al. 2006).

Phylum Cnidaria (Jellyfish and Portuguese Man-of-War)

Cnidarians are animals with gelatinous consistence and dimorphic life cycle: the free form (medusa or jellyfish) and the polyps, which are fixed to the substrates. Four classes are associated with envenomation: Anthozoa (**corals and anemones**), Hydrozoa, Scyphozoa, and Cubozoa.

These animals have highly specialized defense cells called cnidocytes. **These cells contain** nematocysts, composed of small spicules distal to a spiral structure kept under pressure, which

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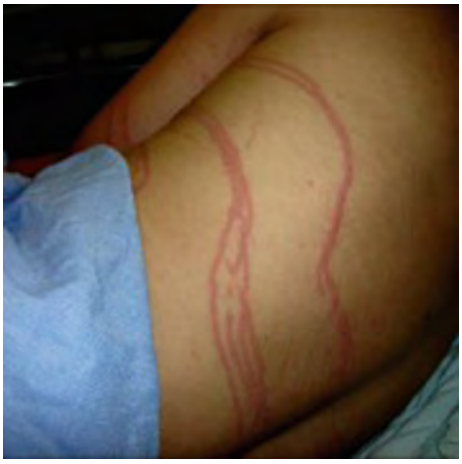

Fig. 5 Linear erythematous plaques caused by contact with a Cubomedusae (*Chiropsalmus quadrumanus*) (Photograph courtesy of Shirley Pacheco de Oliveira)

triggers after changes in the mechanic pressure and/or osmosis and may inoculate venom into the human dermis. Cnidarians with long tentacles can have millions of nematocysts, and many nematocysts remain intact in the victim's skin, without initially discharging its contents (Haddad Jr 2008, 2013).

The venom of nematocysts contains tetramine, 5-hydroxytryptamine, histamine, and serotonin, as well as thermolabile high molecular weight toxins capable of changing the ionic permeability and causing cardiac dysfunction. The venom can cause hemolysis and renal failure in late phases (Burnett et al. 1986). Nontoxic proteins can trigger allergic processes of varying severity (Burnett et al. 1986). The concentration and potency of the venom also are variable among the species (Burnett et al. 1986). The signs and symptoms of the envenomation depend on a toxic effect (immediate) and an allergic effect (immediate and delayed). The points of contact present intense pain and burning sensation (**it is not a burn**, because the manifestations are a result of the action of the toxins). **The skin lesions show an immediate papulous and erythematous linear rash (urticariform) with crossed lines.** On the points of contact, there may be horripilation, probably by alteration of the sympathetic nervous system. In 1–2 h, the **place** may present vesicles, blisters, and even superficial necrosis (Fig. 5). The pain subsists for hours and severe envenomation**s cause systemic phenomena due to general disorganization of nerve activity and direct toxic action in the muscles, which are responsible for cardiopulmonary failure and deaths.**

The Cubomedusae (*Chironex fleckeri*, *Chiropsalmus quadrumanus*, *C. quadrigatus*, *Tamoya haplonema*, *Carybdea alata*, *Carukia barnesi*, and others) and the Portuguese man-of-war (*Physalia physalis* and *P. utriculus*) can cause **envenomations** of this magnitude (Fig. 6; Burnett et al. 1986; Cazorla-Perfetti et al. 2012; Guess et al. 1982; Haddad Jr et al. 2002, 2003, 2010; Resgalla et al. 2011; Risk et al. 2012). There are hundreds of documented deaths caused by contact with Cubomedusae worldwide; most of them are caused by the species *Chironex fleckeri*, in the Indo-Pacific region. There are also reports of deaths related to accidents with Portuguese man-of-war (*Physalia physalis*), Cubomedusae *Chiropsalmus quadrumanus* in the Atlantic Ocean, and *Carukia barnesi*, also present in the Indo-Pacific region and responsible for the complex syndrome Irukandji, causer of serious imbalance in the autonomic nervous system (Burnett et al. 1986; Cazorla-Perfetti et al. 2012; Guess et al. 1982; Haddad Jr et al. 2002, 2003, 2010; Resgalla et al. 2011; Risk et al. 2012).

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Fig. 6 Cnidarians associated with important envenomations in humans. *Left*: *Tamoya haplonema. Center*: *Physalia physalis* (Portuguese man-of-war). *Right*, *above*: *Olindias sambaquiensis. Below*: *Chiropsalmus quadrumanus* (Photograph courtesy of Álvaro Migotto and Fábio Lang da Silveira)

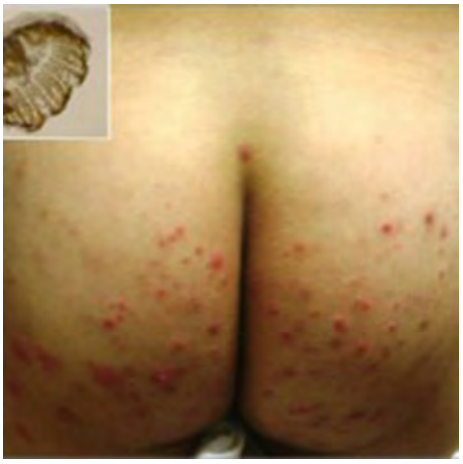

Fig. 7 Classic erythematous papules located under the swimsuit caused by *Linuche unguiculata* (in detail) (Photograph courtesy of Fabio Langda Silveira and André Rossetto)

After the **envenomation**, immediate allergic reactions can occur, such as angioedema and anaphylaxis. There are also late allergic reactions: persistent lesions after 48 h, new lesions at distance, recurrent reactions (four or more), contact dermatitis, or late onset of new lesions. Ingestion of jellyfish is observed in oriental cuisine and was associated with gastrointestinal and skin allergies (Burnett et al. 1986).

Contacts with **some** jellyfish **can present mild pain and rounded marks of their body and short tentacles**. Various jellyfish provoke this kind of envenomation, and the species *Olindias sambaquiensis*, *Chrysaora lactea* (in South of Brazil, Uruguay, and Argentina), and *Pelagia* noctiluca (East Atlantic, including Europe) can cause envenomations. *Olindias sambaquiensis* and ***Chrysaora lactea*** are constantly associated with injuries in South America, accounting for about 80 % of accidents in these regions (Haddad Jr et al. 2002, 2003, 2010; Resgalla et al. 2011).

The larvae **and the adult forms** of the jellyfish *Linuche unguiculata*, a small scyphomedusa, are involved in the genesis of the seabather's eruption, a pruritic erythematous papular eruption that

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develops in areas covered with swimsuits **in West Atlantic Ocean** (Fig. 7; Burnett et al. 1995; Haddad Jr et al. 2001; Rossetto et al. 2007, 2009).

Envenomation by anemones can be severe, although usually cause mild manifestations. Erythematous, irregular, and painful plaques and papules arise at points of contact with the short tentacles of anemones. The fire corals (*Millepora* sp.) are in fact hydrozoans that cause severe and extensive **envenomations** and may lead to medical emergencies. True corals cause minor accidents, but **they** can cause deep wounds in bathers. There is a report of a fatal accident by fulminant hepatic failure after contact of a diver with an anemone (*Condylactis* sp.) (Garcia et al. 1994; Marques et al. 2002).

Accidents by some hydrozoans, true corals, anemones, and fire corals show no typical pattern. The marks are irregular and present rounded or oval shape (Haddad Jr et al. 2010). These accidents should be reminded to divers approaching the underwater substrate, and they are characterized by pain and burning sensation, besides the rapid onset of lesions.

The most common complication of accidents by cnidarians is the residual hyperpigmentation. **However, keloids and atrophy** of subcutaneous tissue and gangrene may arise. Cuts by corals can develop a foreign body granulomatous reaction (Haddad Jr 1999, 2000, 2008; Haddad Jr et al. 2002, 2003, 2010).

Immunologic methods to aid diagnosis are few: **there is an enzyme-linked immunosorbent assay (ELISA) serum test that shows the etiology of seabather's eruption (**Burnett et al. 1995). Histological exams can help diagnose late allergic phenomena, so as **the** contact tests. Some species of **cnidarians can** be identified from the recovery of nematocysts in the human skin by means of adhesive tape method.

There is controversy about the treatment of envenomation by cnidarians: in all accidents compresses of iced sea water or cold packs with protection of a thin cloth should be used, so that freshwater does not reach the site wounded. This measure has potent analgesic effect and always should be applied even on the beach, as a routine first aid. The application of freshwater into the skin triggers loaded nematocysts by osmosis. However, there is a randomized trial that shows the immersion of the affected area in hot water (45 °C) for 20 min would be more effective than cold-water immersion. Our vision is that which interferes with the nociceptive activity of the venom are extreme temperatures, since the cold water also has good analgesic effect, evidenced by earlier studies (Fenner 1997; Haddad Jr 1999, 2000, 2008; Haddad Jr et al. 2002, 2003, 2010; Loten et al. 2006).

When the animal involved is a Cubomedusae (*Chironex fleckeri*, *Carukia barnesi*, *Tamoya haplonema*, or *Chiropsalmus quadrumanus*), the application of 5 % acetic acid (vinegar) to inactivate nematocysts still intact on the skin or in the tentacles not removed is fundamental. In the envenomation by Portuguese man-of-war (*Physalia physalis)* or *Olindias sambaquiensis*, the orientation is not as secure, since that in vitro experiments show that the nematocysts of some specimens of Portuguese man-of-war fired when placed in solutions of vinegar or alcohol. In our clinical experience, however, the application of vinegar proved to be a beneficial measure for any accident caused by cnidarians **in the Brazilian shores (**Haddad Jr 1999, 2000, 2008; Haddad Jr et al. 2002, 2003, 2010).

Other measures such as the use of antihistamines, urine, alcohol, or Coca-Cola® do not have scientific support and should not be applied, under worsening of the condition that at least remains untreated. Patients with systemic manifestations (**arterial** hypotension/hypertension, cardiac arrhythmias, pulmonary edema) should be referred urgently to a hospital, **so as the patients whose pain** persists after first aid measures (in these cases, an intramuscular **ampoule of** dipyrone

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can be administered to control the pain). Cardiac arrhythmias should be treated with intravenous use of verapamil.

The envenomations caused by cnidarians are easy to identify, following the classic pattern of erythematous crossed lines that arise soon after the contact, accompanied by intense pain. There is a pattern in the clinical manifestations caused by cnidarians in Brazil and all the Atlantic Ocean in the South, North, and Central America: **few, long, and crisscrossed lines suggest contact with** Cubomedusae and Portuguese man-of-war (severe envenomation, excruciating pain, and systemic phenomena). The presence of the **purple floating** of the Portuguese man-of-war denounces the animal. Rounded skin lesions with impression of small tentacles without systemic phenomena are suggestive of accidents caused by *Olindias sambaquiensis* and ***Chrysaora lactea***, very **common hydrozoans** on the Atlantic coast of South America (Haddad Jr et al. 2010).

Conclusion and Future Directions

Envenomations caused by sponges are rare and observed in **marine life** researchers and collectors of sponges for commercial purposes, but lesions associated to cnidarians **are common** in the beaches around the world. The great number of bathers and sometimes the excessive presence of jellyfish (blooms) are responsible for hundreds of injuries in isolated points **without means of prevision or prevention of the envenomations**. The therapeutic measures are not totally established, and new studies are necessary to identify the different species of organisms, the severity of the envenomations, and the specific treatments in each place.

Cross-References

► Classification and Distribution of the Aquatic Venomous and Poisonous Animals
► Phylum Porifera and Cnidaria
► Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Review

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Phyla Molluska: The Venom Apparatus of Cone Snails

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Abstract

Predatory marine gastropods of the genus *Conus* comprise ~700 species that have evolved a highly specialized envenomation apparatus. This recently evolved genus has developed potent venoms separately for prey capture and defense. Both strategies utilize a complex but different cocktail of venom peptides that is injected through a hollow harpoon to rapidly immobilize prey or deter predators. Many of the anatomical, histological, and functional aspects of the envenomation process have been elucidated. However, the role of several interconnected organs and the processes that allow the deployment of separate predatory and defensive stings require further study.

Introduction

Predatory marine gastropods of the genus *Conus* belong to the taxonomic class Neogastropoda, which comprises three superfamilies, Muricoidea, Cancellarioidea, and Conoidea. Within the Conoidea branch are three families of venomous marine snails, the Turridae, Terebridae, and Conidae (also known as the Conacea or Toxoglossa group). Cone snails thrive in tropical and subtropical waters around the globe but are most plentiful in the Indo-Pacific region, with a major hot spot of biodiversity centered around the Philippines (Fig. 1; Pimm et al. 2014). Other regions of exceptional diversity are also found in the Atlantic (e.g., Cape Verde Islands) and Indian (e.g., Madagascar) Oceans. Cone snail species have diverse shell pattern, size, and feeding habits, with vermivorous, molluscivorous, and piscivorous species being the three main groups (Fig. 2). Despite only 30–50 Ma of evolution, ~700 species are described worldwide. One driver for this explosive speciation is the separate evolution of potent venom for prey capture and defense (Dutertre et al. 2014a). Importantly, both strategies utilize a sophisticated venom apparatus that delivers a complex cocktail of toxins intramuscularly to rapidly immobilize prey or deter predators. Several species are potentially dangerous to humans, and about three dozen deaths are attributed to *C. geographus* stings. The lethal dose was recently estimated between 0.038 and 0.029 mg/kg, which is in the range of the reported mouse LD_{50} for the deadliest snakes on the planet (Dutertre et al. 2014b).

Visible external features of the animal comprise the foot, body (covered in part by the mantle), the siphon, eyes, and extendable rostrum and proboscis (Fig. 3). To access the venom apparatus in the laboratory, the animal must be taken out of its shell and the mantle lifted up to expose the internal organs. The first detailed report on cone snail anatomy was undertaken by Shaw (1914). His drawings of *C. tulipa* and *C. textile* venom apparatus were remarkably interpreted and remain even by today's standard, some of the most accurate descriptions of cone snail anatomy. The complete venom apparatus can be divided into several functional organs, including a muscular bulb, venom gland or venom duct, radular sac, and

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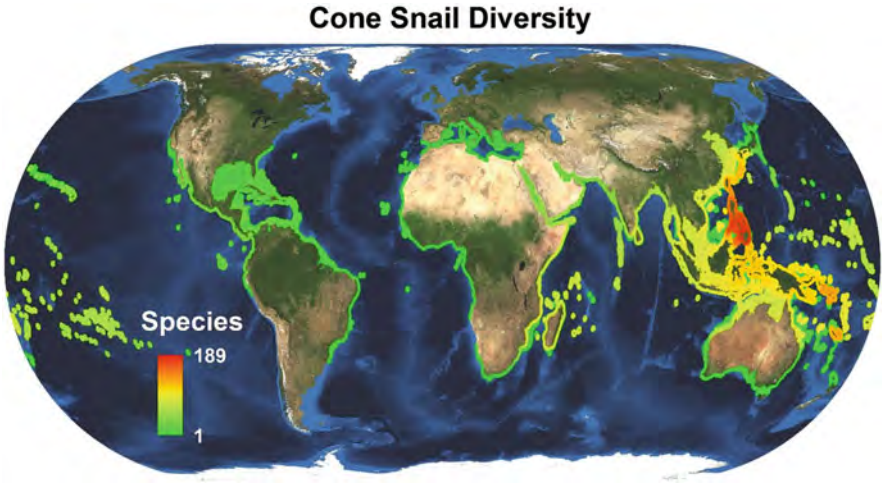

Fig. 1 Cone snail diversity and distribution. Cone snails are found in all tropical regions of the globe but are particularly abundant in the Indo-Pacific area centered in the Philippines (Image courtesy of Clinton Jenkins)

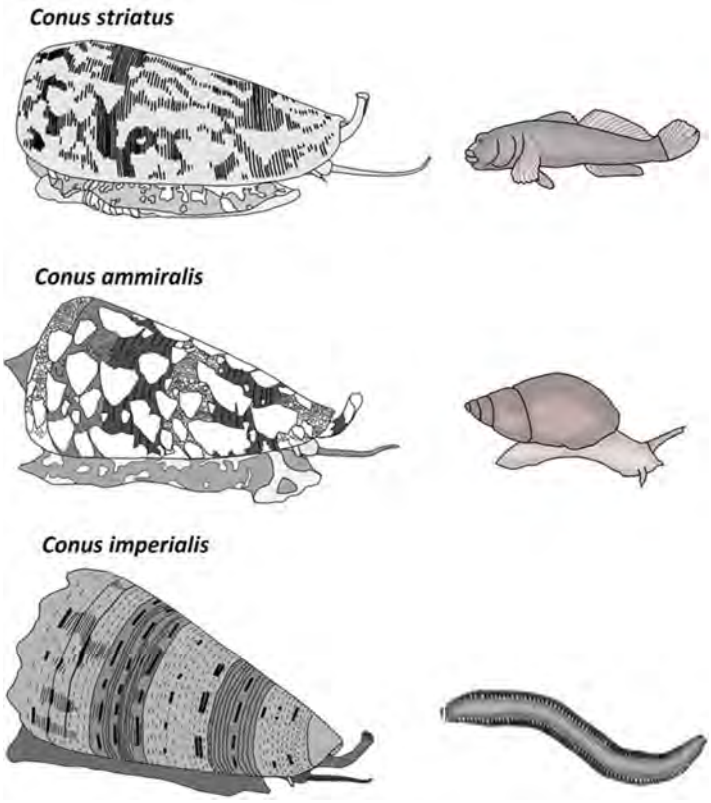

Fig. 2 Cone snail prey preference. Cone snails are generally divided into three main groups based on diet. Representative piscivorous (e.g., *C. striatus*), molluscivorous (e.g., *C. ammiralis*), and vermivorous (e.g., *C. imperialis*) are illustrated

proboscis (Fig. 3). The salivary gland may also play a role in the production of toxins and/or enzymes that facilitate prey capture and digestion. In this chapter, the venom apparatus of cone snails and its role in envenomation are reviewed, whereas the mechanisms that remain to be elucidated are discussed.

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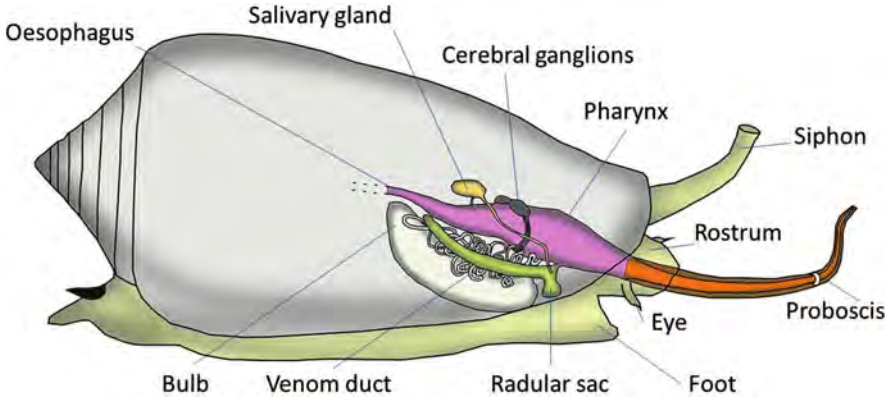

Fig. 3 General anatomy of the venom apparatus of a cone snail. These predatory mollusks have evolved a sophisticated venom apparatus that integrates specialized organs including the proboscis, radula sac, venom duct, and bulb

The Venom Apparatus

The Muscular Bulb

At the proximal end of the venom apparatus is found a large, bean-shaped organ originally called "gland of Leiblein" and wrongly assumed to be the "poison gland" by Shaw (1914). Later, Hermitte demonstrated that this "poison sac would appear to be merely a storage sac and propulsive organ for poison probably secreted by the coiled tube" (Hermitte 1946). Indeed, histological sections confirmed that this organ consists of longitudinal, oblique, and mostly circular muscles together with connective tissues (Endean and Duchemin 1967; Hermitte 1946). Furthermore, injection of extracts from this tissue did not produce observable effects in mice (Whysner and Saunders 1963). A canal or lumen, which is coated by a nonsecretory cubical epithelium, lies in a transversal position and appears as direct projection of the proximal venom duct. More recent investigation of this muscle bulb using proteomic, genomic, and morphologic data revealed high levels of proteins involved in rapid muscle movement, whereas proteins characteristic of venom biosynthesis were in low abundance (Safavi-Hemami et al. 2010). These results suggest that, through a burst of muscle contraction, the venom bulb could prime the proboscis with either predatory or defensive venom prior to prey injection.

The Venom Duct

The venom duct appears as a long and coiled tube, ~0.5–1.5 mm in diameter, located in the body cavity between the venom bulb and the pharynx/esophagus (Fig. 3). The length of this tube varies according to the species, but there is a general trend following diet preference, where piscivorous species have a relatively short duct (~two to three times the length of the shell) and vermivorous and molluscivorous have a long duct (up to six times the length of the shell). The venom duct is the actual "venom gland," producing the conotoxins and enzymes injected into the prey or predator. The classification and function of these conotoxins are the focus of intense research due to their utilization as pharmacological tools and their therapeutic potential (Lewis et al. 2012). It was recognized early that the venom production is not homogenous along the duct. For instance, injection of *C. geographus* venom extract from the distal part into mice had no effect, whereas extract from the proximal part was lethal (Whyte and Endean 1962). Endean and colleagues observed a gradient of "immature to mature venom bodies" from the bulb end to the pharynx end (Endean and Duchemin 1967). Later, molecular biology and proteomic techniques confirmed the segregation of conotoxin synthesis, with certain types being produced only in certain sections of the venom gland (Garrett et al. 2005; Tayo et al. 2010). Transcriptomics further demonstrated the regionalization of the duct with clear patterns of conotoxin gene superfamily expression

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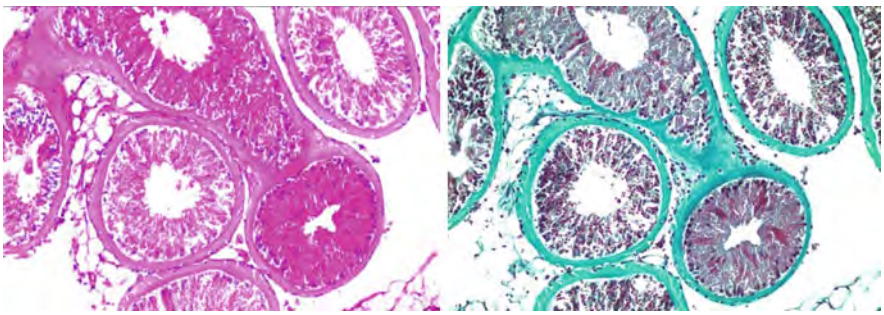

Fig. 4 Cross sections of the venom duct of a cone snail. The *left* panel was stained with Periodic acid–Schiff (*PAS*), whereas the right panel was stained with Gomori's trichrome (the muscle fibers stain *red*, collagen stains *green*, and the nuclei stain *blue*/*black*) (Images courtesy of Brett Hamilton)

(Hu et al. 2012). However, the function of this regionalization remained a mystery until the recent discovery that cone snails can rapidly switch between distinct venoms in response to predatory or defensive stimuli (Dutertre et al. 2014a). Predation- and defense-evoked venoms originate from the distal and proximal regions of the venom duct, respectively, allowing separate venom evolution for each purpose and rationalizing approaches to discover invertebrate- and vertebrate-selective conotoxins.

Histochemistry and Transmission Electron Microscopy of the Venom Gland

Transverse sections of the venom gland show that the inner duct is lined by a thick syncytial epithelium, which almost entirely occupies the lumen (Endean and Duchemin 1967). Indeed, several layers of elongated columnar cells containing visible cytoplasmic granules reduce the lumen to a thin star-shaped area (Fig. 4). The external wall of the duct consists of connective tissues (collagen) and longitudinal muscle fibers, with an intermediate layer of circular muscles.

Transmission electron microscopy of the proximal section of the duct revealed a complex epithelium that could be involved in active transport rather than secretion (Marshall et al. 2002). On the other hand, the distal duct shows at least two different cell types, which suggest holocrine secretion with prominent intracellular granules. The same granules were found to fill the lumen of the duct.

The Radular Sac

The highly modified radula used to inject venom is produced in a specialized tissue, called the radular sheath or, more simply, the radular sac. This organ displays a typical wishbone or Y shape, with a short and a long arm (Hermitte 1946). The longer arm contains varying number of teeth at different stage of maturation, all oriented along its axis, with the tips pointing toward the blind end, whereas the shorter arm acts more as a storage for fully mature teeth (Marsh 1977). The size of this organ varies according to the size of the radula, and in general, piscivorous and molluscivorous cones have a large and well-developed radular sac, whereas it is relatively small in worm hunters. The number of teeth present in the radular sac also differs between species, according to the types of diet, with more than 70 (~20 in the short arm and ~50 in the long arm) found in the molluscivorous *C. textile* and *C. marmoreus*, but only 19 (1 in the short arm and 18 long arm) in the worm-hunter *C. miles* (Dutertre and Lewis 2011). The walls of the radular sac consist of connective tissues and smooth muscle fibers, with the lumen lined with epithelium made of columnar cells. From histological sections, it appears likely that this epithelium is responsible for producing the chitinous radula teeth (Endean and Duchemin 1967).

The morphology of the radula itself is remarkable, being both hollowed to let through the venom and sharp and barbed to penetrate deep into the prey's tissues and tether prey. Often referred to as a hypodermic needle (Hermitte 1946), the radula reveals its complex structure only under a microscope.

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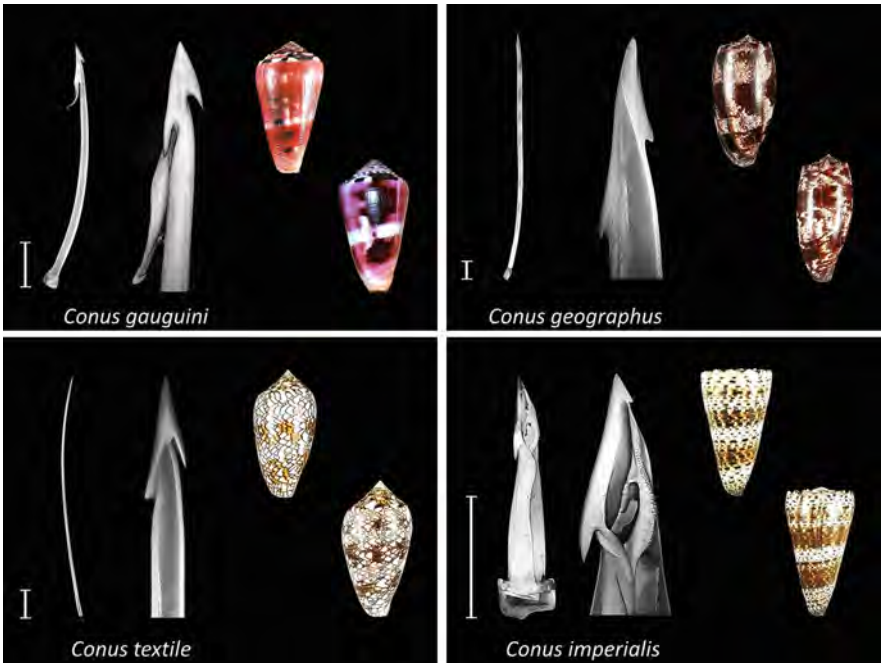

Fig. 5 Cone snail radula. The radula of each species is adapted to the type of prey it hunts. In hook-and-line hunting piscivorous species (e.g., *C. gauguini*), the radula is well developed into hollowed mini-harpoon, to both inject venom and tether the prey. Net hunting piscivorous species (e.g., *C. geographus*) and mollusk hunters (e.g., *C. textile*) also produce a long slender radula, whereas worm hunters (e.g., *C. imperialis*) have a short thick radula. Scale bar represents 1 mm

Typical radula types are shown in Fig. 5. The radula of piscivorous species such as *Conus gauguini* shows a long shaft and a well-developed anterior section. The tip of the radula comprises a blade, a barb, and a long accessory process that is hooked at the distal end to mimic a mini-harpoon. Other piscivorous species such as the deadly *C. geographus* use a simpler radula devoid of the long accessory process, resembling those of the molluscivorous species such as *C. textile*. Finally, the worm hunters such as *C. imperialis* produce a short, thick radula. The degree of sophistication of each radula type is used by taxonomists to help separate species. For instance, a recent systematic classification of Conoidean relied on the morphology of radula in combination with phylogenetic studies to propose new subfamilies within the genus *Conus* (Tucker and Tenorio 2009).

Scanning Electron Microscopy of the Radula Teeth

While the overall shape and diversity of *Conus* radula teeth have been known for centuries thanks to light optical microscopy, it was only through the greater depth of focus obtained with scanning electron microscopes that the many surface details, as well as their putative function, could be revealed. The first scanning electron microscopic image of a *Conus* radula was published by Kohn et al. (1972). The pictures showed the radula tooth of *Conus imperialis* as being a sheet of chitin rolled into a tube of 2½ whorls. Complex serrations along the adapical edge of the inner fold, together with the cutting edges of the external adapical fold and barbs, are proposed to facilitate penetration and help secure the tooth inside the prey (Fig. 6).

Subsequent investigations of many other "snail spears and scimitars" using scanning electron microscopy provided a scheme for coding the many varied characters to be used in taxonomic and phylogenetic studies (James 1980; Kohn et al. 1999). Important subsidiary characters include serrations length, presence of barbs, blades, cusp, and spur as well as the relative width of the tooth base and the ratio of the total radula length/shell length. A ratio smaller than 20 separates the vermivorous from the

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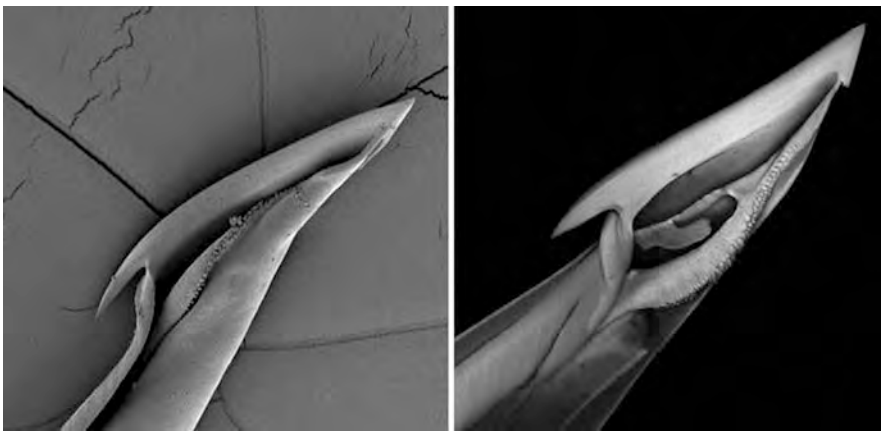

Fig. 6 Comparative scanning electron and confocal microscopic images of *Conus imperialis* radula. Obvious differences are visible in the overall shape and position of the complex serrations along the adapical edge of the inner fold situated at the apex of the radula. On the *left* panel image (SEM), the inner fold appears deformed, effectively obstructing the lumen of the radula. This SEM vision is incompatible with the copious amount of thick venom containing large granules that *Conus imperialis* forcefully injects into its prey. On the *right* panel image (confocal), the inner fold is moved away to widely open the lumen of the radula. Professor Dietrich Mebs is acknowledged for the permission to use this SEM picture

molluscivorous and piscivorous species. However, closely related species and those with similar diet habits can show nearly identical radula structure. Remarkably, a comparative analysis of the radular teeth of several Indo-Pacific molluscivorous species exclusively based on shell and radula morphology could correctly pair 53 out of 55 species studied (Nishi and Kohn 1999). Furthermore, subspecies and geographical variants could also be distinguished through discrete radular character measurements (Nishi and Kohn 1999). Interestingly, a recent radular morphology study of several Indian Conidae enabled the correction of some erroneous descriptions in the literature (Franklin et al. 2007).

Despite the numerous advantages of scanning electron microscopy, this technique is now challenged by more recent technologies such as confocal microscopy. Indeed, the harsh conditions for sample preparation required for SEM can significantly alter the shape and position of some fragile structures. In the case of *Conus* radula, the repeated cleaning and strong drying conditions prior to SEM analysis can distort the shaft and even modify the folds, interfering with the correct interpretation of function. To illustrate this, Fig. 6 compares images of the radula of *Conus imperialis* obtained with SEM and confocal microscopy. The obstructed lumen of the apex of the radula seen in the SEM image appears as an obvious artifact when compared to the confocal image, which provides an undistorted view of a functional radula.

The Salivary Gland and Other Foregut Glands

The salivary gland appears as a diffuse tissue located near the pharynx, from which two thin salivary ducts arise and terminate on each side of the short arm of the radula sac. Histological sections show that the salivary ducts are lined with a ciliated cuboidal epithelium, whereas the gland cells containing granules are grouped in small clusters (Endean and Duchemin 1967). Ciliary movements are responsible for the expulsion of secretions into the lumen of the duct. Histochemistry analysis of these granules demonstrated the presence of polysaccharides and proteins (Marsh 1971). Interestingly, protease activity was detected in the salivary gland, but the role of the secretion of proteolytic enzymes into the short arm of the radular sac remains to be investigated. Recently, conotoxin-like sequences were identified in a salivary gland cDNA library, raising the possibility that this organ may participate in venom production and therefore be part of the venom apparatus (Biggs et al. 2008). However, a more recent transcriptomic analysis of *C. geographus* salivary gland failed to reveal conotoxin-related transcript (Dutertre et al. 2014a). While

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the participation of the salivary gland secretions to venom production may be species dependent, it seems more likely that these salivary gland conotoxin-like mRNA arise from pseudogenes, possibly evolutionary relics inherited from a common developmental origin of both the salivary gland and the venom duct (Page 2012).

In addition to this primary salivary gland, a secondary (or accessory) salivary gland, as well as snout gland, has been reported in some *Conus* species (Marsh 1971). As opposed to the diffuse aspect of the salivary gland, the secondary salivary gland has a well-defined tubular shape narrowing down into a fine duct, which prolongs anteriorly to the ventral side of the proboscis and opens close to the mouth (Marsh 1971). A very detailed correlated light and electron microscopic study has revealed a structure composed of a luminal epithelium, a fibromuscular layer, a submuscular layer, and a capsule (Schultz 1983). The secretion of the secondary salivary gland produces polysaccharide and proteins, which remain to be identified at the molecular level and biochemically characterized. The snout gland is found closely associated with the radular sac, and its secretion consists of strongly acidic sulfated mucosubstances, again of unknown function (Marsh 1971).

The Proboscis

To propel the venom-filled radula into the prey, cone snails use a pleurembolic proboscis (Miller 1989). The proboscis is an extensible organ that is generally not visible when enclosed within the rostrum. As a general safety recommendation, it is often advised that live cone snails should be handled from the posterior end (larger end), assuming the proboscis cannot be extended further than the length of the shell. However, maximal elongation of the proboscis can easily reach 1.5 times the length of the shell in many species (Greene and Kohn 1989). At the proximal end, the proboscis is connected to the pharynx, whereas the distal end forms the anterior extremity of the alimentary tract, in other words the true anatomical mouth of the animal. Histological sections show that the wall of the proboscis is made of circular, crossed helical, and longitudinal muscle layers, whereas the lumen is lined with a single layer of cuboidal cells (Endean and Duchemin 1967). Between the muscle layers and the lumen are found nerves and haemocoel, which allow their hydrostatic function (Greene and Kohn 1989). A particular differentiation of the proboscis can be seen about 2/3 from the proximal end, in the form of a subapical sphincter muscle. It has been suggested that this sphincter may hold the radula tooth and prevent it from moving backward (Greene and Kohn 1989). It could also function to pressurize the venom to facilitate injection into the envenomed animal. The tip of the proboscis is covered with sensory papillae, which are thought to relay information on prey type and prey location upon initial contact (James et al. 2014). Remarkably, an amputated proboscis can fully regenerate in just 10 days allowing recovery from inadvertent damage. Such an injury occurs in the wild when predatory fish sections the proboscis they have mistaken for a worm prey (personal observations). Unable to catch prey, the cone snail would not survive without proboscis regeneration, which thus appears as a key evolutionary adaptation.

Future Directions

Mechanism of Envenomation

Whereas the anatomy of the cone snail venom apparatus is seemingly well characterized, the mechanism of envenomation is only superficially understood. A presumed chemical stimulus from prey will usually trigger an immediate extension of the proboscis. Then, upon contact with the appropriate tissue, the envenomation proceeds at an explosive pace. In fish-hunting and mollusk-hunting cone snails, a high-speed ballistic mechanism propels the radula into the prey's tissues at a velocity of 0.66–3 m s^{-1} (Salisbury et al. 2010; Schulz et al. 2004). A constriction near the tip of the proboscis is thought to

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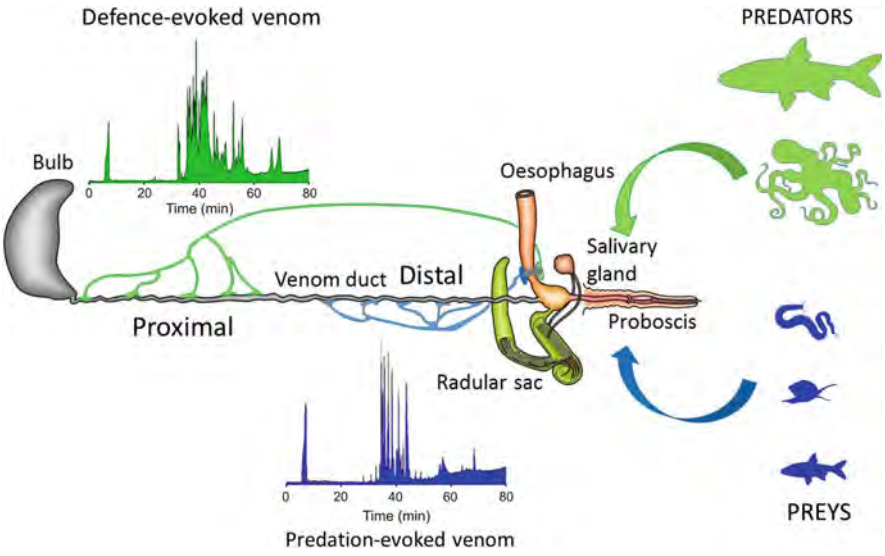

Fig. 7 Proposed mechanism for the separate release of predation- and defense-evoked venoms. Predatory (*blue*) or defensive (*green*) stimulus is detected by the mechanical, visual, and/or olfactory sensors of the cone snails. This information is relayed to the cerebral ganglia surrounding the esophagus, where it is integrated and transmitted to the appropriate neuronal circuit. The release of distal venom peptides to produce the predation-evoked venom or proximal venom peptides for the defensive response will be triggered through stimulation of the innervation of the corresponding part of the venom duct. The muscular bulb is proposed to flush the released peptides out of the lumen, allowing the rapid switch between the two types of venoms (Dutertre et al. 2014a)

provide the physical resistance needed to build the necessary pressure for the radula tooth propulsion, whereas the muscular sphincter may regulate venom flow and pressurization.

One of the most intriguing enigma that remains to be elucidated concerns the loading of a tooth from the radula sac to the tip of the proboscis. Invagination of the proboscis has been put forward as a probable mechanism (Hermitte 1946). However, field and laboratory observations of live cone snails do not support this hypothesis, at least in the case of mollusk hunters. Indeed, the latter have the remarkable ability to inject multiple times their prey in short intervals, and successive injections can occur without complete retraction of the proboscis. Therefore, a single tooth could be pushed from the short arm of the radula sac to the tip of the proboscis via coordinated contractions of several muscles, including the radula sac itself, pharynx, and the proboscis. In addition, the identity of the organ generating the pressurization of the venom prior to injection has not been unambiguously determined. The large muscle bulb at the proximal end of the venom duct appears as the likely candidate, but the pharynx could also participate. Finally, it has been noted that venom extracted from the gland appears as a thick paste, in contrast to the liquid venom injected. The origin of the liquid that allows this dilution of the venom duct secretions is also unknown.

Predatory Versus Defensive Venom

A recent study has uncovered one of the most important aspects of *Conus* biology that had eluded past investigations (Dutertre et al. 2014a). Like other venomous animals, cone snails were thought to inject the same cocktail of toxins for both predation and defense, presumably by exploiting the conserved pharmacology across prey and predators. This paradigm had never been challenged, but in a surprising twist, cone snails were found to possess the remarkable ability to rapidly switch between two different types of venom composition in response to predatory or defensive stimuli (Fig. 7). For instance, *C. marmoreus* can alternate between nonoverlapping venom mixtures within minutes (Dutertre et al. 2014a).

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More surprising was the absence of all well-known paralytic conotoxins in the predatory-evoked venom of *C. geographus*, although they have been described as facilitating prey capture. Instead, these deadly toxins dominated the defense-evoked venom, consistent with the often lethal outcome following *C. geographus* sting in humans. A specialized venom duct was the key evolutionary innovation to allow this unique feature, with the distal and proximal regions producing the predation- and defense-evoked venoms, respectively (Fig. 7). Molecular evolution analysis of gene superfamilies indicated that both predatory and defensive conotoxins are rapidly evolving under the influence of positive selection. This specific defensive envenomation strategy appears widely utilized by all types of cone snails, including various worm-, mollusk-, and fish-hunting species. Therefore, defensive use of venom may have had a more important role in venom evolution and cone snail specialization than previously thought (Dutertre et al. 2014a). For instance, the evolution of molluscivory and piscivory diets may have originated in the defensive strategies deployed against cephalopods and fish predators.

Conclusion

In conclusion, a large number of studies have uncovered the anatomy, histology, and function of a number of the various organs that make the venom apparatus of cone snails so remarkable. However, many important mechanisms remain to be elucidated in order to fully understand the envenomation process of cone snails and how this differs between predatory and defensive stings.

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Marine and Freshwater Toxins
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Venomous Marine Fish: Evolution of the Venoms. Chondrichthyes (Cartilaginous Fish)

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Abstract

The authors discuss the presence of the toxins and venoms in fish, a common recourse of these animals **used** for their defense, and cite the most important families of marine and freshwater venomous fish with commentaries about the mechanisms of inoculation, biochemical characteristics of the venoms, and clinical/therapeutical aspects of the envenomations. The authors emphasize their studies on toadfish, freshwater and marine stingrays, scorpionfish, and marine and freshwater catfish, the fish most frequently associated with human injuries.

Evolution of the Venom in Fish and Mechanisms of the Inoculation of the Toxins

Introduction

Venomous fish are those that present glandular structures producers of venom and an apparatus capable to inoculate these secretions, as the rays of fins, bony stings, body spines or teeth (Haddad Jr 2008; Cardoso et al. 2009). The marine stingrays and catfish are good examples of venomous fish. In contrast, the poisonous fish can store or produce toxins that they accumulate in some tissues, as the skin, liver, reproductive system, or muscles, not being capable of active inoculation (Haddad Jr 2008). The pufferfish represent this kind of passive defense.

The venomous fish are present in worldwide environments. The most important are the rays of the Dasyatidae, Gymnuridae, Myliobatidae, Rhinopteridae, Hexatrygonidae, Urolophidae, Plesiobatidae and Urotrygonidae families (the marine stingrays), Ariidae (marine catfish), **Scorpaenidae (scorpionfish and lionfish**, among other venomous fish), **Synanceiidae (stonefish)**, Batrachoididae (toadfish), Trachinidae (weever fish), and, in the freshwater environments, the Pimelodidae, Heptapteridae, and Ictaluridae **families** (freshwater catfish) and Potamotrygonidae (freshwater stingrays) (Haddad Jr 2008; Cardoso et al. 2009).

The mechanisms of envenomation are variable among the species, involving different apparatuses and venomous glandular tissues, which cause different inoculation points, not allowing uniform prevention measures, as we see in the **snakebites**, for example (Haddad Jr 2008). Fish can present venom glands in places as different as the rays of the fins, caudal stings, and spines in preopercular or dorsal positions.

Evolution of the Fish's Venom

The skin of fish is responsible for various important functions. Among others, it promotes the protection of internal structures, the protection against infections and other diseases, and the hydric

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balance (Quay 1972). The epidermis of the fish does not present the corneal stratum. It is composed of a layer of **squamous cells** of varied thickness and presents other cells, like goblet cells (secretory cells that are responsible for the cuticle), eosinophilic square-shaped cells, leukocytes, and macrophages. The cuticle is the mucous external layer that reduces the turbulence during swimming and also protects against infections once it presents antibodies.

The epidermal secretory cells in fish are of two types: the first secrets slime, which is a very important factor to reduce turbulence, being a basic part of the mucous cutaneous covering of the skin or cuticle. The main mucous cells are the goblet cells, which present a large vacuole within which the globules of secretion are stored. The goblet cells are predominant in the epidermis and discharge their secretion in the surface through a small canal (Quay 1972).

The proteinaceous cells are the other secretory cells present in the skin of fish. There are various types of proteinaceous cells, with different morphology, but it is not difficult to distinguish the proteinaceous cells from the mucous cells using colorations like the hematoxylin and eosin (Quay 1972). The proteinaceous cells are responsible for the production of toxins, and they do not have canals to take their secretion until the surface. So, they cannot discharge their content in the surface of the skin, having need of cutaneous lesion for that to happen. The main cells of proteinaceous lineage are the clavate cells (Halstead 1970; Cameron and Endean 1973).

The toxins produced by the proteinaceous cells in the skin are the crinotoxins and they are not associated with venom apparatus. Some experiments showed that the crinotoxins of catfish are produced for clavate (proteinaceous) cells of the epidermis. The fish that present crinotoxins can kill another fish when in the same aquarium. Additionally, some crinotoxins are toxic to vertebrates (Halstead 1970; Cameron and Endean 1973).

The crinotoxins can be liberated in the water or after lesions in the skin, and they are an important mechanism of defense for the fish that produce them. A great number of species can produce crinotoxins in the proteinaceous cells of the epidermis, as the families Myxinidae, Petromyzontidae, Muraenidae, Canthigasteridae, Grammistidae, Ostraciontidae, Diodontidae, Tetraodontidae, Batrachoididae, Synanceiidae, Ictaluridae, and Plotosidae (Quay 1972). The functions of the crinotoxins are the protection against other organisms, as bacteria, fungi, and other invertebrates, or predators (Cameron and Endean 1973).

The development of crinotoxins and their function in the protection of the fish that produce them had been important points in the evolution of the systems of defense of the fish. The thickening of the epidermis with an increase of number of the proteinaceous cells in the proximity of acute and harmful structures, for example, the rays of fins of some fish, conferred greater effectiveness to the defense of the fish and to the system of inoculation of toxins, with obvious advantages for the venomous fish in their environment. The crinotoxins passed to be called of venoms and the adapted structures of inoculation, venom apparatuses (Quay 1972; Halstead 1970; Cameron and Endean 1973).

Chondrichthyes (Cartilaginous Fish): Marine Stingrays, Venomous Sharks, and Chimaeras

Composition and Actual Knowledge About the Venoms

Gymnuridae, Myliobatidae, Hexatrygonidae, Urolophidae, Rhinopteridae, Dasyatidae, Plesiobatidae, and Urotrygonidae Families

The marine stingrays are cartilaginous fish that present one to four **bony** stingers in caudal position, constituted of vasodentine (Figs. 1 and 2). The stingrays are bentonic fish and the injuries **occur**

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Fig. 1 The stingrays of the Dasyatidae family are the most common stingrays associated with human envenomations. Note the sting in a medium position in the tail (Photo: Vidal Haddad Junior)

Fig. 2 Detail of the retroserrated sting of a stingray of the Dasyatidae family (Photo: Vidal Haddad Junior)

when the victim step on the animal, which whip with the tail in **a** defense mechanism, introducing deeply the **sting(s)**, which in determined stingrays can reach up to 20 cm of length (Haddad Jr 2008; Cardoso et al. 2009; Halstead 1970; Haddad Jr et al. 2013).

Injuries caused by marine stingrays occur when bathers enter in shallow water over great distances but rarely occur very close to the **sand**. This kind of envenomation is common in fishermen, especially in professional fishermen separating fish and shrimps in the nets. The trauma is **always** important causing the break of **the** epithelium sheath. **This epithelium** contains a glandular tissue in the groves of the sting that flows for the wound.

The size and effectiveness of the stings to promote the envenomation is **variable**, The family Gymnuridae (butterfly or butter stingrays) presents a small sting near to the base of the tail, and the envenomation is rare and rarely **is** severe, once the movement of whip does not allow a **violent blow. This fact minimizes** the trauma and the capacity of inoculation of the venom (Fig. 3). The other families, especially the Dasyatidae (nail or sandpaper stingrays – Figs. 1 and 2), have more developed stings, localized in medial or distal positions in the tail, causing severe injuries and important **envenomation** in the victim. The main species of stingrays capable of causing severe envenomation are *Dasyatis guttata*, *D. say*, *D. centroura*, *D. americana* (Americas), *Dasyatis brevicaudata* (and other species in Indo-Pacific) (Haddad Jr 2008; Halstead 1970; Haddad Jr et al. 2013), *Dasyatis pastinaca* and *Myliobatis aquila* (Europe), *Rhinoptera bonasus* (cownose

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Fig. 3 Stingray of the Gymnuridae family. The stinger is very small, so as the tail (Photo: Vidal Haddad Junior)

Fig. 4 The cownose stingray (Rhinopteridae family) (Photo: Vidal Haddad Junior)

stingray – Fig. 4), and *Aetobatus narinari* (the spotted stingray). The last two species are present worldwide.

The **envenomation** caused by marine stingrays is severe, causing intense pain, local inflammation manifested by edema and erythema, and systemic manifestations not associated with the venom, which has local effects, but associated to pain, as malaise and cold sweating (Haddad Jr 2008; Haddad Jr et al. 2013). Skin necrosis can be observed but is less common than in envenoming by freshwater stingrays (Haddad Jr 2008; Haddad Jr et al. 2013). The necrosis **causes** chronic ulcers which take months until healing and cause serious problems to the activities of the fishermen. Other venomous cartilaginous fish are the sharks of the *Squalus* genus (dogfish) and *Heterodontus* genus. They present two spines in anterior position to the dorsal fins and can cause painful stings in fishermen. The chimaeras, a curious cartilaginous fish, also present a venomous spine in the same position, but envenomations by chimaeras are very rare (Haddad Jr 2008). The venoms of these species are poorly studied and there is no additional information about their action.

Conclusion and Future Directions

The evolution of the venoms and venomous apparatus in fish points to mechanisms of defense and not attack. However, the toxins are a valuable path to experimental studies and development of new drugs and antivenoms for the envenomations. The studies about cartilaginous fish and specially the

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stingrays are good models for these studies, and the severity of the sting and the action of the venoms justify the scientific interest in these fish.

Cross-References

- ► Classification and Distribution of the Aquatic Venomous and Poisonous Animals
- ► Intoxications Caused by the Ingestion of Seafood and Fish
- ► Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Review
- ► Venomous Freshwater Fish
- ► Venomous Marine Fish: Osteichthyes (Bony Fish)

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Venomous Marine Fish: Osteichthyes (Bony Fish)

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Abstract

The authors discuss the presence of the toxins and envenomations caused by marine bony fish, a very common cause of injuries in fishermen and bathers. The most important families of marine venomous fish are commented, and the biochemical characteristics of the venoms and clinical/therapeutical aspects of the envenomations are cited. The authors emphasize their studies on toadfish, scorpionfish, and marine catfish, the fish most frequently associated with human injuries.

Introduction

The most venomous fish are included in the bony fish, such as the stonefish (Synanceiidae) and the scorpionfish (Scorpaenidae). They can cause severe envenomations and death in humans. However, a great number of bony fish present toxicity **and there are more venomous fish than reptiles, as an example (**Haddad 2008). The most important **fish** in frequency, severity of the envenomations, and importance of the venoms for experimental and clinical studies will be commented in this chapter.

Ariidae and Plotosidae (Marine Catfish). Synanceiidae (Stonefish). Scorpaenidae (Lionfish, Scorpionfish, and Stonefish). Batrachoididae (Niquims (*Thalassophryne* sp.)). Trachinidae (Weever Fish). Clinical Aspects of the Envenomations. Actual Studies on the Characteristics of the Venoms

Ariidae Family

The catfish are the most important fish associated to human injuries, both in marine and freshwater environments (Haddad 2008; Cardoso et al. 2009; Quay 1972; Halstead 1970; Cameron and Endean 1973; Haddad and Martins 2006). The Ariidae family is the most representative family **of catfish** around the world (Fig. 1). The *Genidens*, *Cathorops*, and *Bagre* genders are associated with the majority of the injuries in the South Atlantic Ocean, but there are **many** dangerous species around the world, such as the striped catfish *Plotosus lineatus* of the Indo-Pacific region (Plotosidae family) (Haddad 2008; Cardoso et al. 2009; Quay 1972; Halstead 1970; Cameron and Endean 1973; Haddad and Martins 2006; Haddad et al. 2008, 2009). The injuries occur when the fishermen manipulate fish and shrimps in their nets and when swimmers step on small catfish discarded at the beaches by amateur or professional fishermen (Haddad 2008).

Marine catfish cause envenomation through serrated bony stings localized in anterior position to the dorsal and pectoral fins, which can be locked in extended position, that increases the risk of

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Fig. 1 Marine catfish (*Genidens genidens*). Note the three stings in the dorsal and pectoral fins (Photo: Vidal Haddad Junior)

Fig. 2 A serrated sting of a catfish in the finger of a fisherman. The catfish are the fish that provoke the majority of human injuries around the world (Photo: Vidal Haddad Junior)

serious **trauma** (Fig. 1). The stings are covered by an integumentary sheath with glandular venomous tissue. This **epithelium breaks** when the sting penetrates in the victim and it liberates the venom in the wound. **The victim presents** intense pain, blanching in the point of the puncture, malaise and vomiting (due the pain), and **(rarely)** skin necrosis (Haddad 2008; Cardoso et al. 2009; Quay 1972; Halstead 1970; Cameron and Endean 1973; Haddad and Martins 2006) (Fig. 2). The catfish **envenomation** causes local effects and courses with intense pain and complications such as secondary infections and broken fragments in the wound stings (Haddad 2008; Cardoso et al. 2009; Quay 1972; Halstead 1970; Cameron and Endean 1973; Haddad and Martins 2006; Haddad et al. 2009).

The yellow catfish (*Cathorops spixii*) is one of the most abundant species of catfish in the southeastern coast of Brazil. The venoms used in the studies are found in the glandular epithelium which covers the sting (a) or found in the body mucus produced by cells denominated as *club cells* (a) as described by Figueiredo et al. for the evaluation of the inflammatory reaction induced by the main venoms (mucus and sting) of *C. spixii*, allowing the examination of the kinetics of leukocyte recruitment into peritoneal cavity and the mediator production that take place during this type of response (Figueiredo and Menezes 1978). Initially, it was described that both mucus and sting

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venoms of *C. spixii* induce an increase in vasopermeability in the peritoneal cavity in Swiss male mice dependent on the local production of LTB4 (leukotriene B4). In addition, while sting venom promotes a classical inflammatory reaction characterized by the influx of neutrophils at 2 h followed by macrophages after 24 h, mucus venom sustains in the peritoneal cavity of mice the recruitment of neutrophils until 24 h after injection with a late infiltration of macrophage 7 days after injection. In particular, mucus venom can affect the phenotype of these phagocytic cells, inducing their maturation through the increase of the expression of molecules responsible for the antigen presentation as MHC class II and of the production of IL-12p70 cytokine involved in Th-cell activation (Junqueira et al. 2007).

Recently, to gain a better understanding in the inflammatory responses induced by both venoms of the catfish *C. spixii*, an investigation was made on the peptide and protein components of fish skin mucus in comparison with those in the sting venom using LC−MS/MS approach and the biological functions of both types of components in the microcirculation of mice using an intravital microscopy. Initially, the electrophoretic analysis of both samples revealed seven bands in the sting venom and nine bands in the skin mucus. Sting venom and skin mucus presented common bands with high mass, around 40–60 kDa and 13–15 kDa. Moreover, an interesting and different feature of sting venom was observed with the presence of three bands of 26, 60, and 70 kDa which were lacking in the skin mucus. After fractionation using reversed-phase binary HPLC, it was revealed that sting venom has 11 fractions, while the skin mucus has 13 fractions. The peptide fractions are more intense in the skin mucus, and the proteic components were more intense in the sting venom. Moreover, 13 common proteins were identified in both the samples such as an H2ab protein (gi148227934), a chain B crystal structure of oxyhemoglobin (gi209156416), the enzyme APOBEC-2 (gi209736158), a protein similar to melanotransferrin precursor (gi16343451), and the WAP65 (gi158021040), an acute phase protein involved in the inflammatory response increasing the number of leukocytes rolling and adhering to the endothelium. Peptide fractions from the sting venom (1–5) and skin mucus (1–7) analyzed by MALDI-TOF mass spectrometry revealed a higher number of components in the sting venom compared with the fractions collected from the skin mucus. Therefore, two peptides from skin mucus presented as pure components with 1515.62 and 1515.51 Da. Only two peptides from the sting venom and two peptides from the skin mucus showed activity against *E. coli* and only one peptide of skin mucus induced lysis of human erythrocytes. Three peptide venom fractions induced a venular stasis; moreover, another induced constriction of arteriolar vessels. Regarding the fractions obtained from skin mucus, two peptides induced hemorrhage, and two others produced an enlargement of arteriolar wall diameter. Collectively, the envenomation provoked by *C. spixii* may be the result of bioactive peptides while the inflammatory process is mainly due to the action of proteins present in sting venom and skin mucus (Ramos et al. 2012).

Scorpaenidae Family

The fish of the family Scorpaenidae and Synanceiidae are the most venomous fish in the world. Three genders provoke envenomation in humans more frequently: *Synanceia* (stonefish), *Pterois* (lionfish), and *Scorpaena* (**scorpionfish**) (Haddad et al. 2003a). The stonefish of the Indo-Pacific region cause very serious envenomation, with reports of dozens of deaths (Fig.3; Halstead 1970).

The black scorpionfish (*Scorpaena plumieri*) and the red scorpionfish (*Scorpaena brasiliensis*) are the most common and the main fish of the genus *Scorpaena* associated to injuries in humans in the Americas (Haddad et al. 2003a; Haddad and Lastória 2004; Fig. 4). The lionfish (Fig. 5), an original inhabitant of the Indo-Pacific region, is now disseminated in North and Central America.

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Fig. 3 *Synanceia verrucosa*, one of species of stonefish. The venom is located in the rays of fins, especially in the dorsal fin (Photo: Vidal Haddad Junior)

Fig. 4 *Scorpaena plumieri*, the black scorpionfish. This very venomous genus is responsible for severe accidents in the Atlantic Ocean (Photo: Vidal Haddad Junior)

Fig. 5 The lionfish is a venomous fish of the Scorpaenidae family, recently found in the Atlantic Ocean (Photo: Vidal Haddad Junior)

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Lionfish can provoke very painful injuries, with common development of bacterial infections (Haddad 2008; Haddad et al. 2003a; Haddad and Lastória 2004).

The venom apparatus of the Scorpaenidae and Synanceiidae families are composed by 12–13 rays of the dorsal fin, three rays of the anal fin, and two rays of the pelvis spines with venomous glandular tissue in the grooves of upper two-thirds of the ray. **As so** the envenomation caused by catfish, the venom flows for the wound when the ray penetrates in the skin of the victim and the epithelium is broken.

The envenoming by scorpionfish is also very serious and causes intense, excruciating pain. There are mild alterations in the point of the puncture(s) but the signs and symptoms are systemic and severe, on the contrary of the other venomous fish (Haddad 2008). It is possible to observe malaise, fever, local adenopathy, respiratory and cardiac alterations, hallucinations, and seizures (Haddad et al. 2003a; Haddad and Lastória 2004). In a series of 23 injuries, all the patients presented intense pain and systemic alterations, confirming the gravity of the envenomation (Haddad et al. 2003a).

In a documented case, they described human envenomation by stonefish with pulmonary edema, developing within 35 min of envenomation that was confirmed radiographically (Lehmann and Hardy 1993). The injection of the venom from *Synanceia trachynis* into several animal species produced evidence of increased vascular permeability (Wiener 1959). In particular, acute pulmonary edema was observed in mice that were given the venom by intravenous injection. *S. plumieri* venom injection in the footpad or peritoneal cavity of mice induces a sustained inflammatory response in the lungs characterized by an increase of vascular permeability, neutrophil influx into the BAL fluid, and the production of the pro-inflammatory mediators such as IL-6, KC, and matrix metalloproteinases which result in venom deposition in the airway, lung hemorrhage, and alveolar macrophage activation (Boletini-Santos et al. 2008). In addition, the local response induced in footpad of mice by this venom was analyzed and demonstrated that the formation of edema was dependent on the kallikrein–kinin system (Menezes et al. 2012).

Batrachoididae Family

There are various species of fish of the Batrachoididae family around the world, and the *Thalassophryne*, *Porichthys*, *Batrachoides*, and *Opsanus* genders are the most important, especially the species *Thalassophryne nattereri*, the niquim or miquim, often associated with accidents in the north and northeast regions of Brazil (Fig. 6; Haddad et al. 2003b). These fish stay motionless in sandy or muddy bottoms, and they are numerous in estuarine areas, causing accidents when stepped by bathers and fishermen in shallow waters.

The *Thalassophryne* toadfish present the most sophisticated venom apparatus known in fish (Haddad et al. 2003b). The venom is inoculated through a system of two dorsal and two preopercular hollow spines when a basal and well-constituted unique gland is pressured and injects deeply its content in the victim (Fig. 7).

Thalassophryne nattereri (niquim) is a venomous fish of the Batrachoididae family, and in Brazil, it is known by the frequency and severity of the accidents provoked in fishermen and bathers in estuarine areas of the north and northeast regions (Fonseca and Lopes-Ferreira 2000; Faco et al. 2003). The clinical manifestations are erythema, edema, and mild necrosis without systemic signs and symptoms. The venom displays proteolytic and myotoxic activities, but it is devoid of phospholipase A_2 activity (Lopes-Ferreira et al. 1998). The local pathogenesis induced by injection of the venom includes edema, erythema, and severe pain followed by intense necrosis and a markedly poor healing response (Lopes-Ferreira et al. 2001). The inefficient healing is very important for the evolution of the injury, which is devoid of specific treatment and drug therapies (Haddad et al. 2003b; Lopes-Ferreira et al. 2000). Investigations have shown that the dramatic

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Fig. 6 *Thalassophryne nattereri*, the toadfish associated with a great number of injuries in fishermen in estuarine waters of South America (Photo: Vidal Haddad Junior)

symptoms of *T. nattereri* envenomation and nociception as well as the edematous response could be related to kallikrein–kinin cascade, since only the administration of the novel tissue kallikrein inhibitor (phenylacetyl-Phe-Ser-Arg-*N*-(2,4-dinitrophenyl)-ethylenediamine – TKI) reduces these clinical manifestations but not PKSI-527 or SBTI, which are specific and nonspecific plasma kallikrein inhibitors, respectively (Lopes-Ferreira et al. 2004).

Analyses of the cremaster muscle showed that the venom elicits a peculiar alteration in the microcirculation with intense vascular congestion, thrombosis in venules, and focal transient constrictions in arterioles, without inducing hemorrhage (Lopes-Ferreira et al. 2002). The injection of venom into footpad of mice increases the mRNA for IL-1, IL-6, and TNF genes as well as the release of these soluble cytokines. Moreover, absence of leukocyte infiltration in the intraplantar region of footpad of mice after venom application was also reported (Lopes-Ferreira et al. 2001; Lima et al. 2003). In addition, Pareja-Santos and colleagues showed that *T. nattereri* venom alters the extracellular matrix structure of mouse footpad tissue by the activation of matrix metalloproteinases (MMP-2 and MMP-9), in addition to decreasing collagen fiber content during the healing phase. It was also shown that the venom affects the cytoskeleton organization and pseudopodia formation of epithelial cells in in vitro system. This scenario indicates an ambiguous role of the venom in the inflammatory process (Magalhães et al. 2005). On the one hand, it displays a potent pro-inflammatory activity illustrated by the detected chemoattractant upregulation, and on the other hand, it affects the ability of tissue healing due to the extracellular matrix disorganization caused by the MMP upregulated activity and by defective infiltration of inflammatory cells. Nevertheless, the reasons that the venom favors delayed local inflammatory response or whether a particular group of toxins in the venom directly controls the traffic of leukocytes into inflamed tissue are ill defined.

Combined proteomic and transcriptomic approaches applied to analyze *T. nattereri* venom complexity revealed the identity of the major toxins as a galactose-specific lectin belonging to the family of C-type lectins named nattectin and the family of new proteins natterins with molecular mass range of 30–45 kDa. From the venom gland of *T. nattereri*, five major natterin genes have been

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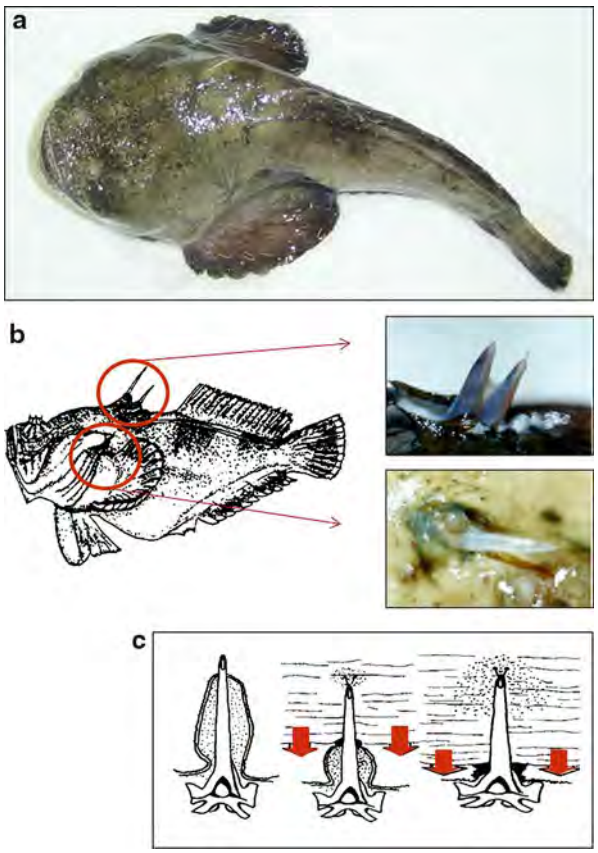

Fig. 7 ***Thalassophryne nattereri*** and venom apparatus. Specimen of *T. nattereri* collected in Mundaú Lake in the state of Alagoas, Brazil (**a**). Picture representing the location of the spines: 2 in the dorsal region and 1 in each side (**b**). Venom apparatus: the pressure on the gland located at the base of spikes promotes the venom passage into the canal of the spine (**c**) (Source: Photo and illustration kindly provided by Dr. Monica Lopes-Ferreira)

identified which are named natterin 1–4 and natterin P. Natterin 1 and 2 have a sequence identity of 84 %, whereas natterin 3 and 4 are to around 40 % identical with natterin 1 and 2. Natterin P has 84 % sequence identity to natterin 4. On the functional level, natterins are shown to have a kininogenase activity and therefore are able to activate inactive kininogens into active kinins, which were associated with nociceptive and edema-inducing effects (Lopes-Ferreira et al. 2004; Magalhães et al. 2006; Piran-Soares et al. 2007).

The venom of *Thalassophryne nattereri* and their toxins have been widely studied for various aspects such as the toxic effects in human victims (Fonseca and Lopes-Ferreira 2000) and in experimental models (Faco et al. 2003; Lopes-Ferreira et al. 1998, 2000, 2001, 2002, 2004, 2011; Lima et al. 2003; Pareja-Santos et al. 2009; Magalhães et al. 2005), the biochemical and pharmacological aspects (Lopes-Ferreira et al. 2004, 2011; Magalhães et al. 2006; Piran-Soares et al. 2007), and the ability to induce neutralizing antibodies to the major toxic effects in mice (Lopes-Ferreira et al. 2000; Komegae et al. 2011). The search for an effective therapy for envenomation caused by venomous fish *T. nattereri* demonstrated the importance of specific antibodies produced in high and constant levels in rabbits and horses in the neutralization of toxic activities (Lopes-Ferreira et al. 2000) and proved experimentally that animals with high serum titers of specific antibodies show lower responses of nociception, edema, or necrosis when subjected to a second contact with the venom. Besides the confirmation of the persistence of IgG antivenom in mice, patients injured by fish have high levels of IgG antivenom for up to 6 months after the accident (Komegae et al. 2011).

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The initial works for the biochemical characterization and molecular identification of *T. nattereri* venom toxins and the evaluation of the inflammatory response in injured tissues led us to the construction of a useful methodological tool for better understanding of the importance of the cellular innate response in containing toxins and consequently in restoring the integrity of damaged tissue. In this murine model, it was demonstrated that the venom produces a delayed and deficient cellular infiltrate and affects the ability of tissue regeneration due to the disorganization of the extracellular matrix caused by increased matrix metalloproteinase (MMP) activity, which hinders the infiltration of inflammatory cells (Magalhães et al. 2005). Furthermore, these studies allowed to identify the venom of *T. nattereri* or their toxins as good immunogenic molecules able to modulate the innate and adaptive arms of the immune system.

To gain new insights into the mechanisms of venom pathogenesis and to further elucidate the role of its major toxins, the natterin family and nattectin were undertook in in vitro and in vivo investigations using these isolated toxins. The extracellular matrix components and the integrin β1 subunit are targets for the natterins and nattectin. The extracellular matrix degradation or remodeling activities exerted by these toxins affect cell–cell and cell–extracellular matrix adhesion and survival and impair inflammatory cell migration into inflamed tissues. Nattectin has a specific ability to bind types I and V collagen constituents of the extracellular matrix and venular basement membrane and improves integrin-mediated HeLa cell adhesion and resistance to apoptosis by its binding to RGD-dependent integrins, especially the β1 subunit. Natterins bind and cleave type I and IV collagen, disrupting cell attachment and HeLa cell survival, and have cytotoxic effect on both adherent cells or at in suspension, showing direct induction of necrosis that is followed by cell detachment (Saraiva et al. 2011).

Nattectin, a basic and non-glycosylated monomeric protein with 15 kDa, which contains the QPD (Gln-Pro-Asp) sequence in the carbohydrate recognition domain, recognizes Gal-β(1–3)-*N*-acetylgalactosamine and showed a Ca^{2+}-independent hemagglutinating activity. Nattectin acts on endothelial cell membranes inducing activation, and subsequent release of chemotactic factors such as IL-1β, LTB4, KC, MMP-9, and MMP-2 induced persistent neutrophil mobilization in mice (Lopes-Ferreira et al. 2011). The macrophages recruited into peritoneal cavity by nattectin produce lipidic mediators and IL-12 p70. Macrophages derived from 7-day nattectin mice were CD11c + $CD11b^{low}Ly6^{high}F4/80R^{high}$ and express high levels of MHC class II and CD80 molecules. Culture of peritoneal exudates derived macrophages from 7-day nattectin mice and immature BMDCs with nattectin markedly increased the surface expression of CD40, CD80, CD86, and MHC class II in a dose-dependent manner and the production of MMP-2 and MMP-9 distributed in nucleus and cytoplasm of cells that was associated with strong activity in the culture supernatant. Nattectin-treated DCs secreted IL-12 p70 and IL-10. The nattectin-treated BMDC or macrophage-derived DCs were highly efficient at Ag capture. The specific immune response elicited by nattectin was characterized by the production of specific antibodies IgG1 and mainly IgG2a with IL-10 and IFN-γ synthesis by splenic cells. These results enable us to address that nattectin induces the recruitment of $Ly6C^{high}$ monocytes into the peritoneum, which exhibit a pro-inflammatory profile, where they differentiate into proliferating $F4/80R^{high}$ macrophages. Macrophage-derived DCs mature in the presence of the cytokine milieu generated against nattectin, exhibiting T-cell co-stimulatory molecule expression and induced a Th1-polarized response (Ishizuka et al. 2012).

Moreover, nattectin was described as an important immunomodulatory agent that controls the process of macrophage activation and M1/M2 polarization. The polarization of peritoneal and bone marrow-derived macrophages induced by nattectin to M1 profile is dependent on Th1 cytokines (IL-12 and IFN-γ) and negatively regulated by Th2 cytokines (IL-4, IL-10, and IL-13). Also it was revealed that IL-4 plays a dual role in this polarization: a regular action of IL-4 was seen in the

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negative regulation of the CD40 expression, but an unexpected positive regulation was seen in the expression of CCR7 and MHC class II. Finally, the in vivo studies showed that the influx of neutrophils and small peritoneal macrophage – F4/80^{low}MHCII^{hi} induced by nattectin is totally dependent on IL-4 and IFN-γ cytokines. Furthermore, the induction of IL-6 release is negatively regulated by IL-4 and positively regulated by IL-12 and IFN-γ. Together, the results allowed us to expand the knowledge about the regulation of macrophage activation, as well as confirmed the ability of nattectin, a fish C-type lectin, as an important immunomodulatory agent (Grund et al. 2006).

Studies for the better understanding of the capacity of the *T. nattereri* venom to modulate the immune system had started in 2006 and identified in mice that lower doses of venom induce a Th1 and Th2 and humoral memory immune response identified by the production of IL-5 and IFN-γ by splenic cells and by the production of total IgE antibodies and venom-specific IgG1 and IgG2a. Also, it was observed that the B lymphocytes expressed low levels of CD45R/B220 molecule (Grund et al. 2012).

The presence of the specific T response with high levels of specific IgG antibodies confirmed that B cells with effector or memory functions have been generated and also the presence of B cells negative for B220 molecule point to the differentiation of long-lived antibody-secreting cells (ASC), which represent a major source of protective antibodies and ensure long-term immunity. Additional studies to better understand the memory compartment generated by venom confirmed that *T. nattereri* venom can trigger a chronic humoral response in BALB/c mice (Grund et al. 2013). Up to 74 days of immunization, it was observed an increased production of specific IgG2a antibodies that diminish after 120 days. In contrast, the levels of specific IgG1 and IgE increases dramatically after 120 days, as compared to levels observed between days 21–74. These results indicate that persistent interaction of venom proteins with the cells of the germinal center of the spleen promotes a shift toward Th1 to Th2 response. This study also confirmed a hierarchical relationship of ASC development, since the venom induced dependent on time and on the micro-environment (peritoneum, spleen, or bone marrow) cells with different expression of B220 and especially in the inflamed peritoneum the differentiation and maintenance of ASC terminally differentiated (B220^{neg}). It was observed that chronic humoral response was accompanied by an intense inflammatory process in the peritoneum, characterized by mast cells, eosinophils, and mainly neutrophils. Although this study demonstrated for the first time the important role of IL-17A along with IL-5 in the differentiation and maintenance of ASC with B220^{neg} phenotype, the treatment of the animals with neutralizing anti-IL-17A or anti-IL-5 before immunization and a booster dose with the *T. nattereri* venom completely prevented the differentiation and maintenance of ASC B220^{neg} in the inflamed site and the production of IgE, in contrast, inducing differentiation of ASC with high levels of B220 molecule and the preferred synthesis of IgG2a. In addition, in this study, it was observed that the memory phase of the humoral response elicited by the venom is composed of effector memory T lymphocytes (TEM) CD4+ CD44^{high}CD40L^{pos}Ly6C^{pos} in the peritoneum producing IL-4, IL-5, IL-17A, and central memory T lymphocytes (TCM) CD4 + CD44^{high}CD40L^{neg}Ly6C^{pos} in spleen and in bone marrow producing IL-5 and IL-23. Together, it was observed that the chronic humoral response induced by the venom has a complex modulation and is marked by different subtypes of T lymphocytes producing typical cytokines that promotes a microenvironment rich in co-stimulatory molecules and mediators such as cytokines.

Moreover, it confirmed the existence of a hierarchic process of differentiation: activated memory B cells dependent on antigen progressively acquire increased levels of CD138 and decreased levels of CD45R/B220 to finally arrive at ASC with B220^{neg} phenotype, which are IgG1-secreting cells dependent on IL-17A. Only Bmem from peritoneal cavity or bone marrow of venom-immunized

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mice presented the capacity to generate functionally active ASC. IL-17A or IL-21/IL-23/IL-33 improves the ability of venom to induce intracellular IgG of peritoneal derived ASC. Cognate stimulations with venom and IL-17A are sufficient to downregulate the expression of CD45R/B220 (Komegae et al. 2013a).

The generation of long-lived antibody-secreting cells (ASC) and memory B cells is a critical event for an effective vaccine and the choice of adjuvant can influence these processes. Various cellular and molecular mechanism involved in the protease action that determine Th2 responses have been identified. However, direct or indirect actions in the regulation of the induction, survival, and longevity of ASC in differential compartments remain largely unknown. It was investigated whether the proteolytic activity of venom proteins such as natterins is determinant for the modulation of the memory immune response in mice, promoting the differentiation of memory B cells to terminally differentiated end-stage cells. Komegae et al.provide a new evidence that the Th2-polarized humoral response induced by proteases needs a key component – the participation of long-lasting Bmem and ASC (Grund et al. 2013). Collectively, it was showed that (1) in the early phase of response against natterins, the proteolytic activity positively controls the formation of B1b cells in the spleen and BM, of Bmem in all compartments, and of ASC $B220^{pos}$ and ASC $B220^{neg}$ in the spleen and the production of Th2 Abs such as anaphylactic IgG1 and IgE. Also in the early phase, the proteolytic activity of natterins negatively controls the formation of B1a in the peritoneal cavity, of B2 in all compartments, and of ASC $B220^{pos}$ in BM and ASC $B220^{neg}$ in the peritoneal cavity; (2) in the chronic phase of memory response to natterins, the proteolytic activity controls the exit of B2 and Bmem from the peritoneal cavity and the exit of ASC $B220^{pos}$ and ASC $B220^{neg}$ from both the niche peritoneal cavity and BM, inducing the maintenance of B1a and B2 in the spleen and BM and B1b, Bmem, and ASC $B220^{neg}$ in the splenic niche. Finally, high-affinity specific IgG1 and IgE Abs response dependent on the protease activity of natterins is produced by memory B cells and ASC generated in the GC after innate-like B1b or B2 activation but not by peripheral innate-like B cells that have not entered into GC.

Finally, there is a study to gain better insights into the role of TLRs and MyD88 in the development and differentiation of memory B cells, especially of ASC, during the Th2-polarized memory response induced by natterins (Komegae et al. 2013b). In vivo findings demonstrated that the anaphylactic IgG1 production is dependent on TLR2 and MyD88 signaling and that TLR4 acts as an adjuvant accelerating the synthesis of high-affinity IgE. Also, TLR4 (MyD88 independent) modulated the migration of innate-like B cells (B1a and B2) out of the peritoneal cavity and the emigration from the spleen of B1b and B2 cells. TLR4 (MyD88 independent) modulated the emigration from the spleen of Bmem as well as ASC $B220^{pos}$. TLR2 triggered to the egress from the peritoneum of Bmem (MyD88 dependent) and ASC $B220^{pos}$ (MyD88 independent). Additionally, TLR4 regulates the degree of expansion of Bmem in the peritoneum (MyD88 dependent) and in BM (MyD88 independent) as well as of ASC $B220^{neg}$ in the spleen (MyD88 independent). TLR2 regulated the intensity of the expansion of Bmem (MyD88 independent) and ASC $B220^{pos}$ (MyD88 dependent) in BM. Finally, TLR4 signals sustained the longevity of ASC $B220^{pos}$ (MyD88 independent) and ASC $B220^{neg}$ into the peritoneum (MyD88 dependent) and TLR2 MyD88-dependent signaling supported the persistence of B2 cells in BM, Bmem in the spleen, and ASC $B220^{neg}$ in the peritoneum and BM. Terminally differentiated ASC$B220^{neg}$ required the cooperation of both signals through TLR2/TLR4 via MyD88 for longevity in the peritoneum, whereas Bmem required only TLR2/MyD88 to stay in the spleen, and ASC $B220^{pos}$ rested in the peritoneum dependent on TLR4 signaling. Also it was confirmed that the longevity of ASC $B220^{neg}$ into the inflamed peritoneal cavity induced by natterins is strongly supported by upregulated CXCR4 expression dependent on SP1R signals in B lymphocytes and its recirculation through lymphoid

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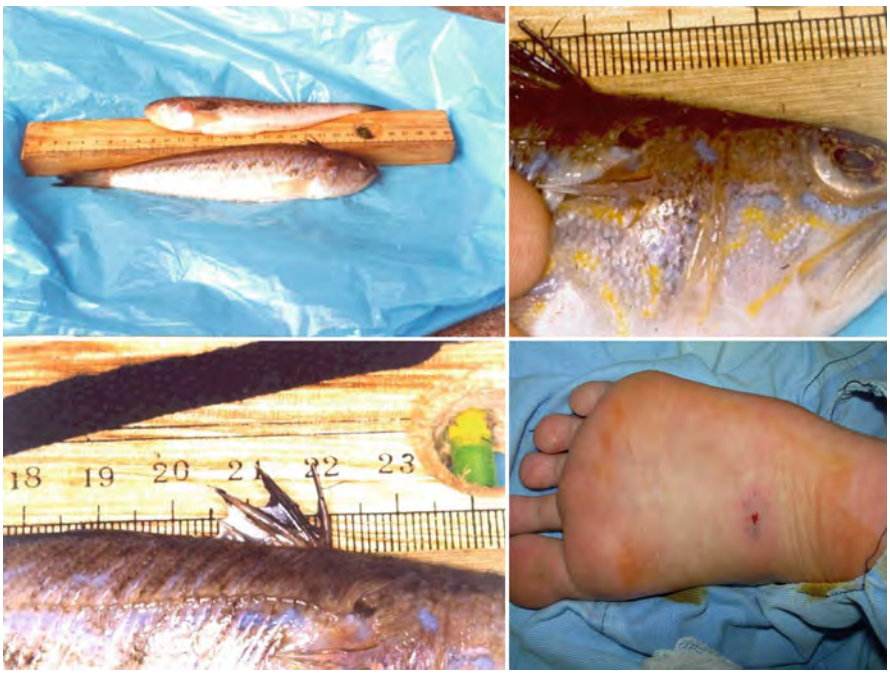

Fig. 8 The weever fish are found in the Mediterranean and African coast and are responsible for the majority of injuries caused by venomous fish in Europe (Photo: Vidal Haddad Junior)

organs. The data sustain that earlier events on memory B-cell differentiation induced in secondary immune response against natterins, after secondary lymph organ influx and egress, may be the key to determining peripheral localization of innate-like B cells and memory B cells such as ASC $B220^{pos}$ and ASC $B220^{neg}$.

In conclusion, the understanding of the requirement of TLR/MyD88 and the co-participation of lymph- or blood-derived S1PR signals for the in vivo differentiation and survival for long time of memory B cells and especially of the long-lasting subtype (ASC) induced by proteases derived from *T. nattereri* venomous fish with kininogenase activity allow a further clarification of the role of proteases in the development and maintenance of chronic Th2 disorders.

Trachinidae Family

The weever fish of the Trachinidae family present a thermolabile and proteic venom, inducer of skin necrosis and intense pain (Haddad 2008). In experimental studies there cardiorespiratory depression phenomena. The venom has neurotoxic effect similar to that of sea snakes (Skeie 1962). These fish are found only in the waters of the Atlantic Ocean in Europe and North Africa, causing the majority of the envenomation by fish in Europe (Fig. 8). The secondary infections can aggravate the manifestations and cause risk of death.

Conclusion and Future Directions

The studies on venoms of marine fish are in a crescent, due to the number of victims around the world and the pharmacological potential of the toxins, both motivating new researches on the species, initial treatments, and prevention of accidents, and in the marine venomous fish are some of the most venomous animals in the world, such as the stonefish and the scorpionfish.

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Cross-References

- ▶ Classification and Distribution of the Aquatic Venomous and Poisonous Animals
- ▶ Intoxications Caused by the Ingestion of Seafood and Fish
- ▶ Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Review
- ▶ Venomous Marine and Freshwater Fish: Osteichthyes (Bony Fish)
- ▶ Venomous Marine Fish: Evolution of the Venoms. Chondrichthyes (Cartilaginous Fish)

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Marine and Freshwater Toxins
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Venomous Freshwater Fish: Catfish and Freshwater Stingrays

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Abstract

The authors comment the actual research on the properties of the toxins of the freshwater venomous fish and their potential use in Human Medicine. They discuss the mechanisms of inoculation, biochemical characteristics of the venoms, and clinical/therapeutical aspects of the envenomations, **with emphasis in their studies** on freshwater stingrays and freshwater catfish, the fish most frequently associated with human injuries **in these environments**.

Introduction

The most important venomous freshwater fish are the catfish, which are common worldwide. The venom is present in a sheath that covers three stingers in dorsal and pectoral positions, and the envenomation is very painful but does not present systemic manifestations, excluding those caused by the intense pain. However, secondary infections and break of the stinger are complications that can occur. Freshwater stingrays present one to four stingers on the tail and they can cause severe envenomations in humans, presenting excruciating pain and skin necrosis.

Pimelodidae and Ictaluridae Families: Freshwater Catfish. Freshwater Stingrays (Potamotrygonidae Family). Clinical and Therapeutic Aspects of the Envenomations. Characteristics of the Venoms

Pimelodidae and Ictaluridae Families

The freshwater catfish are siluriform fish common in rivers and lakes. There are various venomous genders and species, as the South American *Pimelodus* and *Pimelodella* genders **and** the North-American *Ictalurus* and *Noturus* genders. The main **human** populations at risk are amateur and professional fishermen (Fig. 1) (Haddad 2008; Cardoso et al. 2009; Quay 1972; Halstead 1970; Cameron and Endean 1973). There are other species **in the** Pimelodidae family that present venom **on** the stings, but they only provoke **occasional** injuries and they **do** not have epidemiologic importance (Haddad 2008; Haddad and Lastória 2005).

A great number of fish of this family presents bony stings in anterior position to the dorsal and pectoral fins, but only some genera present venom. The venom is localized in **a sheath** that recovers the sting. **The venom** flows to the wound when the sheath is broken **in the** penetration of the sting, similar to the marine catfish.

The penetration of the sting and inoculation of the venom cause intense pain, edema, and erythema. The painful process is intense and fades after near 6 h, but the complications are common,

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Fig. 1 The South American yellow catfish (*Pimelodus maculatus*), as other species of freshwater catfish, causes injuries in fishermen (Photo: Vidal Haddad Junior)

Fig. 2 The envenomation caused by freshwater stingrays is very severe, but the effects are local, causing extensive skin necrosis (Photo: Vidal Haddad Junior)

as retention of fragments of the sting or bacterial infections (Haddad 2008; Haddad and Lastória 2005).

Potamotrygonidae Family

The elasmobranches of the family Potamotrygonidae are the unique stingrays adapted to freshwater environment, and they exist only in South America. There are **three to four** genus of freshwater stingrays and the most important as much in number of species as in associations with human envenomation is the *Potamotrygon* genus (Fig. 2) (Haddad et al. 2004, 2013; Garrone Neto and Haddad 2010).

The freshwater stingrays currently are spreading for the South American freshwater system (especially in Brazil) (Garrone Neto and Haddad 2010). These fish, widely distributed in Amazon and La Plata basins, have physical appearance and habits similar to marine stingrays. They remain buried in sandy bottoms in shallow water, causing many accidents on people crossing rivers (Haddad et al. 2004, 2013; Garrone Neto and Haddad 2010).

Freshwater stingrays have **one to four stingers** on the tip of the tail, which penetrate the victim when the tail is whipped (**usually when the fish is stepped on**). The stinger contains venomous glandular tissue in its groves and the immediate effects are similar to those observed in marine

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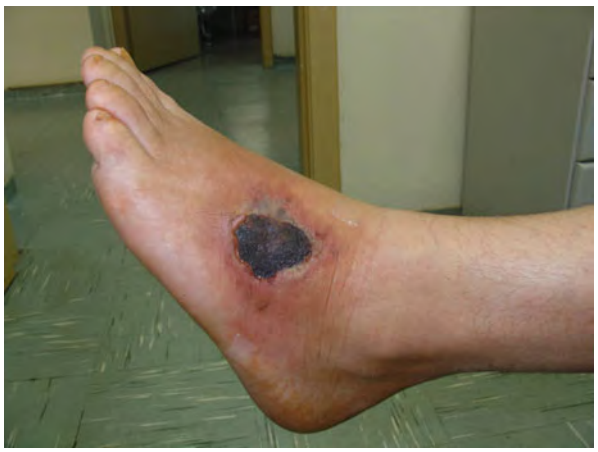

Fig. 3 The envenomation by freshwater stingrays always causes dry necrosis and results in chronic ulcers (Photo: Vidal Haddad Junior)

stingrays (intense pain and edema/erythema at the point of inoculation). After nearly 24 h, the pain diminishes and there is necrosis (Fig. 3). The skin necrosis is the rule in the envenomation by freshwater stingrays, causing chronic ulcers. The complications are common, especially secondary infection and retention of fragments of the stinger (Haddad et al. 2004, 2013; Garrone Neto and Haddad 2010).

Secondary infection installs itself as precipitating or aggravating factor of large ulcers usually on the lower limbs, in the ankle, or foot. Initial measures of treatment are not different from those used in other venomous fish, such as the use of hot water, but the use of systemic antibiotics in all cases should be encouraged, because the prevention or control of infections interferes with evolution. The wound shall be exploited for removal of fragments and intensive washing. Established ulcers may require skin grafts. The early excision of the lesion can be tried empirically, with dubious results (Haddad et al. 2013).

Stingrays of the family Potamotrygonidae are widespread throughout river systems of South America that drain into the Atlantic Ocean. Some species are endemic to the most extreme freshwater environment of Brazil and cause frequent accidents to humans.

Magalhães et al. provided in vivo evidences of toxic effects for the species *P.* cf. *scobina* and *P.* gr. ***o****rbignyi* venoms on target cells in microcirculatory environment (Magalhães et al. 2006). Both stingray venoms induced significant edematogenic and nociceptive responses in mice. The increase in the temperature of storage promoted a crescent decrease of the edematogenic and nociceptive responses induced by both venoms. A strike augments of leukocytes rolling and adherent cells to the endothelium of cremaster mice were induced by both venoms. The data also presented that injection of both venoms induced necrosis and low level of proteolytic activity, without inducing hemorrhage. But when the venoms of both stingray species were injected, added with their respective mucus secretion, the necrotizing activity was more vigorous.

A combination of analytical protocols such as reversed-phase high-performance liquid chromatography (RP-HPLC), followed by a biological microcirculatory screening and mass spectrometry analysis, allowed the identification and characterization of two novel bioactive peptides from the venom glands that cover the stings of *Potamotrygon.* gr. *orbignyi* termed Orpotrin and Porflan (Conceição et al. 2006). Orpotrin isolated from venom glands induces a major vasoconstrictive effect on large arterioles of the microcirculatory network of cremaster muscle of mice under physiological conditions, and the primary sequences of Porflan and bioassay results of natural

peptide or two synthetic analog peptides suggest that these toxins are a new class of fish toxins, directly involved in the inflammatory cell recruitment processes of a stingray sting (Conceição et al. 2009, 2011).

It was described that the mucus and venom of the stingray *Potamotrygon* cf. *henlei* possess a diverse mixture of peptides, enzymes, and pharmacologically active components with antimicrobial activity that inhibited the growth of *C. albicans* and induce a pro-inflammatory response characterized by induction of nociceptive, edematogenic, and proteolytic activities accompanied by a dose-dependent increase in vascular permeability, neutrophil and macrophages recruitment, and secretion of IL-1β and IL-6 cytokines and a higher release of KC and MCP-1 chemokines (Monteiro-dos-Santos et al. 2011).

A protein similar to the β-chain of hemoglobin with a molecular weight of 16072.8 Da was identified, isolated, and partially sequenced from the mucus of *P.* cf. *henlei* and was shown to be responsible for antimicrobial activity against bacteria (*Micrococcus luteus* and *Escherichia coli*) and yeast (*Candida tropicalis*) without hemolytic activity. The topical application of 4 μM of the protein in the microcirculation environment induced an increase in the number of leukocyte rolling without change in numbers of adhered cells (Conceição et al. 2012).

Together these studies also demonstrated for the first time that toxic effects provoked by injection of venoms from Brazilian stingrays in mice show moderate levels of intensity, as well as the presence of proteins and peptides within the mucus that contribute to potentiating the local tissue destruction. The results obtained provide fundamental information for future basic research, clinical diagnosis, and development of new therapies to accident.

Additionally, comparative studies between venoms of and marine venoms of stingrays showed differences in the enzymatic composition, with high levels of hyaluronidase in the freshwater stingray's venom, a fact clearly linked to the great skin necrosis observed in the envenomations (Haddad et al. 2004; Barbaro et al. 2007; Pedroso et al. 2007).

Conclusion and Future Directions

The number of venomous fish that use venom as mechanism of defense is very high and virtually all families have specimens that present toxins in the skin or specialized structures capable to inoculate the venom. The toxins cause human envenomation that should be understood by health teams and are object of studies of their biochemical and toxinological properties. However, this great underwater laboratory is poorly studied and needs more attention for their rich possibilities. The medical knowledge about first aids and complications of an envenomation by fish is not satisfactory and a patient injured by a venomous fish does not have the correct measures for control of the pain. It is the hour of new studies on the field and the hope is to contribute with this idea with the information in this chapter.

Cross-References

► Classification and Distribution of the Aquatic Venomous and Poisonous Animals
► Intoxications Caused by the Ingestion of Seafood and Fish
► Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Review
► Venomous Marine Fish: Evolution of the Venoms. Chondrichthyes (Cartilaginous Fish)
► Venomous Marine Fish: Osteichthyes (Bony Fish)

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Toxinology
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Pufferfish Poisoning and Tetrodotoxin

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Abstract

Pufferfish possess a potent neurotoxin, tetrodotoxin (TTX), and have caused human intoxications all over the world, especially in East Asia, where people have a time-honored food culture of eating pufferfish. TTX is distributed over a wide variety of organisms including flatworms, ribbon worms, mollusks, crustaceans, starfish, fish, and amphibians, and in the East Asian or Southeast Asian countries, TTX poisonings due to marine snails, horseshoe crabs, and gobies have also occasionally occurred, suggesting the possibility of further geographic expansion and/or diversification of TTX-bearing organisms caused by a change in the marine environment. The origin of TTX is likely certain species of marine bacteria, and many TTX-bearing organisms are toxified via the food chain that starts with the bacteria. Recently, valuable information regarding the dynamics of TTX taken up into the pufferfish body through food organisms, and the molecular mechanisms involved is accumulating, while TTX-bearing organisms are assumed to utilize TTX effectively for their survival, although TTX is a fatal toxin to organisms that possess no TTX. The present chapter describes the distribution of TTX in metazoans, and then introduces recent studies to clarify the intake of TTX to pufferfish liver using a pharmacokinetic model, and to reveal the transfer profile of TTX in pufferfish based on several TTX administration experiments. Moreover, after discussing on the potential physiologic functions of TTX in TTX-bearing organisms such as defense, offense, and attraction, human intoxications due to TTX will be summarized.

Introduction

Pufferfish, of the order Tetraodoniformes, includes 2 suborders, 10 families, 93 genera, and 430 species. They widely distribute from the tropical to the temperate waters around the world and inhabit a variety of ecosystems, marine, estuarine, and fresh waters. Pufferfish has morphologically distinct features such as long and tapered bodies, bulbous heads, small mouths, and hard beaks. Additionally, the family Tetraodontidae (pufferfish) has no pelvic fins and ribs. Pufferfish is apparently to defend themselves by various means. Some species have the remarkable ability, inflatability, to expand their body quickly by swallowing huge amounts of water and display their bigger shape against the predators, when threatened. Porcupine fish, the family Diodontidae, has long and pointed spines on the skin to cover its body. Boxfish, the family Ostraciidae, wears in armor. Pufferfish is omnivorous in their eating habitats. Stomach analysis showed fragments of corals, sponges, algae, mollusks, crustaceans, and fish.

Pufferfish is well known to be among the most poisonous fish and contain a considerable amount of tetrodotoxin (TTX). TTX named after the family Tetraodontidae in 1909 by Tahara and was

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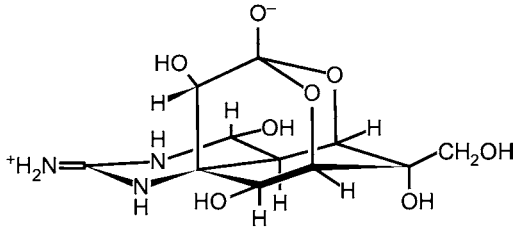

Fig. 1 Structure of TTX

isolated as a crystalline from pufferfish *Takifugu rubripes* ovary in 1950 by Yokoo. The structure of TTX was independently determined in 1964 by three groups, Goto et al., Tsuda et al., and Woodward et al. The synthesis of racemic TTX was achieved in 1972 by Kishi et al. and the total synthesis of TTX was successful in 2003 by Isobe et al. TTX has a unique chemical structure, a heterocyclic guanidine compound, with a molecular formula of $C_{11}H_{17}N_3O_8$ and a molecular weight of 319.27 (Fig. 1) (Moczydlowski 2013). To date, more than 30 of TTX analogs have been isolated and structurally elucidated from wild TTX-bearing organisms such as pufferfish and newts (Yotsu-Yamashita 2001), since Nakamura and Yasumoto (1985) found tetrodonic acid, 4-*epi*-TTX, and 4,9-anhydroTTX from the pufferfish, *Takifugu pardalis* and *Takifugu poecilonotus*, as the minor toxin principles. Although crystalline TTX is insoluble in water as well as in all sorts of organic solvents, it is soluble and thermostable in weakly acidic solutions. In strongly acidic and alkaline solutions, however, the toxin is labile and decomposed to lose toxicity by heating.

TTX is a potent neurotoxin which selectively binds voltage-gated sodium channel in muscle and nerve tissues, resulting in paralysis of the tissues concerned, in a severe case fatality by respiratory paralysis (Narahashi 2001). Fifty percent lethal dose (LD_{50}) of TTX is determined to be 8.7 and 10 μg/kg mice by intravenous and intraperitoneal injections, respectively. Minimum lethal dose (MLD) for human adult is estimated to be approximately 1–2 mg TTX, when pufferfish poisoning occurred (Hwang and Noguchi 2007). TTX analogs also showed similar pharmacological properties to TTX, although lethal potency markedly varied among them due to difference in their sodium channel binding affinity. Recently, attempts were made to apply TTX as a therapeutic agent for severe cancer-related pain and drug addiction (Nieto et al. 2012). Therefore, TTX attracts attention from the viewpoints of not only food safety and toxicology but also medical science.

Distribution of TTX in Pufferfish and Other Organisms

Pufferfish has been known for a long time to have TTX. However, all of pufferfish does not have the toxin. Toxicity of pufferfish remarkably varies among individuals, locations, and seasons even in the toxic species. Furthermore, TTX occurrence is not limited among pufferfish but has been increasing in phylogenetically distinct organisms, from bacteria to vertebrates. Here the distribution of TTX in pufferfish and other aquatic organisms is described.

Distribution of TTX in Pufferfish

Among pufferfish, members of the family Tetraodontidae have TTX but not other families to our knowledge. Table 1 shows toxicity of Japanese marine pufferfish. The toxicity greatly varies among pufferfish species and tissues in the fish; the highest concentration in the liver and ovary, and to a less extent in muscle and testis. It is notable that certain species such as *Lagocephalus lunaris*, *Takifugu pardalis*, and *Takifugu poecilonotus*, have a high level of toxicity in even muscle. In particular,

Table 1 Toxicity of Japanese marine pufferfish

Genus	Species	English name	Japanese name	Toxicity level						
				Ovary	Testis	Liver	Bile	Skin	Intestine	Muscle
Canthigaster	*C. rivulata*	Brown-lined puffer	KITAMAKURA	×	–	○	–	◎	○	×
Sphoeroides	*S. pachygaster*	Bleuback puffer	YORITOFUGU	×	×	×	–	×	×	×
Takifugu	*T. pardalis*	Panther puffer	HIGANFUGU	●	◎	●	●	◎	◎	◎
	T. chrysops	Red-eyed puffer	AKAMEFUGU	●	×	◎	–	◎	○	×
	T. snyderi	Vermiculated puffer	SHOSAIFUGU	●	○	●	–	◎	◎	○
	T. vermicularis	Pear puffer	NASHIFUGU	●	○	◎	–	●	○	○
	T. porphyreus	Purple puffer	MAFUGU	●	×	●	–	◎	◎	×
	T. obscurus	Obscure puffer	MEFUGU	●	×	◎	–	◎	◎	×
	T. poecilonotus	Finepatterned puffer	KOMONFUGU	●	◎	●	–	◎	◎	◎
	T. xanthopteus	Striped puffer	SHIMAFUGU	◎	×	◎	–	×	○	×
	T. exascurus		MUSHIFUGU	◎	×	◎	–	◎	○	×
	T. stictonotus	Spottyback puffer	GOMAFUGU	●	○	●	◎	◎	×	○
	T. flavidus	Yellowbelly puffer	SANSAIFUGU	◎	×	◎	–	○	◎	×
	T. niphobles	Grass puffer	KUSAFUGU	●	○	●	–	◎	●	○
	T. pseudommus		NAMERADAMASHI	◎	×	○	–	○	○	×
	T. rubripes	Tiger puffer	TORAFUGU	◎	×	◎	–	×	○	×
	T. chinensis	Eyespot puffer	KARASU	●	–	●	–	–	–	–
Arothron	*A. firmamentum*	Starry toadfish	HOSHIFUGU	◎	×	×	–	○	○	×
	A. stellatus	Starry puffer	MOYOFUGU	●	–	○	○	×	×	×
Lagocephalus	*L. lagocephalus oceanicus*	Blueback puffer	KUMASAKAFUGU	×	×	×	–	×	×	×
	L. inermis	Smooth-backed blowfish	KANAFUGU	○	×	●	○	×	○	×
	L. sceleratus	Spotted rough-backed blowfish	SEN-NINFUGU	●	–	◎	◎	○	◎	○
	L. lunaris	Green toadfish	DOKUSABAFUGU	●	-	◎	–	○	○	◎
	L. gloveri		KUROSABAFUGU	×	×	×	–	×	×	×
	L. spadiceus	Half-smooth golden pufferfish	SHIROSABAFUGU	×	×	×	–	×	×	×

● Strongly toxic (>1,000 MU/g), ◎ moderately toxic (100–999 MU/g), ○ weakly toxic (10–99 MU/g), × non-toxic (<10 MU/g), – data not available
1 MU (mouse unit) is defined as the amount of toxin that kills a male mouse of ddY strain weighing 20 g, 4 weeks old, in 30 min after intraperitoneal injection
1 MU is equivalent to about 0.2 μg of TTX

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L. lunaris is a notorious species, because it has often caused severe food poisoning incidences in Southeast Asian countries as well as Japan (Nagashima et al. 2011). As to *T. pardalis* and *T. poecilonotus*, the fish collected from certain areas of the northern coast of Japan exhibited unusually higher toxicity in muscle, indicating the local variation in toxicity (Kanoh et al. 1984; Kodama et al. 1984). A fish of highly toxic species is not necessarily toxic, showing a remarkable individual variation in toxicity of pufferfish. Moreover, the seasonal variation in toxicity is also observed. Ikeda et al. (2010) demonstrated maturation-associated changes in toxicity of *T. poecilonotus* collected from Ariake Sea, Kyushu, Japan from October 2006 to December 2007; in females liver toxicity was high during the ordinary period (April–November) and ovary toxicity was high during the maturation period (December–March). Brillantes et al. (2003) reported seasonal variation in toxicity of *L. lunaris* landed in SamutSakrn fish landing in Thailand from August 2000 to August 2001; they were toxic from March to November and showed the highest toxicity in August in all the organs examined (liver, gonads, skin, and muscle).

Distribution of TTX in Other Organisms

Table 2 summarizes phylogenetic distribution of TTX in animals other than pufferfish. TTX is not unique to pufferfish and has been detected in other fish species, goby *Yongeichthys criniger* of the family Gobiidae. Other vertebrates also have TTX. Amphibians (certain newts and frogs) have the toxin (Miyazawa and Noguchi 2001; Noguchi and Arakawa 2008; Hanifin 2010). Newt has been first reported to possess TTX in organism other than pufferfish in 1964. The toxin was identified in eggs of California newt *Taricha torosa* and then in several other *Taricha* species and *Triturus* species as well as *Cynops* species in Japan. TTX appears to be widely distributed in the newts. In newts, TTX was concentrated in the skin and ovaries and detected in low concentrations in the liver unlike in pufferfish. TTX was also identified in the *Atelopus* genus of poison-arrow frogs native to Central and South America. *A. chiriquiensis* has, in addition to TTX, a similar toxin called chiriquitoxin. Tree-frog *Polypedates* sp. in Bangladesh, also have TTX.

TTX was also found in diverse phyla of invertebrates. Some examples of toxic species are starfish, the echinoderms *Astropecten* spp. of the family Astropectinidae, arrow worm *Parasagitta elegans* in the phylum Chaetognatha, and arthropods such as several crabs in the family Xanthidae of the phylum Crustacea and horseshoe crabs in the Limulidae family of the order Xiphosura are also TTX-bearers. In the phylum Mollusca, blue-ringed octopus *Hapalochlaena* spp. has TTX in the posterior salivary glands. These toxic cephalopods likely use the toxin to capture prey. TTX has been also identified in marine snail gastropods in the families of Trochidae, Naticidae, Cymatiidae, Bursidae, Buccinidae, Melongenidae, Nassariidae, and Olividae. Trumpet shell *Charonia sauliae*, ivory shell *Babylonia japonica*, and glans nassa *Nassarius* (*Alectrion*) *glans* in Japan and certain species of the family Nassariidae (*Niotha clathrata*, *Zeuxis simiplicutus*, *Z. siquijorensis*, and related species) in Taiwan and China have intoxicated and even killed people who have eaten them. Other TTX-containing invertebrates include annelid *Pseudopotamilla occelata* in the family Sabellidae of the phylum Annelida, ribbon worm in the families of Lineidae, Tubulanidae, and Cephalothricidae of the phylum Nemertinea, and flatworm in the family Planoceridae of the phylum Plathyhelmintes. Trace amounts of TTX were determined in the algae *Jania adhaerens*, a member of the family Corallinaceae.

Furthermore, occurrence of TTX and/or its related components was demonstrated in certain species of marine bacteria (Chau et al. 2011); *Vibrio* spp. including *V. alginolyticus* and *V. parahaemolyticus*, *Aeromonas* spp. including *A. tetraodonis*, *Shewanella* spp. including *S. alga* and *S. putrefaciens*, *Pseudomonas* spp., and *Plesiomonas* spp. Most of TTX-producing bacteria were obtained from TTX-bearing organisms such as the liver, ovary, skin, gastrointestinal tract of

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Table 2 Phylogenetic distribution of TTX in animals other than pufferfish

Animal	Family	Scientific name	Mainly toxic parts
Frog	Bufonidae	*Atelopus chiriquiensis*	Skin
		A. varius	Skin
		A. zeteki	Skin
		A. limosus	Skin
		A. glyphus	Skin, egg
		A. certus	Skin
		A. oxyrhynchus	(Whole body)*
	Dendrobatidae	*Colostethus inquinalis*	Skin
	Brachycephalidae	*Brachycephalus ephippiun*	Skin, liver
	Rhacophoridae	*Polypedates sp.*	Skin
Newt	Salamandridea	*Taricha torosa*	Egg, ovary
		T. granulosa	Egg
		Cynops pyrrhogaster	Skin, muscle
		C. ensicauda popei	(Whole body)*
		Triturus alpestris	(Whole body)*
		T. cristatus	(Whole body)*
		T. helveticus	(Whole body)*
		T. oregon	(Whole body)*
		Notophthalmus viridescens	(Whole body)*
		Paramesotriton hongkongensis	(Whole body)*
	Ambystomatidae	*Ambystoma tigrinum*	(Whole body)*
Fish	Gobiidae	*Yongeichthys criniger*	Skin, viscera, gonad
Starfish	Astropecinidae	*Astropecten polyacanthus*	Whole body
		A. latespinosus	Whole body
		A. scoparius	Whole body
		A. vappa	Whole body
Arrowworm	Sagittadae	*Parasagitta elegans*	Head
		Flasscisagatta spp.	Head
Crab	Xanthidae	*Zosimus aeneus*	Whole body
		Lophozozymus pictor	Whole body
		Atergatis floridus	Whole body
Horseshoe crab	Limulidae	*Carcinoscorpius rotundicauda*	Egg
Annelid	Sabellidae	*Pseudopotamilla occelata*	(Whole body)*
Octopus	Octopodidae	*Hapalochlaena maculosa*	Posterior salivary gland (adult), whole body (semi-adult)
		H. lunalata	Posterior salivary gland

(*continued*)

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Table 2 (continued)

Animal	Family	Scientific name	Mainly toxic parts
		H. fasciata	Posterior salivary gland
Gastropod	Pleurobranchidae	*Pleurobranchaea maculata*	Outer tissues
	Trochidae	*Umbonium suturale*	Soft part
	Naticidae	*Polinices didyma*	Muscle, digestive gland
		Natica vitellus	Muscle, digestive gland
		N. lineata	Muscle, digestive gland
		N. pseutes	Soft part
	Cymatiidae	*Chalonia sauliae*	Digestive gland
		C. lampus lampus	Digestive gland
		Cymatium echo	Digestive gland
	Bursidae	*Tutufa lissostoma*	Digestive gland
	Buccinidae	*Babylonia japonica*	Digestive gland
	Melongenidae	*Hemifusus ternatanus*	(Whole body)*
	Nassariidae	*Niotha clathrata*	(Whole body)*
		N. lineata	(Whole body)*
		Nassarius condoidalis	(Whole body)*
		N. glans	Muscle, viscera
		Zeuxis siquijorensis	(Whole body)*
		Z. samiplicutus	(Whole body)*
	Olividae	*Oliva miniacea*	(Whole body)*
		O. mustelina	(Whole body)*
		O. nirasei	(Whole body)*
Ribbonworm	Lineidae	*Lineus fuscoviridis*	(Whole body)*
	Tubulanidae	*Tubulanus punctatus*	(Whole body)*
	Cephalothricidae	*Cephalothrix linearis*	(Whole body)*
Flatworm	Plamoceridae	*Planocera multitentaculata*	(Whole body)*
		P. reticulata	(Whole body)*

*means that whole body of the specimen was examined for toxicity assay

marine pufferfish, intestine of starfish, sea urchin, intestine of xanthid crab, posterior salivary gland and other soft parts of blue-ringed octopus, copepod, and marine red calcareous alga (Hwang and Noguchi 2007). Surprisingly, TTX has not been identified in any terrestrial reptile or other animal higher up the evolutionary tree, except that the common garter snake *Thamnophis sirtalis* harbored TTX in the liver when preyed upon toxic newt *Taricha granulosa* (Hanifin 2010).

Accumulation and Pharmacodynamics of TTX in Pufferfish

Over a half century, much effort has been done to investigate the toxification mechanism of TTX-bearing animals inhabiting aquatic and/or terrestrial environments, not only pufferfish but also newts and frogs. However, the underlying mechanism is still not fully elucidated; who produce TTX in the terrestrial environment, how TTX is biosynthesized, why and how the animals concentrate TTX at a high level in specific organs, and so on. Recently attempts were made to estimate

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transfer profile and pharmacokinetics of TTX in the pufferfish by in vivo administration experiments. Here accumulation and pharmacodynamics of TTX in pufferfish are described.

Accumulation of TTX in Pufferfish

Extensive studies on TTX toxification of marine pufferfish and the other toxic organisms have been conducted and led the hypothesis that two routes are implicated; symbiosis or parasitism with TTX-producing bacteria and the marine food chain with bioaccumulation. The former was reported by many researchers who identified the TTX-producing bacteria as described above. The latter was supported by the feeding experiments of the cultured pufferfish in net cages or tanks. Marine pufferfish, *T. rubripes* and *T. niphobles*, showed nontoxicity in the case of the farming with the use of nontoxic artificial diets after hatching (Matsui et al. 1981, 1982; Noguchi et al. 2006). Then, they became toxic and accumulated TTX primarily in the liver when fed with TTX-containing diets (Matsui et al. 1981; Yamamori et al. 2004; Honda et al. 2005a; Kono et al. 2008). These findings strongly suggest that TTX is the exogenous substance for pufferfish and effectively transferred to the liver. However, it remains to be clarified the mechanisms of TTX toxification and TTX metabolism in marine pufferfish; how TTX is absorbed into the fish body, how TTX is transported in the body, and how TTX is concentrated into the specific organs such as the liver and ovary. Furthermore, current studies including the feeding tests failed to estimate the kinetics interpretation of TTX in the body.

Pharmacodynamics of TTX in the *Takifugu rubripes* by Administration into Hepatic Portal Vein

Matsumoto et al. (2008a) examined the TTX toxification mechanism of the marine pufferfish *T. rubripes* by in vivo technique from the standpoint of pharmacokinetics. Innocuous cultured *T. rubripes* specimens (about 1 kg in body weight) were used and did not contain any detectable amount of TTX (<0.01 ng TTX/μL blood and <10 ng TTX/g tissue). The fish was anesthetized with the artificial seawater containing 0.03 % 2-phenoxyethanol and placed on an experimental workbench. During experiments, the fish was swaddled by wet papers to prevent the body surface from xeransis, and breathed by the gill ventilation using the perfusion of the seawater treated with bubble aeration at 20 °C. After the laparotomy of the fish body, hepatic vein was cannulated with a PE50 polyethylene tube. TTX was bolus-injected into hepatic portal vein using a syringe. Blood samples were obtained via the venous cannula using a heparinized syringe at intervals for 300 min after the administration. TTX was immediately extracted with acetic acid, ultrafiltered, and determined by LC/ESI-MS analysis.

Time courses of blood concentration of TTX after a single bolus injection of 0.25–0.75 mg TTX/kg body weight into the hepatic portal vein are shown in Fig. 2. The pharmacokinetic parameters are summarized in Table 3. After the injection of 0.25 mg TTX/kg body weight, the blood concentration of TTX was 3.00 ± 0.58 ng/μL at 3 min and fell to 0.71 ± 0.02 ng/μL at 30 min, and then decreased gradually to 0.14 ± 0.06 ng/μL at 300 min (Fig. 2). The dose-increase from 0.25 to 0.75 mg TTX/kg body weight elevated the blood concentration of TTX. The area under blood concentration-time curves (AUCs) of TTX demonstrated a tendency to increase from 147 ± 33 to 348 ± 40 ng min/μL with the increase from 0.25 to 0.75 mg TTX/kg body weight, although no significant differences were observed in AUCs at the dose ranging from 0.25 to 0.50 mg TTX/kg body weight ($p > 0.05$) (Table 3). Meanwhile, the hepatic portal vein clearance (CL_{hp}), the mean residence time (MRT), and the steady-state volume of distribution (V_{ss}) were constant irrespective to the dose ($p > 0.05$). These results indicate that the pharmacokinetics of TTX in the pufferfish after administration of 0.25–0.75 mg TTX/kg body weight was not saturated.

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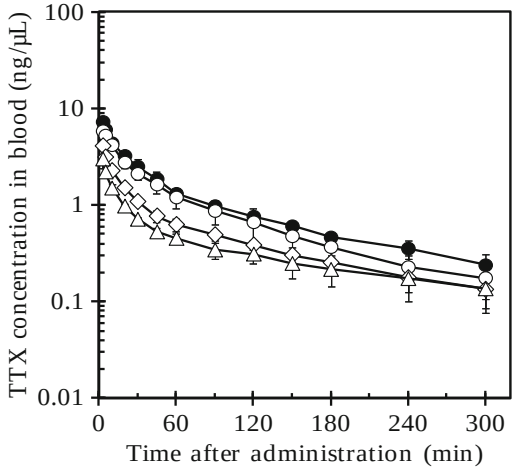

Fig. 2 Time course of the blood concentration of TTX in *Takifugu rubripes* after administration into hepatic portal vein. Pufferfish received a single bolus injection of 0.25(△), 0.37 (◇), 0.50 (○), and 0.75 (●) mg TTX/500 μL/kg body weight. Each point and vertical bar represent the mean ± standard error of the three individual experiments performed in triplicate

Table 3 Pharmacokinetic parameters after administration of TTX into the hepatic portal vein in *Takifugu rubripes*

Dose (mg/kg)	Body weight (kg)	AUC (ng•min/μL)	CL_{hp} (μL/min/kg)	MRT (min)	V_{ss} (mL/kg)
0.25	0.94 ± 0.10^{a}	147 ± 33^{a}	1848 ± 336^{a}	173 ± 53^{a}	284 ± 22^{a}
0.37	1.16 ± 0.11^{a}	176 ± 32^{a}	2264 ± 447^{a}	120 ± 23^{a}	$254 \pm 13^{a,b}$
0.50	1.07 ± 0.06^{a}	293 ± 64^{a}	1890 ± 426^{a}	103 ± 18^{a}	182 ± 20^{b}
0.75	1.02 ± 0.04^{a}	348 ± 40^{b}	2217 ± 281^{a}	115 ± 19^{a}	$249 \pm 29^{a,b}$

Values are the mean ± SE of three individual experiments performed in triplicate. Different superscripts (a and b) in the same columns indicate significant differences among the administrated groups ($p < 0.05$ by Turkey's test)
CL_{hp} hepatic portal vein clearance, MRT mean residence time, V_{ss} steady-state volume of distribution

Bioavailability of TTX in the *Takifugu rubripes* by Administration into Gastrointestinal Tract

TTX absorption through gastrointestinal tract was examined in *T. rubripes* (Matsumoto et al. 2008a), because it is thought that marine pufferfish accumulates the toxin mainly by the oral route (Matsui et al. 1981; Yamamori et al. 2004; Honda et al. 2005a; Kono et al. 2008). The materials and methods for the administration of TTX into gastrointestinal tract were the same as those into hepatic portal vein, except for the injection route.

Figure 3 shows the time courses of blood concentration of TTX after a gastrointestinal administration of 0.25, 0.50 or 1.00 mg TTX/kg body weight. After the administration of 0.25 mg TTX/kg body weight, the blood concentration of TTX was transiently increased to 0.46 ± 0.10 ng/μL until 30 min and thereafter decreased gradually to 0.09 ± 0.02 ng/μL at 300 min. Comparison of the AUCs following direct injection into the gastrointestinal tract and hepatic vein elucidated that the bioavailability of TTX was 62 %. In the cases of administration at the dosage of 0.50 and 1.00 mg TTX/kg body weight, the blood concentration of TTX increased to 0.45 ± 0.06 and 0.53 ± 0.11 ng/μL until 150 min after administration, respectively, and then modestly decreased to about 0.3 ng/μL 300 min after administration in both cases. The AUCs were estimated to be 236 ± 41 and 234 ± 19 ng in/μL and the bioavailability was 84 % and 42 % after the administration of 0.50 and

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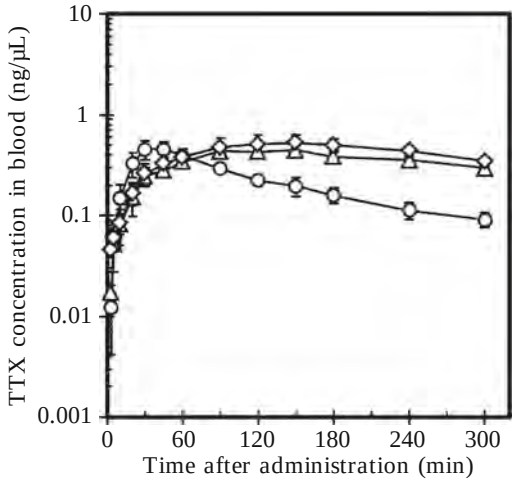

Fig. 3 Time course of the blood concentration of TTX in *Takifugu rubripes* after gastrointestinal administration. Pufferfish received a single bolus injection of 0.25 (○), 0.50 (△), and 1.00 (◇) mg TTX/500 μL/kg body weight. Each point and vertical bar represent the mean ± standard error of the three individual experiments performed in triplicate

1.00 mg TTX/kg body weight, respectively. These results suggest the high gastrointestinal absorption of TTX by a saturable mechanism in *T. rubripes*.

Transfer Profiles of TTX Administered into the Pufferfish

Since pufferfish contains TTX not only in liver but also in other organs such as gonads and skin the distribution of TTX in the *T. rubripes* after administration into hepatic vein was examined (Matsumoto et al. 2008b). TTX solution (0.25 mg TTX/500 μL/kg body weight) was given by a single bolus injection into the hepatic vein. Blood samples were obtained from the hepatic vein cannulated at designed time points (10, 20, 30, 45 and 60 min) after administration. At the time, spleen, kidney, skin, muscle and liver were separated and subjected to TTX determination.

The blood concentration of TTX decreased over time after the injection, from 1,450 ± 45 ng/mL at 10 min to 364 ± 59 ng/mL at 60 min (Fig. 4). TTX concentrations in the spleen and kidney decreased in parallel with the blood concentrations. Briefly, TTX concentrations of the spleen and kidney were 702 ± 87 and 520 ± 31 ng/g at 10 min and decreased to 229 ± 61 and 129 ± 31 ng/g at 60 min after injection, respectively. However, TTX concentrations in the skin and muscle remained at the low level throughout the experiment. The TTX concentration in the skin varied from 79 ± 47 ng/g to 224 ± 68 ng/g, and that in the muscle varied from 19 ± 7 ng/g to 75 ± 34 ng/g ($p > 0.05$). In contrast, the TTX concentration in the liver gradually increased after the injection, from 469 ± 130 ng/g at 10 min to 1,240 ± 90 ng/g at 60 min. These results indicate that TTX effectively transferred into the liver in a short time for 60 min.

Ikeda et al. (2009) investigated transfer profile of TTX intramuscularly administered in the *T. rubripes* during rearing for 1 week after administration. In the experiment, nontoxic cultured juvenile specimens (approximately 4 month-old, 13.2 ± 3.4 g in body weight) were used, intramuscularly administered 0.01 mg TTX/100 μL/individual, and kept in an aerated 90-L tank. Each 5 fish was collected at intervals for 168 h. TTX was determined in blood, liver, skin, and muscle by ELISA or LC/MS.

Figure 5 illustrates changes in the anatomic distribution of TTX, expressed by the relative amount of toxin retained in each tissue to the administered toxin. The amount of toxin remaining in the whole body was about 60 %, although it transiently decreased at 8–12 h. At 1 h after administration,

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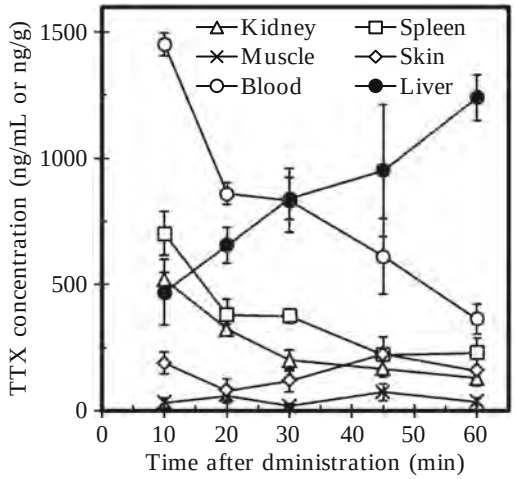

Fig. 4 Time course of TTX concentration in the blood and tissues in *Takifugu rubripes* after administration of 0.25 mg TTX/500 μL/kg body weight into hepatic vein. Each point and vertical bar represent the mean ± standard error of the four individual experiments performed in triplicate

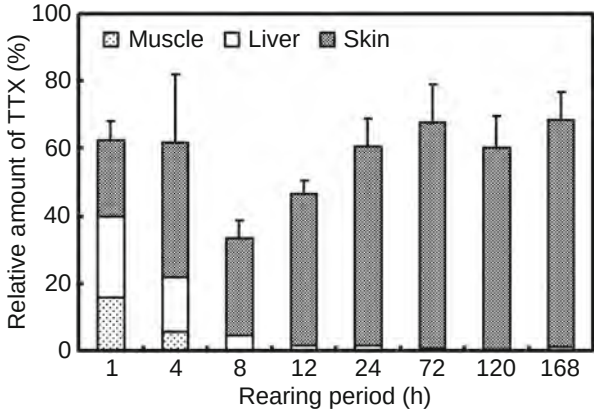

Fig. 5 Change in the relative amount of TTX retained in each tissue of the *Takifugu rubripes* specimens during the rearing period after intramuscularly administration of 0.01 mg TTX/individual (Ikeda et al. 2009). Bar represent standard deviation of five individuals

TTX intramuscularly administered was transferred to liver and skin from muscle via the blood-stream, in which TTX concentration was determined to be 1.2 μg/mL plasma. The toxin amounts in muscle and liver rapidly decreased up to 12 h, in contrast that in skin increased to most of the remaining toxin at 168 h.

Furthermore, the sexual difference in transfer profile of TTX was examined using artificially hybridized specimens of the pufferfish, *T. rubripes* and *T. niphobles* (designated "torakusa") (Wang et al. 2011), since in general *T. rubripes* slowly matures in more than 2 years, and *T. niphobles* within 1 year. The specimens used in the experiment (approximately 10-month old, 71.5 ± 15.1 g in body weight) appeared in a very early stage of maturation. The GSI (gonadosomatic index, the ratio of gonad weight to body weight) of the specimens was 0.40 ± 0.06 % and 5.96 ± 2.41 %, respectively, for the female and male specimens. Each fish was intramuscularly administered TTX (0.029 mg TTX/100 μL/individual) and reared in the tank with an aeration for 3 days. TTX was measured in

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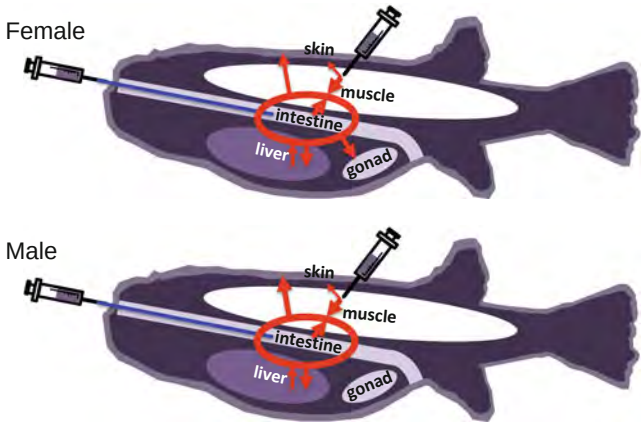

Fig. 6 Presumable transfer of TTX orally administered in the pufferfish

blood, gonads (ovary and testis), liver, skin, and muscle at intervals for 72 h by ELISA or LC/MS analysis.

TTX rapidly transferred from the muscle via the blood to other organs, as in the case of juvenile specimens of *T. rubripes*. Toxin content in the ovary increased to 0.01 mg TTX/g tissue at the end of the 72-h test period, while that in the testis was not detectable, showing the difference between the sexes in TTX transfer in the pufferfish. These results suggest that a part of TTX is first taken up in the liver and then transferred in the ovary in female specimens and in the skin in male specimens (Fig. 6).

Physiologic Functions of TTX in TTX-Bearing Organisms

While TTX is a fatal toxin to organisms that possess no TTX, including humans, TTX-bearing organisms are assumed to utilize TTX effectively for their survival. The intratissue microdistribution of TTX in various TTX-bearing organisms was evaluated using an immunohistochemical technique with an anti-TTX monoclonal antibody, and the information obtained provides several clues to the functions of TTX in TTX-bearing organisms (Arakawa et al. 2010). Although the mechanisms are not fully clarified, here the current estimations of the physiologic functions of TTX are described.

Defense against Predators

In many marine pufferfish, the highest levels of TTX are found in the ovaries (Noguchi and Arakawa 2008), especially in the maturation period (Ikeda et al. 2010). Although the microdistribution of TTX in the oocytes varies depending on the species and maturation stage, *Takifugu vermicularis* accumulate TTX mainly in the yolk granules and yolk vesicles with the progression of maturation (Fig. 7) (Mahmud et al. 2003). In the goby *Yongeichthys criniger*, the amount of toxin in the ovary increases during the spawning period as in pufferfish (Tatsuno et al. 2013). In pufferfish, the toxin that accumulates in the eggs is transferred to the hatched larvae, where it is maintained for a certain period after hatching (Nagashima et al. 2010). Itoi et al. (2014) observed juveniles of generally nontoxic fish that ingest pufferfish (*Takifugu niphobles* and *Takifugu rubripes*) larvae and then promptly spit them out, and presumed that the TTX transferred from the mother works to repel predators, based on the findings that TTX was primarily localized on the body surface of the larvae and that rainbow trout (*Oncorhynchus mykiss*) and arctic char (*Salvelinus alpinus*) are able to sense extremely low levels of TTX with gustatory receptors (Yamamori et al. 1988). Flatworms, ribbon

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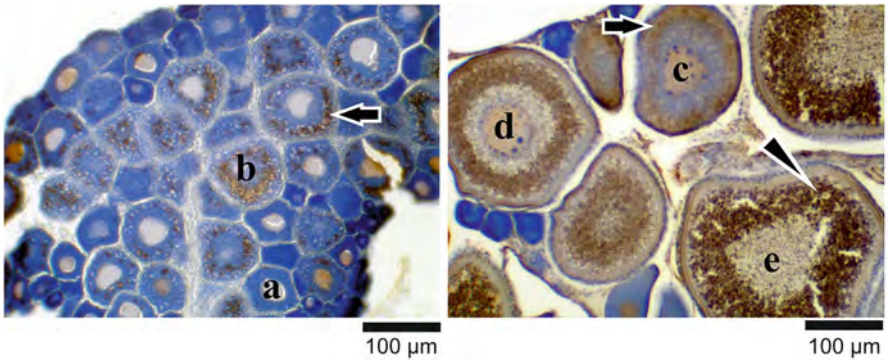

Fig. 7 Immunostained immature (*left*) and mature (*right*) ovary sections of *Takifugu vermicularis*, where TTX is visualized as a *brown* color. Five stages of oocytes, such as the perinucleolus stage (**a**), late perinucleolus stage (**b**), yolk vesicle stage (**c**), and yolk globule stage I (**d**), and yolk globule stage II (**e**) are observed. *Arrow* and *arrowhead* indicate yolk vesicles and yolk globules, respectively

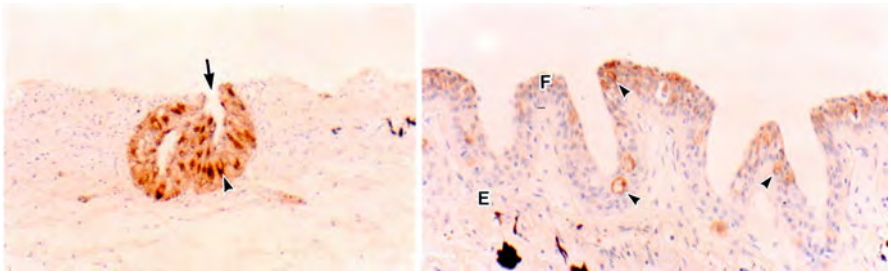

Fig. 8 Immunostained skin sections of *Takifugu vermicularis* (*left*) and *Chelonodon patoca* (*right*) (Mahmud et al. 2003). TTX is localized at cytoplasm of the gland in *T. vermicularis* and sacciform cells in *C. patoca* (*arrowheads*). E, epithelial layer; F, super epithelial layer

worms, and horseshoe crabs also possess high concentrations of TTX in their eggs (Miyazawa and Noguchi 2001; Noguchi and Arakawa 2008), and TTX may act as a repellant, thereby contributing to the survival of the eggs and hatched larvae, like in pufferfish. Adult individuals of marine and brackish water pufferfish, and newts possess TTX-bearing exocrine glands, gland-like structures, or sacciform cells, in their skins (Fig. 8), and secrete TTX in response to external stimuli (Arakawa et al. 2010; Williams 2010). Similarly, *Cephalothrix* ribbon worms harbor TTX in the bacillary cells of the epidermis (Tanu et al. 2004) and excrete TTX from the body surface when stimulated, suggesting that they utilize TTX as a defensive substance to protect themselves against predation (Williams 2010). The small marine snail *Niotha clathlata* also secretes TTX upon stimulation.

Offense to Prey

As described above, TTX may function defensively in many species, but some species are presumed to use it offensively. For example, the blue-ringed octopus possesses TTX in its posterior salivary glands, *Cephalothrix* ribbon worms in the granular cells near the pseudocnides of the proboscis (Tanu et al. 2004), and arrow worms in the head near the raptorial apparatus, and all are thought to utilize the TTX to capture food organisms (Williams 2010). There are several recorded human envenomations by the blue-ringed octopus. A species of the flatworm *Planocera* harbors TTX in the pharynx, and appears to use it to overcome their much larger gastropod prey (Williams 2010).

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Furthermore, Hwang et al. (2007) suggest that some carnivorous marine snails use TTX and paralytic shellfish poison (PSP) offensively against their prey organisms.

Resistance of TTX-Bearing Organisms to TTX

TTX-bearing organisms, such as toxic marine pufferfish, the goby *Y. criniger*, the xanthid crab *Atergatis floridus*, and the newt *Cynops pyrrhogaster*, show extremely high resistance to TTX, i.e., the minimum lethal dose (MLD) of TTX administered intraperitoneally to these animals is 300–1,000 times (more than 10,000 times in the newt) greater than that in mice (Noguchi and Arakawa 2008). In contrast, nontoxic species of marine pufferfish show medium resistance to TTX (MLD, 13–15 times greater than in mice), and general fish show resistance as low as mice. Therefore, TTX-bearing organisms could effectively use TTX as a defensive or offensive substance without being influenced themselves.

The mechanism of TTX resistance in pufferfish and newts has been explained based mainly on the TTX-resistant sodium channels found in the animals, in which the aromatic amino acid commonly located in the p-loop region of domain I in TTX-sensitive sodium channels is replaced by a nonaromatic amino acid, resulting in their extremely low affinity to TTX (Noguchi and Arakawa 2008). *Thamnophis* garter snakes inhabiting North America acquire similar sodium channel mutation-based TTX resistance by interacting with their toxic prey, TTX-bearing *Taricha* newts. Predation by the TTX-resistant garter snakes, in turn, then increases the TTX level of the newts. Such "coevolutionary arms races" are considered to have generated elevated levels of TTX and TTX resistance in some populations of *Taricha* newts and *Thamnophis* snakes (Hanifin 2010). On the other hand, the shore crab *Hemigrapsus sanguineus* possesses TTX-binding proteins in its hemolymph that confer TTX resistance to this nontoxic crab. Pufferfish also possess TTX-binding proteins, and not only the TTX-resistant sodium channels, but also a toxicity-masking mechanism due to such proteins might contribute to their high TTX resistance (Noguchi and Arakawa 2008).

Attractive Effect of TTX on TTX-Bearing Organisms

Toxic small marine snails are endowed with high resistance to TTX, and not only secrete TTX in response to external stimuli, but are also significantly attracted to TTX. More toxic species prefer TTX, while nontoxic species exhibit a negative response, suggesting that TTX functions as an attractant to toxic species, but as a repellant to nontoxic species (Arakawa et al. 2010). Similarly, Saito et al. (2000) found that juveniles of the pufferfish *T. rubripes* were attracted by TTX-containing gelatin. In addition, Okita et al. (2013b) revealed using olfactory ablated test fish that *T. rubripes* juveniles detect TTX by their olfactory organ. Interestingly, general fish that have no TTX may sense TTX by taste and repel it, whereas pufferfish smell TTX-bearing organisms and preferentially prey on them. Matsumura (1995) indicated that mature males of the pufferfish *T. niphobles* were attracted by a very low concentration of TTX, and presumed that the TTX released from the ovulated eggs works as a pheromone to attract male fish. Moreover, TTX may serve as an alarm pheromone for larval newts to avoid cannibalistic conspecifics, or as an attractant for the parasitic copepod *Pseudocaligus fugu* to choose toxic pufferfish hosts (Hanifin 2010; Williams 2010).

Other Functions

Hatchery-raised nontoxic pufferfish (*T. rubripes*) suffer from predation more often than toxic wild juveniles (Shimizu et al. 2008). This might be due to differences in their adaptive swimming behaviors and presence/absence of TTX. Recently, Sakakura et al. (unpublished) found that when

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TTX was administered to hatchery-raised nontoxic juveniles, their ecologic behavior became similar to that of wild juveniles, making it more difficult for predators to prey on them. In addition, Okita et al. (2013a) revealed that when TTX was orally administered to nontoxic *T. rubripes* juveniles, it passed through the blood–brain barrier to reach the brain, and that TTX was similarly localized in the brain in toxic wild juveniles. These findings suggest that TTX is involved in controlling information transmission in the central nervous system of pufferfish. Sodium channel mutations and/or the presence/absence of TTX in the cranial nervous system may strongly influence the physiology and ecology of pufferfish. On the other hand, Honda et al. (2005b) observed that when nontoxic cultured pufferfish were fed a TTX-containing diet, their immune function was activated, although the mechanism remains unclear. TTX seems to act not only as a defensive or offensive substance, but also has various physiologic functions that are essential for the survival of TTX-bearing organisms.

Human Intoxication of TTX

In a human poisoned by TTX, the lips and tongue usually become numb within 10–45 min after ingestion, followed by additional neuromuscular symptoms, such as paralysis of legs and arms, paresthesia, dysphasia, and respiratory distress (Noguchi and Ebesu 2001). Death occurs due to respiratory failure in the most critical cases. When a poisoning occurs, it is essential to transport the patient immediately to a well-equipped hospital. At present, there is no antidote or specific medication for TTX, and no fundamental treatment besides facilitating elimination of the toxin from the body, and managing the respiratory/circulatory system properly using an artificial respirator. Although a monoclonal anti-TTX antibody has been developed, and utilized as a chemical tool for research as described above (Arakawa et al. 2010), it has little effect in clinical use.

The Japanese have been eating pufferfish since ancient times, and have created a unique food culture for pufferfish. Food poisoning due to these fish still frequently occurs, however, and about 2 victims die yearly. Here TTX poisoning of humans, focusing mainly on the cases in East Asia is described.

TTX Poisoning Due to Pufferfish

According to the statistics of Japanese Ministry of Health, Labour and Welfare, 301 incidents of pufferfish poisoning have occurred in Japan, involving 434 patients and 19 deaths during the 10 years from 2001–2010 (Table 4). A typical poisoning case (Arakawa et al. 2010) is described below. In October 2008, a 69-year-old male died at a hospital in the Nagasaki Prefecture. He stated that he cooked a “usubahagi” (a sort of thread-sail filefish) that he caught by himself and ate some of its raw meat (sashimi) after dipping it in a mixture of the liver and soy sauce. Approximately 30 min after ingestion, he felt numbness in his limbs, and after another 30 min, he vomited and became comatose before being transported by ambulance to the hospital. He died approximately 4 h after ingestion. Thereafter, it was determined that the patient cooked a “kinfugu” (local name of pufferfish) with the “usubahagi,” but the liver was missing among the leftovers. Arakawa et al. (unpublished) investigated the leftovers, and found that the “usubahagi” was nontoxic, but the “kinfugu” was actually a highly toxic species, “komonfugu” *Takifugu poecilonotus*, and 600 MU/g of TTX was detected in the skin. Furthermore, 0.7 MU/mL, 2 MU/mL, and 45 MU/g of TTX was detected in the blood, urine, and vomit of the patient, respectively, leading to the conclusion that this was a case of TTX intoxication due to the mistaken ingestion of *T. poecilonotus* liver.

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Table 4 Food poisonings due to marine toxins in Japan (total number of 2001–2010, Ministry of Health, Labour and Welfare)

Causative food	Causative toxin	Number of incident	Number of patient	Number of death
Pufferfish	Tetrodotoxin	301	434	19
Ciguateric fish	Ciguatoxin	41	160	0
Marine snail	Tetramine	41	91	0
Boxfish	PTX-like toxin	6	10	1
Parrot fish	PTX-like toxin	4	7	0
Bivalves	PSP/DSP	2	4	0
Marine snail	Tetrodotoxin	2	2	0
Others		3	19	0
Unknown		34	57	3
Total		434	784	23

PTX palytoxin, *PSP* paralytic shellfish poison, *DSP* diarrheic shellfish poison

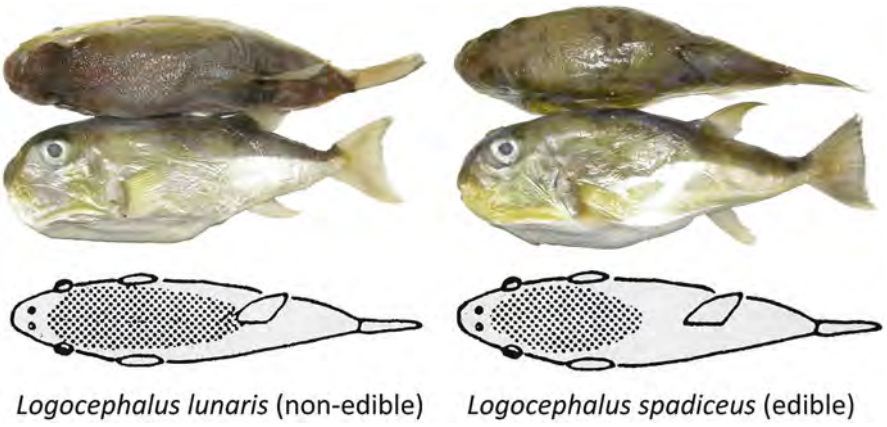

Fig. 9 Pictures of *Lagocephalus lunaris* (highly toxic and non-edible; *left*) and *L. spadiceus* (nearly nontoxic and edible; *right*). They are apparently very similar, but distinguishable based on the distribution of dorsal spines

To clarify the cause of pufferfish poisoning, identification of the causative species, as well as investigation of the leftover fish toxicity are essential. Although species identification is usually based on morphologic characteristics, such as the skin pattern, fin shape, and small spine distribution, several methods using proteins or genes have also been established (Arakawa et al. 2010; Nagashima et al. 2011), and the species can be identified even from a small tissue fragment. Techniques for analyzing TTX have also progressed, and TTX can be detected not only in the leftovers but also in the blood and urine of the patients (Leung et al. 2011; Islam et al. 2011).

Recently, the non-edible pufferfish *Lagocephalus lunaris*, which usually inhabits tropical or subtropical waters, has been frequently mixed up with edible species in Japanese coastal waters. This pufferfish, which appears very similar to the almost nontoxic species *Lagocephalus spadiceus* (Fig. 9), possesses high levels of TTX in their muscles and there were 5 confirmed poisoning incidents in 11 patients due to mistaken ingestion in the Kyushu and Shikoku Islands during 2008–2009 (Arakawa et al. 2010).

In Taiwan and China, including Hong Kong, although pufferfish is not eaten as frequently as in Japan, there have also been many cases of food poisoning cases due to the ingestion of wild

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pufferfish. Some of these cases differed from those in Japan, however, in that poisoning was caused by ingesting puffer roe that had been sold as a fake dried mullet roe called "karasumi," or by ingesting a dried dressed fish fillet produced from toxic pufferfish, and sold as the meat of the thread-sail filefish (Hwang and Noguchi 2007). In countries outside of East Asia, people generally do not have a custom of eating pufferfish, but poisonings due to accidental ingestion of pufferfish occasionally occur all over the world, including Australia (Isbister et al. 2002), Brazil (Silva et al. 2010), Thailand (Brillantes et al. 2003), and Bangladesh. Particularly in Bangladesh, 3 large pufferfish poisoning incidents occurred in 2008, involving 141 patients and 17 deaths (Islam et al. 2011). In the United States, Floridian *Sphoeroides* pufferfish caused 28 cases of food poisonings during 2002–2004, though the main toxic principle of the pufferfish was not TTX, but PSP (Arakawa et al. 2010). TTX poisoning cases occurred due to a prepackaged, ready-to-eat pufferfish product imported from Japan in California in 1996, and due to an imported pufferfish product from Korea that was mislabeled as monkfish in Illinois in 2007 (Cohen et al. 2009). Recently, toxic Indo-Pacific pufferfish, including *Lagocephalus sceleratus*, migrated from the Red Sea via the Suez Canal to the Mediterranean Sea, and poisonings due to the species have occurred in the eastern Mediterranean (Bentur et al. 2008).

TTX Poisoning Due to Marine Snails

In December 1979, a man in the Shizuoka Prefecture, Japan, ingested the digestive gland of the carnivorous marine snail *Charonia sauliae* and was seriously poisoned (Noguchi et al. 2011). He exhibited paralysis of his lips and mouth, and respiration failure, which are the typical symptoms and signs of pufferfish poisoning. TTX was detected for the first time in a marine snail, that is, the leftovers of *C. sauliae*, and the causative agent was therefore concluded to be TTX. Similar poisonings involving three patients occurred thereafter in the Wakayama and Miyazaki Prefectures. In *C. sauliae*, TTX localizes in the digestive gland, and other organs, including the muscle, are nearly nontoxic. TTX or its derivative has been also detected in closely related species, such as the frog shell *Tutufa lissotoma* and the European trumpet shell *Charonia lampas lampus*, the latter of which caused TTX poisoning in Spain in 2007 (Noguchi et al. 2011; Silva et al. 2012).

In China and Taiwan, people have a time-honored custom of eating small necrophagous marine snails, and food poisoning due to the snails has frequently occurred (Hwang and Noguchi 2007; Arakawa et al. 2010; Noguchi et al. 2011). At least 28 incidents were recorded during 1985–2004 in China, and 9 incidents during 1994–2006 in Taiwan, involving 233 patients and 24 deaths in total. In April 2004, a serious incident due to *Nassarius glans* (Fig. 10) occurred in Taiwan, in which 2 of 5 poisoned patients died within 30 min after ingestion. The causative species in China was identified as *Zeuxis samiplicutus* (Fig. 10), while a total of 14 species of Nassariidae, Naticidae, and Olividae, including *N. glans*, were reported as the responsible gastropods for the poisonings in Taiwan.

TTX poisonings due to *N. glans* have also occurred in Japan (Arakawa et al. 2010). In the Nagasaki Prefecture in July 2007, a 60-year-old woman developed a feverish feeling in the limbs, abdominal pain, and an active flush and edema in the face 15 min after ingesting the marine snail. Thereafter, her condition worsened, and she developed dyspnea, whole-body paralysis, and mydriasis; she was finally transported to an emergency hospital. The patient required an artificial respirator for the first 3 days, but recovered enough to take breakfast on the 4th day. She unexpectedly relapsed after lunch, however, and developed respiratory arrest and was again placed on the respirator. She gradually recovered and was discharged from the hospital 3 weeks later. Immediately after the incident, Arakawa et al. (unpublished) investigated the leftover gastropods and detected a maximum of 4,290 MU/g of TTX in the cooked muscles and digestive glands of *N. glans*. Moreover, an extremely high concentration of TTX, a maximum of 10,200 MU/g in the viscera and 2,370 MU/g in

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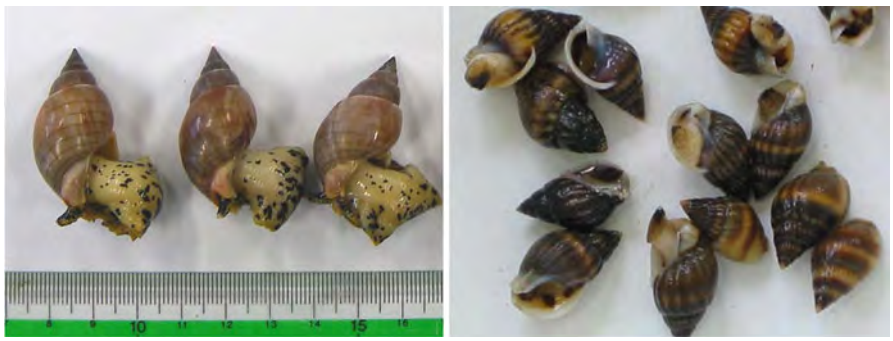

Fig. 10 Necrophagous marine snails, *Nassarius glans* (*left*) and *Zeuxis samiplicutus* (*right*)

the muscle, was detected in *N. glans* specimens collected from the same sea area as the ingested snail. In this case, the once-recovered symptoms recurred after the patient began eating again. The recurrence might have been due to the digestion of a highly toxic, previously undigested tissue fragment of *N. glans* and absorption due to the resumption of meals, again exposing her respiratory center to a high concentration of TTX.

TTX Poisoning Due to Other Organisms

In some Southeast Asian countries, eggs of the horseshoe crab are used as a food and can occasionally cause food poisoning (Arakawa et al. 2010). From 1994 to 2006, 280 patients (5 died among 245 patients with records) with poisoning due to the ingestion of horseshoe crabs were admitted to Chon Buri Hospital, Thailand (Kanchanapongkul 2008). TTX and/or PSP were detected in the eggs and hepatic caecum of the horseshoe crab *Carcinoscorpius rotundicauda*, indicating that either of them or both were the causative agents (Arakawa et al. 2010). In Taiwan, there are some records on TTX poisoning cases due to toxic gobies (Arakawa et al. 2010). As described previously, blue-ringed octopuses possess TTX in the posterior salivary glands, and a few humans have died as a result of bites from the octopus (Williams 2010). Fifteen dogs were suddenly poisoned at the beaches adjacent to Hauraki Gulf, New Zealand, from July to November 2009, all exhibiting similar symptoms, and 5 of them died. McNabb et al. (2010) detected a very high level of TTX in the grey side-gilled sea slug *Pleurobranchaea maculate* found in tide pools near the beach and claimed that the dogs were poisoned with TTX by contact with the sea slugs.

Conclusion and Future Directions

The fact that pufferfish possess a strong lethal toxin has been known since ancient times, and extensive studies have been performed to investigate the properties, mechanisms of action, and epidemiologic features of intoxication, and to determine the distribution, accumulation mechanisms, and physiologic functions in aquatic organisms, of the pufferfish toxin, TTX. As described above, a wide variety of organisms in addition to pufferfish possess TTX, and many of the TTX-bearing organisms, including pufferfish, ingest and accumulate TTX from the outside via the food chain that starts with bacteria. Moreover, valuable information regarding the dynamics of TTX in the pufferfish body and the molecular mechanisms involved is accumulating. Further clarification will make it possible to produce “absolutely nontoxic pufferfish,” which genetically never accumulate TTX even when a large amount of TTX is ingested. On the other hand, functions of TTX in TTX-bearing organisms such as defense, offense, attraction, behavior control, and immunity activation, have also

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begun to draw attention. Regarding these functions, more demonstrative studies, or studies that delve into mechanisms that underlie the observed phenomena are necessary, and we expect that many new and more interesting findings will be obtained through this process.

In Japan, edible species of pufferfish and their body tissues, allowable pufferfish fishing areas, and processing methods for toxic parts were stipulated by the Ministry of Health and Welfare (presently the Ministry of Health, Labour and Welfare) in 1983. Since then, pufferfish poisoning, or deaths due to pufferfish poisoning, has largely decreased. That is, an unusual food culture has been realized based on scientific information that allows people to safely ingest products that contain toxin and are primarily inedible. As the toxification of pufferfish comes from toxic food organisms, pufferfish, whose whole body, including the viscera, is nontoxic, can be produced by culturing with nontoxic diets in an environment where the invasion of TTX-bearing organisms is completely blocked (Noguchi et al. 2006). Although pufferfish liver is currently prohibited from ingestion, even if it is nontoxic, the Saga Prefecture has begun to consider serving the nontoxic cultured pufferfish liver as a food following an individual toxicity assay. A further advanced food culture may be established in the future.

On the other hand, toxic Indo-Pacific pufferfish have invaded from the Red Sea via the Suez Canal to the Mediterranean Sea, raising the risk of TTX poisoning in this sea area. In addition, the pufferfish *L. lunaris*, originally inhabiting tropical to subtropical sea areas, now frequently appears in the temperate coastal waters of Japan. Furthermore, human poisonings due to necrophagous and carnivorous marine snails have suddenly occurred in Japan and Spain, respectively, as well as dog poisonings due to sea slugs in New Zealand. Such facts indicate the possibility of further geographic expansion and/or diversification of TTX-bearing organisms, or of TTX contamination of seafood caused by a change in the marine environment, such as an increase in the water temperature due to global warming. Careful attention must be paid to this point from the food hygiene perspective for the future.

Cross-References

► Ciguatoxin and Ciguatera
► Saxitoxin and Shellfish, and Other Neurotoxins

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Ciguatoxin and Ciguatera

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Abstract

Ciguatera is a disease caused by the consumption of fishes from tropical and subtropical waters that have accumulated lipophilic sodium channel activator toxins known as ciguatoxins (CTXs) to levels sufficient to cause human poisoning. Consumption of these temperature-stable, orally active polycyclic ether compounds leads to the activation of neuronal sodium channels that produces a range of characteristic neurological, gastrointestinal, and cardiovascular signs and symptoms that clinically define the illness. Ciguatera is estimated to affect ~50,000 people annually worldwide after accounting for misdiagnosis and non-reporting. Currently there are no clinically validated treatments and no routine tests that can cost-effectively detect ciguatoxins prior to consumption, with government bans on capture or personal avoidance of risk fish species providing the only effective means to mitigate the risk currently. A recently developed rapid extraction method for ciguatoxins coupled to LC/MS/MS detection has potential for surveillance and confirmation of ciguatera outbreaks.

Introduction

Ciguatera is a circumtropical disease associated with the consumption of fish that is contaminated with clinically effective levels of polyether sodium channel activator toxins (ciguatoxins) originating from blooms of benthic dinoflagellates of the genus *Gambierdiscus*. Despite the potential for other benthic dinoflagellate toxins such as okadaic acid and maitotoxin to be involved, there is no clear evidence for the involvement of other classes of toxins (Holmes and Lewis 2002). Unfortunately, ciguatera remains a poorly managed fish poisoning, although continued advances in knowledge of the disease and its etiology have advanced management prospects. Despite these advances, banning or avoiding risk fish species is the only easily implementable management strategy currently available, albeit more than 400 species of fish from 57 families and 11 orders have been described as CTX carriers (Gillespie et al. 1986). This review updates the recent reviews by Vetter and Lewis (2014); Vetter et al. (2014) particularly in relation to our current understanding of the origin, chemistry, mode of action, and clinical manifestations associated with consumption of ciguatoxins in fish.

Origin and Transfer of Ciguatoxins to Fish

Ciguatera is a patchy and unpredictable risk throughout tropical and subtropical waters (Fig. 1) and is typically found in herbivorous and carnivorous coral reef and associated fishes. The ciguatoxins responsible for ciguatera are produced by blooms of the benthic dinoflagellate *Gambierdiscus* spp. and enter the marine food chain to contaminate fish (Lewis and Holmes 1993) (Fig. 2). In addition to *G. toxicus* (Adachi and Fukuyo 1979), the first species implicated in ciguatoxin production in biodetritus (Yasumoto et al. 1977; Bagnis et al. 1980) and monoalgal cultures (Bagnis et al. 1980; Holmes et al. 1991), other

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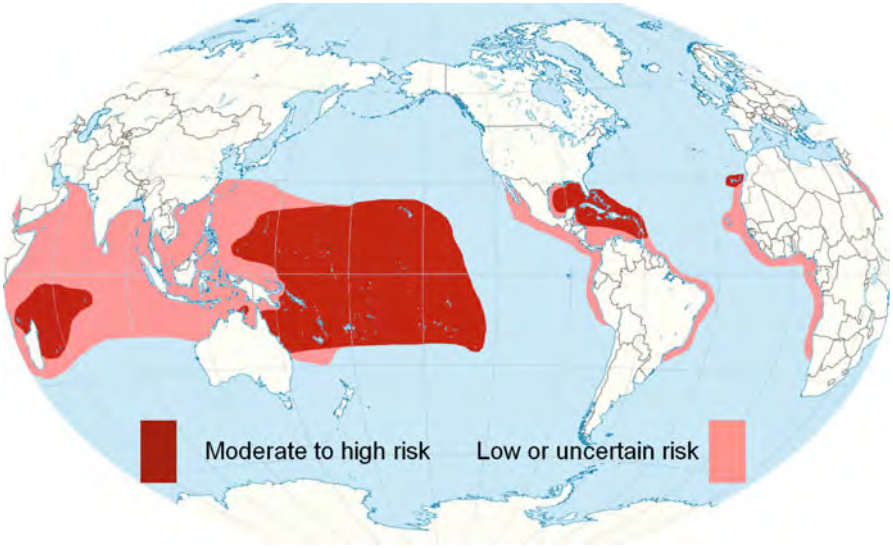

Fig. 1 Distribution of ciguatera globally. Regions of moderate to high risk and low and/or uncertain risk are indicated

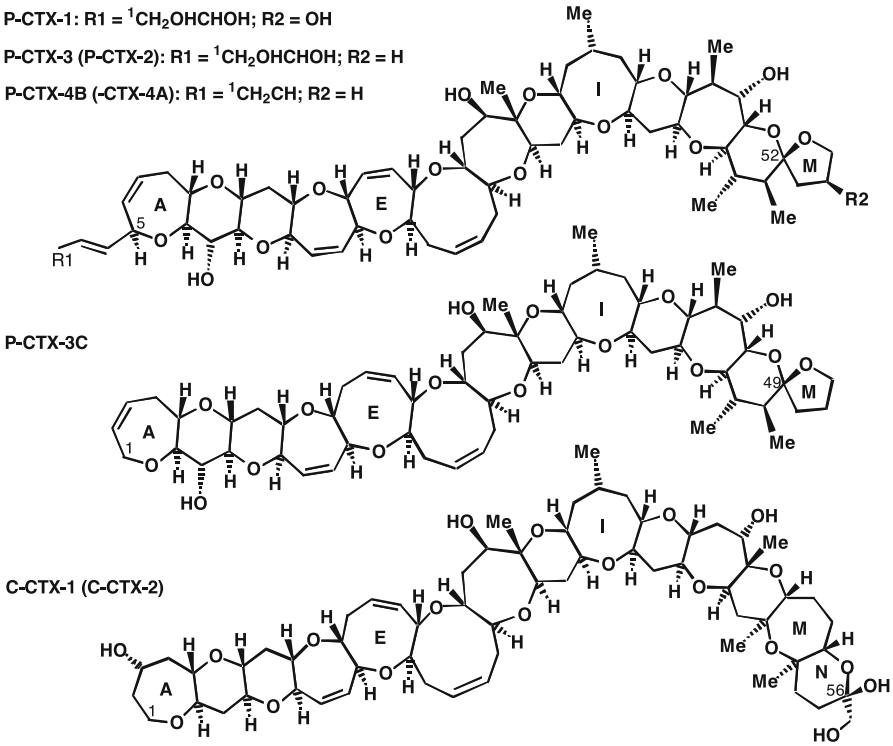

Fig. 2 Structure of Pacific and Caribbean ciguatoxins (*CTXs*). Shown are P-CTX-1 (Murata et al. 1990), P-CTX-3 (Lewis et al. 1991, 1993), P-CTX-4B (Murata et al. 1990), P-CTX-3C (Satake et al. 1993), and C-CTX-1 (Lewis et al. 1998). The energetically less favored epimers, P-CTX-2 (52-epi P-CTX-3; Lewis et al. 1993), P-CTX-4A (52-epi P-CTX-4B; Satake et al. 1996), and C-CTX-2 (56-epi C-CTX-1; Lewis et al. 1998), are indicated in parenthesis. Structures have also been described for 2,3-dihydroxyP-CTX-3C and 51-hydroxyP-CTX-3C (Satake et al. 1998) and additional minor P-CTXs, including the chemical intermediate seco forms (Yasumoto et al. 2000)

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Table 1 Ciguatoxin (CTX)- and maitotoxin (MTX)-like toxicity in mice detected in chloroform (CTX)- and methanol (MTX)-soluble extracts of different *Gambierdiscus* species

Gambierdiscus species	CTX-like activity MU/10^6 *Gambierdiscus* cells	MTX-like activity MU/10^6 *Gambierdiscus* cells
G. toxicus	0	1,000
G. pacificus	0.9 (14)[a]	700
G. australis	0.4	200
G. polynesiensis	120 (1,040)[b]	80

Data from Chinain et al. (1999)
[a]From Caillauda et al. (2011)
[b]From Chinain et al. (2010)
1 mouse unit (MU) = i.p. LD_{50} dose for a 20 g mouse = 5 ng P-CTX-1 equivalents

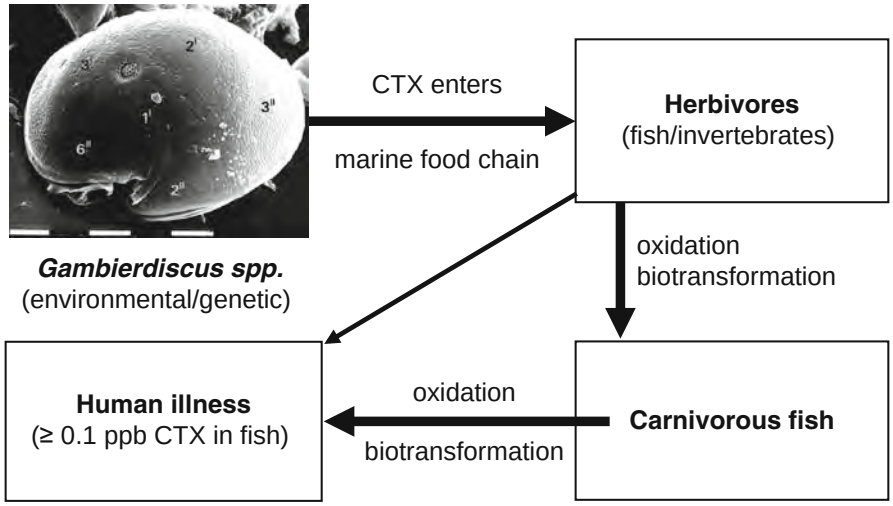

Fig. 3 Food chain transfer of ciguatoxins through the marine food web. An increased risk of ciguatera occurs when dinoflagellates of the genus *Gambierdiscus* bloom and a flux of ciguatoxins enters the food chain. In the Pacific Ocean, fish to convert these less polar ciguatoxins to more oxidized and toxic forms

Gambierdiscus spp. with the potential to biosynthesize ciguatoxin now include *G. polynesiensis*, *G. belizeanus*, *G. australes*, *G. caribbaeus*, *G. carolinianus, G. carpenteri*, and *G. ruetzleri* (Chinain et al. 1999; Litaker et al. 2009). As illustrated in Table 1, levels of ciguatoxin production by cultured *Gambierdiscus* vary widely between species and even between different isolates of the same species. The ciguatoxins produced by *Gambierdiscus* are metabolized to more oxidized forms in fish as they transfer and accumulate through the marine food chain (Lewis and Holmes 1993) (Fig. 3). For example, in the Pacific Ocean, P-CTX-4A produced by *Gambierdiscus* is converted to the more oxidized P-CTX-1, P-CTX-2, and P-CTX-3, as well as the acid-catalyzed spiro isomers of P-CTX-4A (CTX-4B) and P-CTX-2 (P-CTX-3) (see Table 2). Toxins produced by other genera of benthic dinoflagellates have no proven role in ciguatera, with only the palytoxins shown to accumulate to clinically relevant levels to produce a disease that is clinically distinct from ciguatera (Holmes and Lewis 2002).

While the precise environmental trigger(s) that leads to blooms of *Gambierdiscus* remains to be identified, degradation of the reef ecosystem is considered to play an important role in the increased incidence of ciguatera (Lewis and Holmes 1993). Global warming may also contribute to an increased incidence of *Gambierdiscus* blooms and a consequent increase in ciguatera risk in endemic areas,

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Table 2 Size and potency of characterized ciguatoxins

Ciguatoxin	Origin	$[M + H]^+$	Potency (μg/kg)[a]	References
P-CTX-4A	*Gambierdiscus* herbivores	1,061	2	Satake et al. (1996)
P-CTX-4B	*Gambierdiscus* herbivores	1,061	4	Murata et al. (1990)
P-CTX-1 (CTX-1B)	Carnivores	1,111	0.25	Murata et al. (1990), Lewis et al. (1991)
P-CTX-2	Carnivores	1,095	2.3	Lewis et al. (1991)
P-CTX-3	Carnivores	1,095	0.9	Lewis et al. (1991)
P-CTX-3C	*Gambierdiscus*	1,045	2	Satake et al. (1993)
2,3-DihydroxyP-CTX-3C	Carnivores	1,057	1.8	Satake et al. (1998)
51-HydroxyP-CTX-3C	Carnivores	1,039	0.27	Satake et al. (1998)
C-CTX-1	Carnivores	1,141	3.6	Vernoux and Lewis (1997)
C-CTX-2	Carnivores	1,141	1	Vernoux and Lewis (1997)
I-CTX-1	Carnivores	1,141	~0.5	Hamilton et al. (2002b)
I-CTX-2	Carnivores	1,141	~0.1	Hamilton et al. (2002b)

[a]Potency typically determined by intraperitoneal injection (i.p.) of pure ciguatoxin dissolved in 1 % Tween 60/0.9 % saline into 20 g mice

especially in regions where the prevailing water temperature is presently below those optimal for blooms (Llewellyn 2010: Tester et al. 2010). Global warming may also allow ciguatera to spread to currently non-endemic areas (Litaker et al. 2010). Cases of ciguatera, coinciding with the detection of novel toxin-producing dinoflagellates including *G. excentricus* and *G. silvae*, were recently reported in the Canary Islands, and the spread of *Gambierdiscus sp*. to other previously non-endemic areas, in particular the West African and Korean Coast, has been recently reported (Perez-Arellano et al. 2005; Glaizal et al. 2011; Jeong et al. 2012; Nunez et al. 2012; Fraga and Rodriguez 2014). A combination of genetic and environmental factors determines the levels of ciguatoxin entering the marine food chain and explains the unpredictable and sporadic nature of the ciguatera across both time and location.

Many species of reef fish are implicated in causing ciguatera globally. These include the herbivorous Acanthurids and corallivorous Scarids, which are considered key vectors in the transfer of ciguatoxins from *Gambierdiscus* to carnivorous fish in the Pacific Ocean. In the Caribbean, herbivorous fishes are not typically associated with ciguatera, and the herbivore vectors in this region are unclear and may include invertebrates such as crustaceans. While herbivorous species are the first step in the food chain transfer of ciguatoxins, most cases of ciguatera are caused by carnivorous fishes except for the central and eastern Pacific where herbivorous species are an important dietary component. Ciguateric carnivorous fish include the Muraenids and Lutjanids (e.g., moray eel and red bass in the Pacific), Serranids, Epinephelids, Lethrinids, Scombrids, Carangids, and Sphyraenids, which are a particular problem in the Caribbean. Differences in digestive strategies among different herbivores and carnivores are likely to influence the degree and nature of ciguatoxin biotransformation through the marine food chain (Lewis and Holmes 1993). Accumulated ciguatoxins are slowly eliminated over time, with moray eels estimated to depurate ciguatoxins with a half-life of ~260 days (Lewis et al. 1992). This slow elimination is likely related to the high lipophilicity of the ciguatoxins and extensive redistribution into tissues, which is mirrored by a relatively slow elimination half-life (82 h) in rats after oral administration (Ledreux and Ramsdell 2013; Bottein et al. 2011).

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Public Health Impact of Ciguatera

Ciguatera is a global phenomenon affecting ~50,000 people in tropical and subtropical regions annually, assuming a reporting rate of ~20 % which is typically observed for other food poisonings due to either misdiagnosis or non-reporting. Historically, ciguatera outbreaks have been mostly confined to specific regions of reef on susceptible islands, with a patchy distribution across much of the Pacific Ocean and western Indian Ocean and the Caribbean Sea (Fig. 1). The annual incidence of ciguatera in these regions varies from year to year, especially in the smaller atoll island countries of the Pacific, and appears to be on the increase (Skinner et al. 2011). A definitive explanation for such variability remains elusive, but these events presumably directly correlate with the temporal and spatial patchiness of blooms of ciguatoxin-producing *Gambierdiscus* species. More recently, a number of ciguatera cases associated with import of contaminated fish have occurred in non-endemic areas, including New York, San Francisco, Canada, Germany, and France (Geller et al. 1991; Pilon et al. 2000; Epelboin et al. 2014; Zimmermann et al. 2015). Increasing global trade and travel will likely see a further increase in the global spread of ciguatera, highlighting the significant public health implications.

Current difficulties to predict, detect, and treat ciguatera continue to have large socioeconomic impacts, particularly in developing countries where reef fish are an important source of dietary protein. Nowhere are these impacts greater than in the atoll island communities of the Pacific that have few alternatives to fish protein (Lewis 1992). With climate change having the potential to cause regional increases in the incidence of ciguatera (Skinner et al. 2011), it is now more important than ever to understand the mechanisms and impact of ciguatera on human health in order to better manage its detrimental effects to human health and nutrition.

Clinical Presentation and Diagnosis

The symptoms of ciguatera include a wide range of over 30 gastrointestinal, neurological, and cardiovascular symptoms (Table 3). Ciguatera is defined by several large epidemiological studies in the eastern (Bagnis et al. 1979) and western (Gillespie et al. 1986) Pacific, Caribbean (Lawrence et al. 1980), and Indian Ocean (Quod and Turquet 1996). The total number and type of symptoms depends on the quantity and nature of ciguatoxins consumed, which in turn is affected by the geographic origin and type (herbivore or carnivore) of ciguateric fish consumed. For example, gastrointestinal symptoms tend to dominate Caribbean ciguatera, while neurological symptoms tend to dominate Pacific and Indian Ocean ciguatera. Typically, the more severe the intoxication, the more symptoms are reported, with mild cases of ciguatera typically only affecting a fraction of consumers and presenting with only a few symptoms. Consumers of similar-sized portions of the same fish relative to body weight often report a similar number and severity of symptoms, although the specific symptoms involved can vary considerably between individuals.

Gastrointestinal signs and symptoms are typically the first to appear in ciguatera, usually within 30 min to 24 h after ingestion of contaminated fish. These include nausea, vomiting, diarrhea, and abdominal pain, which can lead to dehydration in more severe poisonings. Though slower in onset, the clinical presentation of ciguatera is typically dominated by neurological symptoms. These symptoms tend to be the most distinctive and enduring features of ciguatera and are also subjectively among the most distressing. Ciguatoxins primarily affect peripheral sensory nerves, with paraesthesias and dysesthesias dominating the clinical presentation of ciguatera. Temperature dysesthesia, specifically cold allodynia,is considered pathognomonic and has been described as a sensation of "temperature reversal," with cold temperatures being perceived as hot or burning (Cameron and Capra 1993). However, temperature

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Table 3 Average frequency of common ciguatera symptoms in the Pacific Ocean (Bagnis and Legrand 1987; Bagnis 1979; Baumann et al. 2010)

Symptoms	Frequency (%)
Gastrointestinal	
Diarrhea	64
Nausea/vomiting	54
Abdominal pain	59
Neurological	
Paraesthesias	82
Cold allodynia	85
Arthralgia, myalgia	71
Pruritus	59
Headache	56
Fatigue, asthenia	80
Dental pain	29
Dysuria	23
Perspiration	65
Cardiovascular	
Hypotension	12
Bradycardia	16

discrimination is unaffected in ciguatera, and the symptom is more accurately described as cold allodynia (Vetter et al. 2012). In additional sensory disturbances include diffuse pruritus, metallic taste, fatigue, myalgia, arthralgia, and dental pain (Friedman et al. 2008). Pruritus in particular is a characteristic symptom in the Caribbean and Pacific and the reason why ciguatera is referred to as "la gratte" or "the itch" in French Polynesia. Interestingly, ciguatoxin does not elicit histamine release in skin and in humans; itch was described as a symptom preceding pain at very low concentrations, suggesting that ciguatoxin-induced pruritus is of neurogenic origin (Vetter et al. 2012). Accordingly, the distribution of pruritus often occurs in a stocking-glove pattern affecting predominantly the hands and feet (Mattei et al. 2014b; Zimmermann et al. 2015). Neuronal symptoms of central origin, including cerebellar dysfunction, as well as general weakness or fatigue also occur. Notably, a drop in body temperature, accompanied by chills or sweating, is commonly observed in ciguatera patients (Mattei et al. 2014b) and has been experimentally reproduced in rodents (Bottein Dechraoui et al. 2008; Morey et al. 2008; Peng et al. 1995).

Peripheral neurological symptoms typically develop over several days and can persist for weeks and often months and occasionally for years. In severe cases, cardiovascular symptoms including hypotension and bradycardia are also reported (Chan and Wang 1993; Miller et al. 1999). Only very occasionally are lethal levels of ciguatoxins consumed (Hamilton et al. 2010), possibly because fish accumulating such high levels also succumb to the effects of ciguatoxins. Curiously, ciguatera symptoms including pruritus can recur in some patients after consumption of certain foods, such as nuts, fish, caffeine, and alcohol (Friedman et al. 2008).

Differential diagnosis of ciguatera remains clinical despite advances in detection. A minimum clinical diagnosis should include at least one typical neurological and one other symptoms of ciguatera appearing within 48 h of consumption of a known ciguateric fish and exclude other similarly presenting diseases. Ciguatera is often misdiagnosed as illnesses such as influenza, but it is just as important to exclude other marine poisonings such as palytoxin poisoning, and other unrelated illnesses, before any treatments are considered. Typically, each person exposed to fish of moderate and high toxicity is likely to be affected by symptoms consistent with ciguatera. However, low toxicity fish may only cause disease in a proportion of

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consumers depending on the amount eaten, body weight, and individual susceptibility. Although ciguatera is rarely lethal, fish organs, in particular the liver, may concentrate much high levels of ciguatoxin and consumption of the viscera, and internal organs are accordingly often associated with more severe disease.

Treatment of Ciguatera

Treatment of ciguatera remains largely nonspecific, symptomatic, and supportive. In the absence of a specific treatment, various drugs and herbal remedies have been used to reduce the severity of ciguatera. Given ciguatoxins activate sodium channels, local anesthetics, antidepressants, and anticonvulsants that inhibit neuronal sodium channels and decrease neuronal excitability are expected to have most potential. Case reports of efficacy in treating ciguatera exist for amitriptyline (Bowman 1984; Davis and Villar 1986; Calvert et al. 1987; Ruprecht et al. 2001), nifedipine (Calvert et al. 1987), gabapentin (Perez et al. 2001), pregabalin (Brett and Murnion 2015), fluoxetine (Berlin et al. 1992), and tocainide (Lange et al. 1988), but these approaches are not widely used. Traditional remedies for ciguatera, usually prepared by a specific member of the village, also continue to be explored as possible treatments (Bourdy et al. 1992; Kumar-Roine et al. 2009, 2011; Matsui et al. 2009), although their placebo effect is expected to be significant. Unfortunately, randomized controlled clinical trials to confirm efficacy over placebo are lacking, in part reflecting the difficulty accessing ciguatera cases. However, in vitro efficacy against CTX-induced effects in neuronal cell lines has been reported for a number of compounds, including extracts of *Heliotropium foertherianum* (Boraginaceae) and its active compound, rosmarinic acid (Rossi et al. 2012; Braidy et al. 2014). While these effects typically occur at very high concentrations, and some compounds induce cytotoxic effects on their own (Boydron-Le Garrec et al. 2005), these effects may warrant further investigation in relevant to in vivo models. Recent animal studies examining a wider range of local anesthetics and anticonvulsants identified several that may also be useful at reversing signs of cold allodynia in an intraplantar mouse model of ciguatera (Zimmermann et al. 2013).

Mannitol has been widely used to treat ciguatera poisoning (Palafox et al. 1988; Pearn et al. 1989; Blythe et al. 1992: Palafox 1992) since its use produced spectacular recovery in a ciguatera victim in a coma and who was first suspected to have a cerebral edema. Correctly diagnosed and adequately hydrated cases of ciguatera often respond to an i.v. infusion of mannitol of 1 g/kg over ~30 min, with a second infusion usually effective when on recurrent symptoms that might appear after 24 h of treatment. During the recovery phase, it is recommended that victims avoid fish and alcohol for 3–6 months, and long-term sufferers should establish if allergy-like reactions to certain foods are contributing to recurrences of symptoms and attempt to replace them.

The osmotic effect of mannitol is expected to reverse ciguatoxin-induced edema of the circumaxonal Schwann cells, which likely explains its beneficial action, along with potential neuroprotective effects (Allsop et al. 1986; Pearn et al. 1989; Mattei et al. 1999b; Birinyi-Strachan et al. 2005a). In addition, a number of case reports as well as an open label clinical trial supported the use of mannitol (Bagnis et al. 1992; Blythe et al. 1992; Palafox 1992; Mitchell 2005; Schwarz et al. 2008). However, rat and mouse models of ciguatera failed to show the expected benefits of mannitol (Lewis et al. 1993; Purcell et al. 1999), and a lack of efficacy, particularly in more prevalent milder forms of the disease, was reported in a small double-blind, placebo-controlled trial (Schnorf et al. 2002). Thus, the use of mannitol may need to be carefully reevaluated and perhaps only considered in well-differentiated and more severe forms of the disease. Additional empirical treatment approaches, including the bile acid-binding resin cholestyramine, which may enhance ciguatoxin elimination, have been used in several cases (Shoemaker et al. 2010) with reported benefit, although systematic review or controlled clinical trials assessing treatment efficacy remain lacking.

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Ciguatoxin Chemistry

The ciguatoxins are a family of heat-stable, lipid-soluble, cyclic polyether molecules (Fig. 2). The most potent ciguatoxins arise from less-oxygenated forms produced by *Gambierdiscus*, as discussed above. The ciguatoxins comprise several closely related structural variants depending on their Caribbean, Indian, or Pacific origins, which are denoted C-CTX, I-CTX, and P-CTX, respectively. The chemical structures of a number of Pacific ciguatoxins from fish (Murata et al. 1990; Lewis et al. 1991) and *G. toxicus* (Murata et al. 1990) were first elucidated using 500–600 MHz NMR on 0.2–0.5 mg quantities of pure material. Of these, P-CTX-1 is the most toxic and, along with closely related congeners, is responsible for the neurological symptoms in the Pacific (Table 2). More recently, the major ciguatoxins from the Caribbean (Vernoux and Lewis 1997; Lewis et al. 1998) and Indian Oceans (Hamilton et al. 2002a, b) have been isolated. However, only the Caribbean form was isolated in sufficient quantity to be structurally characterized, using NMR at 750 MHz (Lewis et al. 1998). In addition to structural isomers with different extents of oxidation, ring open *seco* forms have also been identified in trace amounts in *Gambierdiscus* and fish extracts (Yasumoto et al. 2000), but these likely represent unstable intermediates between more and less energetically favored epimers that interchange readily under acidic conditions (Lewis and Holmes 1993).

Ciguatoxin Mode of Action

The overall toxicological effects of ciguatera arise as a result of increased neuronal excitability, spontaneous and repetitive action potential firing in excitable cells, and increased neurotransmitter release caused by ciguatoxin effects on neuronal potassium and voltage-gated sodium channels. Ciguatoxins act at low or sub-nM concentrations in vitro and are similarly lethal in mice by the oral or i.p. route at doses as low as 0.25 μg/kg (Lewis et al. 1991). The pathophysiological effects of the ciguatoxins are defined by their ability to cause the persistent activation of voltage-sensitive sodium channels (Na_v) and to inhibit neuronal potassium channels, leading to increased neuronal excitability and neurotransmitter release, impaired synaptic vesicle recycling, modified Na^+-dependent mechanisms, elevation of $[Ca^{2+}]_i$, and spontaneous firing in numerous neuronal types, as well as edema of Schwann cells and axons (reviewed by (Molgó et al. 1992)).

At the molecular level, the ciguatoxins are some of the most potent known activators of voltage-gated sodium channels (Na_v) (Table 2), of which nine distinct mammalian isoforms ($Na_v1.1$–1.9) have been described. Na_v are expressed widely in excitable cells and are responsible for mediating the depolarizing phase of action potentials. These large membrane-spanning proteins are comprised of four homologous domains (I–IV), each consisting of six transmembrane regions (S1–S6). Like the structurally related brevetoxin, ciguatoxins bind to site 5 of most Na_vs, with critical residues for the interaction mapped to the S5 of domain I and S6 of domain IV (Poli et al. 1986, 1997; Sharkey et al. 1987; Baden 1989; Lewis et al. 1991; Gawley et al. 1992; Trainer et al. 1994). Ciguatoxins cause a hyperpolarizing shift in the voltage dependence of sodium channel activation, allowing sodium channels to open at resting membrane potentials (Bidard et al. 1984). These effects increase Na_v activation and accumulation of intracellular Na^+ at resting membrane potential that underlies the numerous Na^+-dependent effects induced by ciguatoxin in vivo and in vitro (Legrand and Bagnis 1984; Molgó et al. 1992; Hidalgo et al. 2002).

The effects of ciguatoxin on Na_v channels have been assessed in detail in both neuronal and heterologous expression systems. In parasympathetic neurons, P-CTX-1 increased the open probability of tetrodotoxin (TTX)-sensitive sodium channels, leading to gradual membrane depolarization and action potential firing, consistent with an increase in neuronal excitability through modulation of the voltage dependence of channel activation and the induction of persistent Na^+ current (Hogg et al. 1998). Similarly,

in dorsal root ganglion neurons, P-CTX-1 led to a hyperpolarizing shift in the voltage dependence of activation and a TTX-sensitive leak current presumably arising from persistent channel opening as well as a proportion of Na_Vs that open spontaneously at normal and hyperpolarized cell membrane potentials (Strachan et al. 1999; Vetter et al. 2012). In addition, a hyperpolarizing shift in steady-state inactivation of TTX-sensitive channels and an increase in the rate of recovery from inactivation of TTX-resistant channels were observed (Strachan et al. 1999). Studies of P-CTX-1 at the single channel level show effects consistent with alterations to whole cell current (Hogg et al. 1998). Interestingly, at the TTX-resistant current in rodent sensory neurons, the major effect of P-CTX-1 is an increase in the rate of recovery from inactivation (Strachan et al. 1999). In heterologous expression systems, P-CTX-1 and CTX3C elicited pronounced effects on the TTX-resistant $rNa_v1.8$ and caused a shift in the voltage dependence of activation as well as fast inactivation to more negative potentials (Yamaoka et al. 2009). Similar effects were observed for $rNa_v1.2$, $rNa_v1.4$, and $rNa_v1.5$, with CTX-3C causing a hyperpolarizing shift in the voltage dependence of activation and inactivation and significantly accelerating the time to peak current particularly for $rNa_v1.2$, but also for $rNa_v1.4$ and $rNa_v1.5$ at higher concentrations (Yamaoka et al. 2004).

In addition to profound effects on Na^+ conductance, ciguatoxins also inhibit neuronal potassium channels (Hidalgo et al. 2002; Birinyi-Strachan et al. 2005b; Schlumberger et al. 2010; Perez et al. 2011, 2012). In dorsal root ganglion neurons, inhibition of the $I_{K(DR)}$ and the I_{KA} currents in particular leads to prolonged action potential and afterhyperpolarization (AHP) duration and contributes to the increased neuronal excitability, altered membrane potential, and spontaneous action potential firing induced by P-CTX-1 (Birinyi-Strachan et al. 2005b). Similarly, P-CTX-4B inhibited potassium channels in frog myelinated axons with an IC_{50} of approximately 12 nM, but was less potent than P-CTX-1B at inhibiting Na_v channels (Schlumberger et al. 2010). A direct comparison of the effects of several ciguatoxins in cerebellar ganglion neurons showed that all ciguatoxin inhibited potassium currents, with P-CTX-1B being approximately equipotent at inhibiting I_K and I_A, while CTX-3C was particularly potent at inhibiting I_K but had little effect on I_A (Perez et al. 2011). In mouse taste cells, CTX-3C had no significant effect on potassium channels, while gambierol, a related polyether presently only identified in *Gambierdiscus* cultures, potently blocked potassium currents (Ghiaroni et al. 2006). The activity of gambierol at neuronal potassium channels was also confirmed in heterologous expression systems, where $K_v1.2$ and $K_v3.1$ were particularly susceptible to inhibition, but no activity was found at K_v2, K_v4, $K_v1.6$, hERG, and insect *Shaker*IR (Cuypers et al. 2008; Kopljar et al. 2009). The residues involved in this activity were mapped to T427 in transmembrane domain S6, although it remains to be confirmed if the same site is involved in inhibition of potassium channels by the ciguatoxins (Kopljar et al. 2009).

Overall, inhibition of neuronal potassium channels by ciguatoxins is likely to act in concert with effects on Na_v to increase neuronal excitability (Molgo et al. 1991, 1992, 1993a, b; Lewis et al. 2000). For example, while intraplantar administration of P-CTX-1 and P-CTX-3 elicits profound cold allodynia, injection of the related site 5 toxin brevetoxin and the sodium channel activator veratridine only elicits spontaneous pain (Zimmermann et al. 2013); nodal swelling induced by P-CTX-1B in frog neurons is partially dependent on K^+ efflux (Mattei et al. 2014a), and block of voltage-gated potassium channels by P-CTX-1 contributes to increased neuronal excitability in rat sensory neurons (Birinyi-Strachan et al. 2005b). Thus, effects on neuronal potassium and sodium channels by the ciguatoxins lead to various cellular and physiological consequences, including spontaneous action potential discharge, release of neurotransmitters, increase of intracellular Ca^{2+}, and axonal and Schwann edema. It is these effects that are believed to underlie the complex of symptoms associated with ciguatera.

Consistent with this pharmacological activity, ciguatera can be distinguished clinically from scombroid fish poisoning, a preventable intoxication that results mainly from consumption of *Scombridae* fish containing unusually high levels of histamine due to inappropriate storage or handling of fish. Ciguatera

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also differs from tetrodotoxin intoxication, one of the most lethal seafood toxins associated with the consumption of most puffer fish species (family *Tetraodontidae*). Even though puffer fish are easily recognized, there are still many cases of tetrodotoxin poisoning reported yearly. Interestingly, puffer fish toxins selectively block sodium channels in excitable membranes, an action that antagonizes the action of ciguatoxin. This blockade of sodium channels prevents action potentials from propagating along axons, nerve terminals, and muscle fibers, which leads to inhibition of nerve impulse-evoked neurotransmitter release at chemical synapses.

Mitigation

Several approaches can be taken to minimize the impact of ciguatera. At the distributor/individual level, the risk of ciguatera can be minimized or avoided by excluding the purchase or sale of warm water fish, allowing only small servings of individual risk fish ($\leq$50 g), or in the future by testing risk fish prior to consumption. At the industry/government level, ciguatera could be minimized through the introduction of bans on high-risk fish, restrictions on fish captured in high-risk areas, or in the future the large-scale screening of high risk. A number of in vivo methods have been established to detect ciguatoxins by oral dosing of flesh (meat-eating fly larvae and chicken) or intraperitoneal injection of crude extracts of fish (mouse) (Hoffman et al. 1983; Labrousse and Matile 1996; Vernoux et al. 1985) (see Lewis 1995). Alternative in vitro assays offer much potential to replace such assays, particularly sodium channel and antibody assays. Unfortunately, the simple detection of ciguateric fish is made difficult by a number of factors, including the low levels of ciguatoxins present in ciguateric fish ($<$0.05 ppb for P-CTX-1), the meager quantities of CTX available for research, the multiple structural forms that are present even within a single fish, the absence of any useful chromophore, and the difficulties synthesizing even fragments of these molecules (Sasaki et al. 1994; Eriksson et al. 1999; Yamashita et al. 2015).

The occurrence of ciguateric fish is sporadic and generally unpredictable. It is therefore not possible to test a "representative" sample of fish to determine the toxin potential of the remaining untested fish, even for a batch of fish caught on the same day from the same reef. This limits the scope for effective public health management of ciguatera. A membrane immunobead assay, originally developed by Hokama's group as a simple, rapid, sensitive, and specific detection method for CTX and its related polyethers (Hokama et al. 1998), is sold by Oceanit as Cigua-Check. While no false-negative results are reported, further validation of the test is required to confirm the method is robust and accurate, including confirmation that it can statistically distinguish between the positive and negative controls provided in the kit. Recently, antibodies were produced to a synthetic fragment of P-CTX-1 that may prove suitable for the simple detection of ciguatoxins in fish (Pauillac et al. 2000; Oguri et al. 2003). Unfortunately, affinity for the IJKLM antibody of CTX-3C was relatively poor and the ABCDE antibody would not be expected to interact with the major pacific CTX (P-CTX-1). The recent synthesis of the ABCDE rings of P-CTX-1 (Kobayashi et al. 2004) and total syntheses of CTX1B and 54-deoxy-CTX1B (Yamashita et al. 2015) provide an opportunity to improve the targeting of the sandwich assay. Synthesis of fragments of C-CTX is needed before antibodies suitable for detecting the C-CTX can be developed (see Fig. 2). Analytical MS methods have been developed with the required sensitivity, but are unlikely to be cost-effective for routine screening (Lewis et al. 1999). Nonetheless, this approach is routinely used for surveillance and confirmation of ciguatera outbreaks based on the high sensitivity and specificity of this method, especially in conjunction with the accelerated extraction procedure developed by Lewis et al. (2009) (Figs. 4 and 5).

Sodium channel assays have also been developed to detect ciguatoxins using cytotoxicity (Manger et al. 1993, 1995), fluorescent Na^+ assays (Louzao et al. 2004; McCall et al. 2014) in neuronal cells, or

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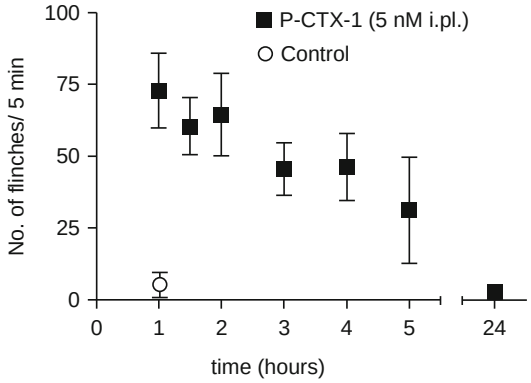

Fig. 4 A mouse model of ciguatoxin-induced cold allodynia. Local injection of P-CTX-1 (5 nM) in the hind paw of mice (intraplantar; i.pl.) produced long-lasting symptoms of frank cold allodynia, with increased licking, lifting, flinching, and shaking of the paw evoked by exposure to a surface cooled below 15 °C (Figure adapted from Vetter et al. (2012))

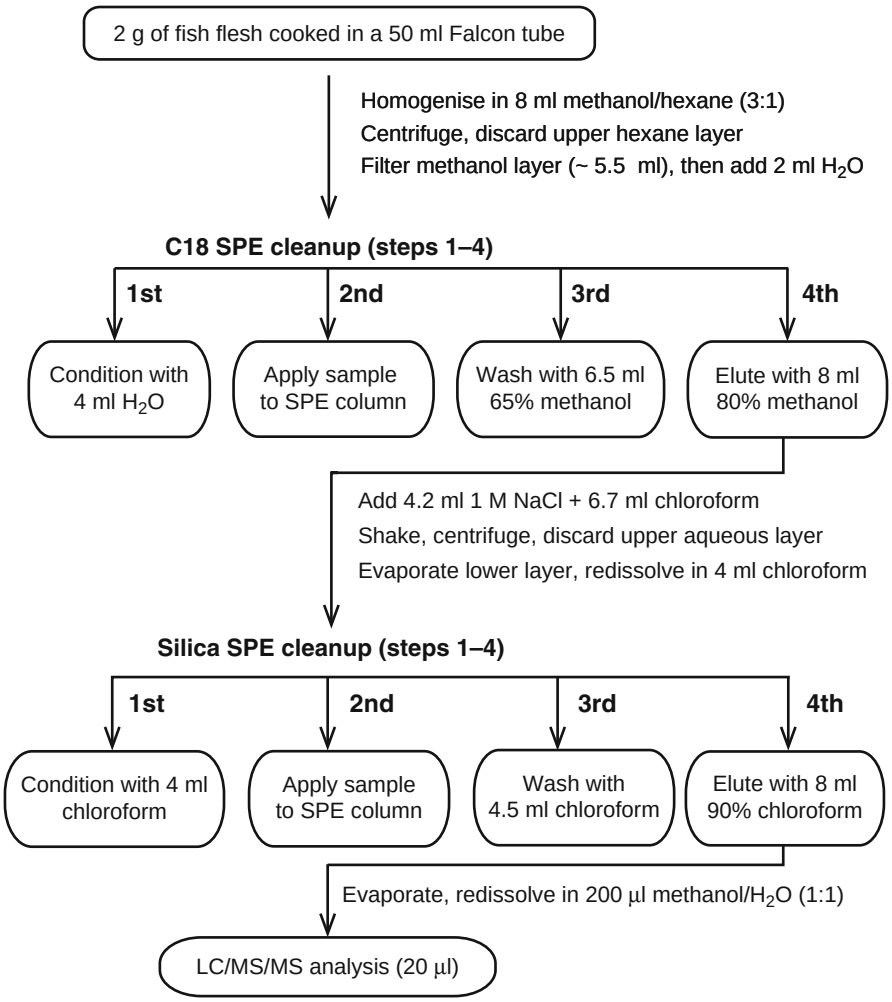

Fig. 5 The ciguatoxin rapid extraction method (*CREM*). CREM accelerates extraction of ciguatoxins using a simplified extraction protocol and as required one or two orthogonal solid phase extraction (*SPE*) cleanup steps prior to LC/MS/MS analysis. Given the similar physical and chemical properties of the different Pacific Ocean, Caribbean Sea, and Indian Ocean ciguatoxins (Lewis 2001), CREM is expected to have applicability to the extraction of different classes of CTXs prior to analysis (Figure adapted from Lewis et al. (2009))

displacement of [^{3}H]PbTx-3 binding to brain tissue (Lombet et al. 1987; Lewis et al. 1991; Poli et al. 1997) as measures of ciguatoxin content. These assays can detect sub-picogram levels of P-CTX-1, give an estimate that reflects potency, and have the potential to be developed into high-throughput assays. While it can be expected that such assays are able to detect multiple ciguatoxin variants as well as other neurotoxic ichthyosarcotoxins, rigorous validation is required to confirm the potential of such assays to be developed into rapid, cost-effective screens for public health protection. In addition, clinical diagnostic tests that detect the presence of ciguatoxin in patients are needed to overcome the present limitations of differential diagnosis.

Conclusions

Ciguatera, the most common nonbacterial seafood poisoning, is, despite its prevalence, often considered a medical curiosity in light of the perplexing and unique array of neurological symptoms associated with consumption of ciguateric fish. However, given the increased incidence in endemic areas, possibly as a consequence of climate change and degrading reef health, as well as increased spread to non-endemic areas as a result of global trade and tourism, ciguatera is rapidly becoming a disease with circumglobal public health and economic impact.

The pathophysiological effects of the ciguatoxins are due to their ability to cause the persistent activation of voltage-sensitive sodium channels (Na_v) that, in combination with inhibition of neuronal potassium channels, leads to increased neuronal excitability and neurotransmitter release, impaired synaptic vesicle recycling, and modified Na^+-dependent mechanisms in numerous cell types. While the mechanisms underlying ciguatoxin-induced cold allodynia have been characterized in detail, the pathophysiological mechanisms and pathways contributing to other ciguatoxin-induced symptoms are less clear. Improved understanding of the mechanisms leading to the symptomatology of ciguatera as well as better in vivo models should lead to novel treatment approaches.

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Marine and Freshwater Toxins
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Effects of Cyanotoxins: Sea and Freshwater Toxins

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Abstract

Cyanobacteria produce a wide range of toxins with different chemical composition and molecular targets. Cyanobacterial neurotoxins comprise a variety of compounds acting either on acetylcholine receptors or acetylcholinesterase, on voltage-gated sodium channels and on excitatory neuronal synapses. Acute intoxications can be lethal and they mainly present as paralytic shellfish poisoning. Chronic exposure may lead to neurodegenerative disorders. Hepatotoxins and neurotoxins present two main groups of cyanobacterial toxins that affect human health. Nodularin and microcystins are hepatotoxins characterized by the presence of non-proteinogenic β-amino acid ADDA in cyclic penta- or heptapeptide structure. Best known acute lethal intoxications of humans have occurred in Brazil, but chronic exposure to these toxins leading to multi-organ failure is a more serious and widespread health problem. Microcystins are also tumor promoters, and there are several reports showing harmful effects of long-term exposure to microcystins in highly populated regions of China and other parts of the world. Cyanobacterial toxins must be considered as a serious threat, and high safety measures must be followed in monitoring the quality of water and food used in human nutrition, medical care, and recreational activities.

Introduction: Cyanobacteria and Their Toxins

Cyanobacteria developed several billions of years ago, and at that time the oxygen they released into the atmosphere was the most devastating toxic substance to life on earth, although there is no evidence that this gave any advantage to cyanobacteria. Presently, this function of cyanobacteria is, from the human point of view, beneficial as they bind nitrogen, trap CO_2, and contribute to our oxygen supply. On the other hand, cyanobacteria present serious hazard to human health because they release potent cyanobacterial toxins. About 2000 species of cyanobacteria inhabit aqueous bodies worldwide, and it seems they can survive under most environmental conditions (Fig. 1).

Substances that they synthesize, including cyanotoxins, serve a role in cyanobacterial bloom growth and competition with other organisms in surface waters. Cyclic cyanopeptides comprise a large and heterogeneous group of biologically active substances that often contain unusual amino acids in their structures. The importance of many of these substances for cyanobacteria still remains to be uncovered, but it seems that the interplay of the effects of cyanobacterial neurotoxins and cyclic hepatotoxic and non-hepatotoxic cyanopeptides is important for the regulation of cyanobacterial bloom growth and decay as well as for the interactions with other photosynthetic organisms. Under favorable conditions such as water eutrophication, these planktonic cyanobacteria readily multiply in marine and freshwater environments forming toxic and nontoxic blooms.

From anthropocentric view, cyanobacteria are harmful organisms producing two main classes of toxins: neurotoxins and hepatotoxins. Acute intoxications with neurotoxins are relatively common and

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Fig. 1 Cyanobacterial bloom (*green*) and pollen (*light brownish* color) in an artificial recreation pond in Koseze, Slovenia

usually present as paralytic shellfish poisoning. Acute intoxications with hepatotoxic cyanopeptides are relatively rare, but life-threatening human intoxications and fatalities (Pouria et al. 1998) have also occurred. Due to the serious health risk, cyanotoxin monitoring in drinking and recreational waters is extremely important, but monitoring programs are not standardized (Farrer et al. 2015). Despite the harmful effects of cyanobacterial toxins, they have a great potential to be used as leading substances for the development of new drugs. This review describes a brief and simple overview of the effects of major cyanobacterial toxins with an emphasis on the effects of microcystins on mammalian cells and organs.

Cyanobacterial Neurotoxins

Cyanobacterial neurotoxins cause a variety of syndromes, and they have also been involved in several human fatalities. Cholinergic receptors and voltage-gated sodium channels are the main targets of the best known cyanobacterial neurotoxins. An excellent review on neurotoxic cyanobacterial neurotoxins has been published (Araoz et al. 2010).

Neurotoxic Lipopeptides

Antillatoxin, jamaicamides, and kalkitoxin are neurotoxic lipopeptides produced by marine cyanobacteria. Antillatoxin A and, to a lesser extent, antillatoxin B are **sodium channel activators**. The increased intracellular sodium concentration causes neuronal damage but also promotes neurite outgrowth in immature cortical neurons in 30–100 nM concentrations (Jabba et al. 2010).

Jamaicamides, hermitamide, and kalkitoxin possess an opposite action on the molecular level: they **inhibit voltage-gated sodium channels**. Prolonged exposure of cerebellar granule cells to kalkitoxin results in delayed neurotoxicity that can be prevented by application of *N*-methyl-D-aspartate (NMDA) receptor antagonists. The mechanism of this action of NMDA antagonists is unclear as the direct

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interaction of kalkitoxin with NMDA receptors has not been proven. Jamaicamide and hermitamide are structurally similar; therefore, it is not surprising that they share the same molecular targets. Besides the sodium channel-blocking activity, jamaicamides are toxic to H-460 human lung cells and to mouse neuroblastoma cells (Araoz et al. 2010).

Neurotoxic Alkaloids

Neurotoxic alkaloids can be divided in three groups: (1) anatoxins acting on cholinergic system, (2) saxitoxins that block voltage-gated sodium channels, and (3) unusual, non-proteinogenic amino acids.

Anatoxins

Anatoxins present a serious health hazard as they can be passed up the food chain and cause severe neurotoxicity. They have also been named as a "very fast death factor."

Anatoxin-a and homoanatoxin-a are powerful agonists at muscular and neuronal nicotinic acetylcholine receptors (AChRs). An enhancement of dopamine release from rat striatal synaptosomes anatoxin-a has been described as being primarily due to a presynaptic membranes depolarization which then triggers the opening of voltage-gated calcium channels (Soliakov and Wonnacott 1996; Marchi et al. 1999; Feuerbach et al. 2005).

Anatoxin-a(s) is structurally and functionally different from anatoxin-a and homoanatoxin-a. It is an organophosphate produced by cyanobacteria with a similar action as the chemical warfare agents sarin and soman. Anatoxin-a(s) irreversibly binds to acetylcholinesterase (AChE), an enzyme responsible for the cessation of ACh action in cholinergic synapses. This leads to the accumulation of ACh in the cholinergic synapses causing hyperexcitability followed by inhibition of synaptic transmission due to desensitization of nAChR.

Anatoxins and homoanatoxin have caused serious intoxications worldwide. Recently, it has been shown that homoanatoxin was present in the giant clams harvested in New Caledonia during a ciguatera outbreak, and it may be responsible for the serious neurotoxic effects observed in the local population that had consumed the contaminated clams. Severe neurotoxic effects with fatal outcome have also been observed in the Netherlands. Three dogs and several birds that had been swimming in a lake infested with cyanobacteria died of neurotoxicosis.

Saxitoxins

Saxitoxins comprise over 50 structurally related molecules produced by dinoflagellates and by some freshwater cyanobacteria. They are a common cause of paralytic shellfish poisoning (PSP). Initially, it has been shown that saxitoxin interferes with normal functioning of neuromuscular system (Kao and Nishiyama 1965), and later it has been found that it blocks the voltage-gated sodium channels (French et al. 1984). Together with tetrodotoxin, it became an invaluable tool in the study of excitatory membrane phenomena. Saxitoxin binds to nerve and muscle sodium channels with 20–60 times higher affinity than to sodium channels in the heart muscle and in denervated skeletal muscle. Its action is similar to tetrodotoxin, another dinoflagellates toxin. Both toxins cause paralysis and victims die from asphyxiation.

Commercially available saxitoxin reduces Ca^{2+} currents through L-type Ca^{2+} channels in ventricular myocytes in a voltage-independent manner, but the mechanism of its action on L-type Ca^{2+} channels has not been further elucidated (Su et al. 2004).

Toxic fish and amphibians possess saxitoxin-binding proteins that may protect them against the action of saxitoxins. Such proteins have been found in the bullfrog and in puffer fish. They may serve as a protective agent and/or for accumulation and excretion of saxitoxin. Saxiphilin found in bullfrog is a transferrin homologue belonging to a large group of cysteine proteases characterized by the presence of a

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thyroglobulin type 1 domain. The saxitoxin-binding glycoprotein from puffer fish is chemically unrelated to saxiphilin and binds tetrodotoxin and saxitoxin with high affinity; Kd ≈ 15 nM.

Nonproteinogenic Amino Acids

Intoxications with unusual neurotoxic amino acid beta-*N*-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (DAB) probably cause slowly progressing neurodegenerative disorders. Intoxications of sheep with DAB that developed neurological disorders and bird fatalities caused by BMAA support this view and also indicate that they are potent ecotoxins. DAB accumulates in the liver and causes hyperammonemia; therefore, neuropathy secondary to liver failure has been initially considered as the mechanism of DBA neurotoxicity.

BMAA produced by cyanobacteria is toxic in acute intoxications, but there is also a possibility that it may cause symptoms seen in soldiers returning from the Persian Gulf War that have developed slowly progressing neurological disorders. During the spring rains, cyanobacteria in cyanobacterial crusts in the sand can produce cyanotoxins, including BMAA and DAB. Cyanobacterial crusts that hold the desert sand together are most abundant in the Qatar region. Military activities lift the desert dust and inhalation of cyanotoxins may cause chronic intoxication. This mechanism of intoxication with delayed neurotoxic effect has been implicated in the development of amyotrophic lateral sclerosis (ALS) in soldiers within 10 years after their military service in the Gulf region (Cox et al. 2009).

There have been reports suggesting that BMAA may also be directly involved in the development of amyotrophic lateral sclerosis/Parkinson/dementia syndrome (APD), but the data were apparently misleading. Now it seems that APD is not caused by BMAA but by cycasin, a methylazoxymethanol-beta-D-glucoside present in cycad plants in Guam. Flour from seeds of the plants was used as a traditional food, and it has been shown that cycasin causes deoxyribonucleic acid (DNA) damage in the brains of experimental animals. Similar brain gene changes are also present in certain human neurological diseases (Kisby et al. 2011; Spencer et al. 2012).

Dermatotoxic and Hepatotoxic Cyanobacterial Alkaloids

Cyanobacteria produce a wide range of neurotoxic alkaloids and alkaloids toxic to the skin, kidney, liver, and several cell lines. Many of them are cytotoxic, genotoxic, and carcinogenic or they possess tumor-promoting properties. Several cyanobacterial indole alkaloids possess antiviral and antifungal activity.

Lyngbyatoxin

Lyngbyatoxin is a cyanotoxin isolated from *Lyngbya majuscula*. It causes seaweed dermatitis characterized by inflammation and formation of vesicles on the exposed skin. Several reports have shown that it is a potent tumor promoter. In vivo experiments in mice have shown that lyngbyatoxin causes lung inflammation and damages intestinal capillaries resulting in intestinal bleeding (Ito et al. 2002).

Aplysiatoxin

Aplysiatoxin was first described as a constituent of a marine mollusk *Stylocheilus longicauda* (Kato and Scheuer 1974), the main food of *Stylocheilus longicauda*, belonging to the family *Aplysiidae*. Aplysiatoxin is another cyanobacterial alkaloid that causes skin irritation and exhibits tumor-promoting activity. It also stimulates survival and proliferation of melanocytes in culture. This finding is interesting in the context that the incidence of melanomas is increasing worldwide. It is not known how much, if at all, cyanobacterial tumor promoters may contribute to the onset of melanoma in exposed skin.

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An analogue of aplysiatoxin (aplog-1) with tumor-inhibiting activity has been proposed as a therapeutic agent for the treatment of Alzheimer's dementia and human immunodeficiency virus (HIV) infection and possibly as an anticancer agent (Nakagawa et al. 2009, 2011; Irie et al. 2012; Kamachi et al. 2013). Aplysiatoxin competes with another tumor promoter, phorbol ester, for the binding to a "phorbol-12,13-dibutyrate" receptor. Binding of aplysiatoxin to this receptor triggers a cascade of events. The activated receptor triggers the metabolism of phosphoinositides on the cytoplasmic side of cellular membranes releasing lipids and lipid metabolites from cellular membranes. The released lipid metabolite diacylglycerol (DAG) in the presence of calcium ions activates protein kinase C, which is responsible for many of the observed actions of aplysiatoxin, including its tumor-promoting activity. In mice acute intoxication with aplysiatoxin causes capillary bleeding, which is similar to the effect of lyngbyatoxin. In lungs a pathology similar to the disseminated intravascular coagulation results in bleeding accompanied by fibrin deposits that are also present in the pulmonary artery. As protein kinase C phosphorylates lipocortin, it makes membrane phospholipids available to the action of phospholipase A2 (PLA2). The released arachidonic acid is metabolized by cyclooxygenase, yielding eicosanoids including thromboxane A2. Thromboxane A2 is involved in hemostasis, so the finding of blood clots in the vasculature of intoxicated animals is not surprising.

Cylindrospermopsin

Cylindrospermopsin is hepatotoxic alkaloid produced by a number of freshwater cyanobacteria worldwide. The first published outbreak of a hepatitis-like disease with additional complications such as kidney failure and bloody diarrhea caused by cylindrospermopsin occurred in Palm Island in Australia. The cause of the "Palm Island mystery disease" (Byth 1980) was unknown at that time, and it was attributed to either toxocariasis or chronic copper intoxication. Six years later, it became clear that the symptoms are most probably caused by a toxin produced by the cyanobacteria *Cylindrospermopsis raciborskii*, later shown to be cylindrospermopsin. The toxin is easily soluble in water due to its zwitterionic nature. Because of its high water solubility, cylindrospermopsin, once in the body, can be rapidly distributed throughout the extracellular fluid. Unlike microcystins that enter cells via organic anion-transporting proteins (OATPs), cylindrospermopsin does not need a special transporter to enter cells. It is toxic to hepatocytes in culture. The mechanism of its action is a reduction in glutathione synthesis, induction of DNA strand breaks, inhibition of protein synthesis, and interference with the cell cycle (Shen et al. 2002). Cylindrospermopsin interferes with a proteinaceous structure that assembles on centromeric DNA and connects to the spindle microtubules. Many components of this complex are highly conserved from yeast to humans; therefore, cylindrospermopsin may be toxic to a large variety of living organisms. The uracil group in cylindrospermopsin is probably crucial for the genotoxic and pharmacological effects of the toxins since the replacement of uracil group abolishes its toxicity. Cylindrospermopsin also alters the expression of several genes responsible for cylindrospermopsin metabolism and causes changes in the mRNA expression of several proteins, including tumor suppressor protein p53. Cytochrome P450 (CYP450) is also involved in the genotoxicity of cylindrospermopsin as CYP450 inhibition abolishes genotoxicity induced by that toxin (Humpage et al. 2005). Recent studies suggest that the induction of reactive oxygen species (ROS) by cylindrospermopsin contributes to its genotoxic potential (Straser et al. 2013).

In vivo experiments on mice and in vitro experiments on isolated rat hepatocytes in culture have shown that the liver is the primary target of cylindrospermopsin. A 1–2 μM concentration of the toxin causes apoptosis and necrosis of hepatocytes in culture. In mice, acute intoxication with extracts from different strains of *C. raciborskii* causes degeneration of the liver as the primary target organ, but it also affects the kidney, thymus, and lung. In acute as well as in chronic human intoxications, cylindrospermopsin produces similar effects. Liver injury is prominent, but concomitantly kidney failure, cardiac problems, lung inflammation, and bleeding form intestines develop. Patients usually recover within a few days to

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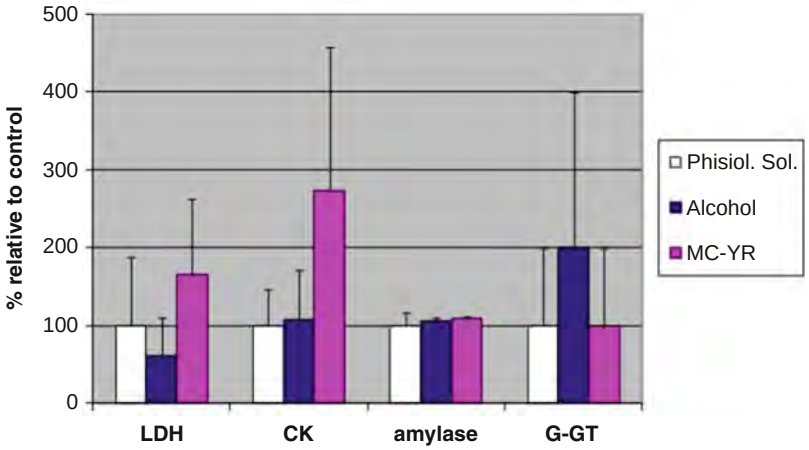

Fig. 2 Serum enzymes after subchronic exposure of rats to low doses of microcystin-YR (*MCYR*). Note a 50 % increase in serum lactate dehydrogenase (*LDH*) and 150 % increase in creatine kinase (*CK*) concentration (From PhD Thesis Irena Horvat Znidarsic)

3 weeks. The effects of chronic exposure of humans to very low doses of cylindrospermopsin are poorly documented, but in experimental animals chronic exposure to increasing sublethal doses of cylindrospermopsin for 10 months causes acanthocytosis and increased hematocrit. Despite the known genotoxic potential of cylindrospermopsin, it has not been yet clearly established whether it has carcinogenic or tumor-promoting activity.

Hepatotoxic Cyclic Cyanopeptides

Considering the number of cyanobacterial freshwater blooms and the toxins produced by cyanobacteria, microcystins and nodularin are by far most studied toxins. Microcystin-producing cyanobacteria comprise most of the freshwater bloom-forming genera. Toxic cyanobacterial blooms appear worldwide, and contaminated drinking water has caused death of stock and wildlife in many countries. Toxic cyanopeptides can be roughly divided into penta- and heptapeptides. All of these peptides contain up to five non-proteinogenic amino acids. The most characteristic is the β-amino acid (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) with specific absorbance at 238 nm. Nodularins are cyanobacterial pentapeptides and microcystins are heptapeptides. Acute intoxication with nodularin or microcystins causes severe liver damage with dilated sinusoids and extensive intrahepatic hemorrhage. Since their primary target is the liver, they are considered to be potent hepatotoxins (Theiss et al. 1988). Microcystins impair liver function and releases several enzymes from hepatocytes in the extracellular space (Fig. 2).

Nodularin and microcystins cannot freely cross cell membranes to enter into a cell; therefore, they must use a transport mechanism in the membranes of target cells. The uptake of cyanobacterial peptide hepatotoxins occurs primarily by an energy-dependent transport process involving hepatic bile acid carrier belonging to the family of organic anion-transporting proteins (OATPs), more precisely the rat OATP1B2 and human OATP1B1, OATP1B3, and OATP1A2 (Fischer et al. 2005). These transporters are most abundant in the liver, but they are also present in the brain, heart, kidney, lung, and intestinal epithelial cells. Organotropism, the specific affinity of hepatotoxic cyanopeptides to various organs, follows the distribution of OATPs in the cells of different organs. OATP1B3 is also present in the membranes of several types of cancer cells. This makes microcystins and nodularins potential leading

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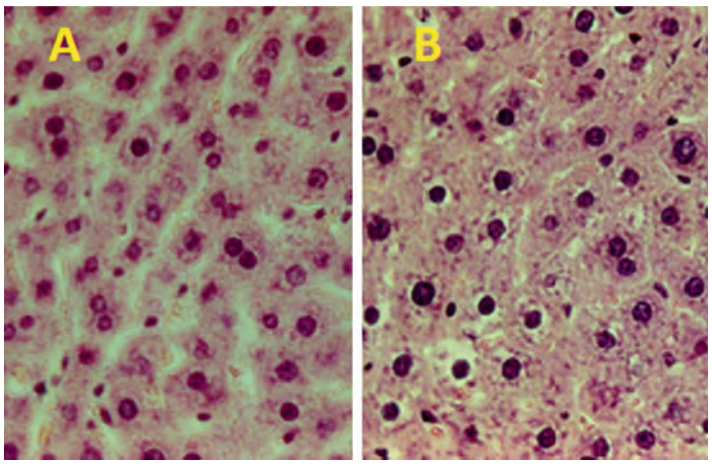

Fig. 3 Liver degeneration after 3 weeks exposure of adult rats to sublethal doses of microcystin-LR (*MCLR*). In control rats, histology shows normal sinusoids (**a**). After the treatment with MCLR, the sinusoid structure is destroyed (**b**)

substances for the development of novel and tissue-specific anticancer drugs. Interestingly, cyanobacteria also produce peptides that may diminish the toxic potential of microcystin and nodularin. A nontoxic nostocyclopeptide M1 prevents the uptake of microcystins and nodularin by inhibition of OATP1B3- and OATP1B1-mediated transport (Herfindal et al. 2011). Microcystins and nodularin share the same molecular targets and mechanisms for their toxic effects. Their toxic effects will be described together, with microcystin-LR (MCLR) as an example.

Patho-biochemical and Genotoxic Basis of Microcystin and Nodularin Toxicity

The initial steps in the toxicity of microcystins are the inhibition of protein phosphatases and oxidative stress. This is followed by genotoxic effects and changes in cell morphology. Among protein phosphatases, the inhibition of serine/threonine protein phosphatase type 1 and even more so type 2A is most pronounced. This leads to hyperphosphorylation of several cellular proteins and activation of protein kinases, resulting in dysregulation of a number of cellular processes and regulatory pathways. Inhibition of protein phosphatases by cyanobacterial toxins affects the cytoskeleton, leading to morphological changes in the liver and in cultured hepatocytes (Fig. 3), the primary target of microcystins (Eriksson et al. 1990; Yoshizawa et al. 1990).

Oxidative stress and genotoxicity are important mechanisms of cell damage caused by microcystins. Cyanobacterial lyophilisate and microcystins cause inflammation of the liver resulting from lipid peroxidation and malondialdehyde production (Guzman and Solter 1999). MCLR-induced oxidative stress decreases the activity of antioxidant enzymes and increases the expression of heat shock protein 70. Superoxide dismutase abolishes the disruption of cytoskeleton by MCLR; therefore, the formation of superoxide radical seems to be the initial step in ROS formation. This view is supported by data showing that MCLR increases the expression of cytochrome oxidase P450 2E1 (CYP2E1) mRNA, and cytochromes are one of the sources of superoxide anion in living organisms. CYP2E1 inhibitors prevent ROS generation meaning that this enzyme is essential in ROS generation at least in hepatoma HepG2 cell line. Superoxide anion is only indirectly involved in the damage of cell components. Metal ions, especially iron, are involved in Fenton reactions catalyzing the formation of hydroxyl radical from superoxide anion. Hydroxyl radical then directly damages membrane lipids, microtubules, and genetic material. A single dose of 100 μg/kg of MCLR damages intestinal mucosa by lipid peroxidation. The role of ROS in microcystin toxicity is further supported by the fact that iron chelators prevent disruption of cytoskeleton in hepatocytes by preventing the formation of hydroxyl radical.

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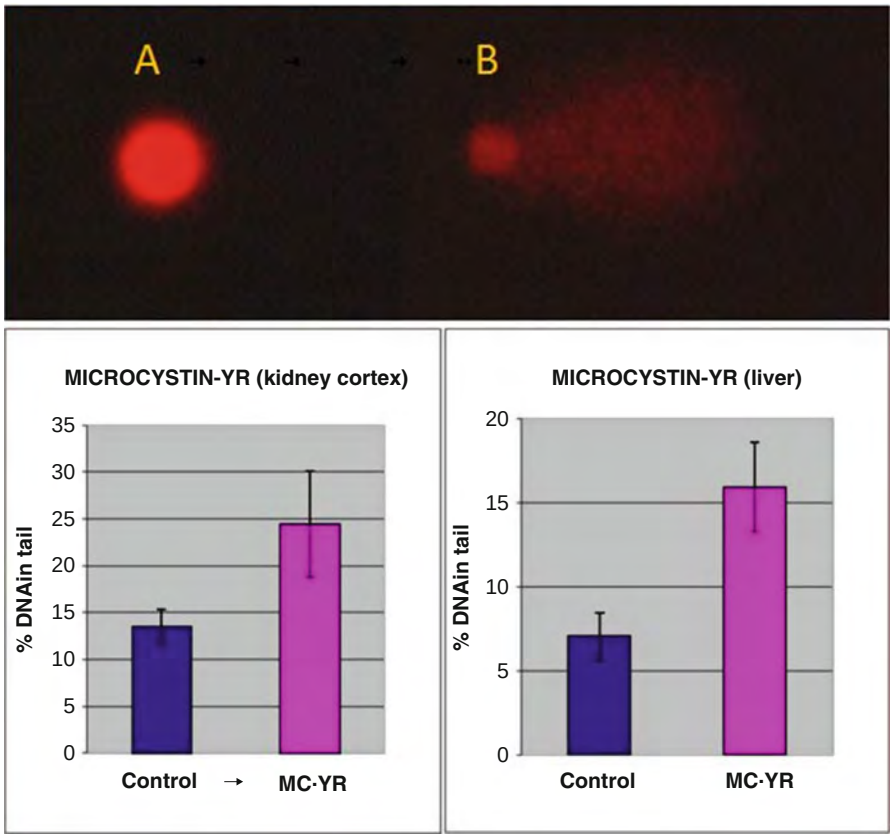

Fig. 4 Microcystin-YR (*MCYR*) induces DNA damage in the liver and kidney cells. Single cell gel electrophoresis assay shows normal compact and rounded shape of the nuclear DNA (**a**) which changes into a comet shape (**b**) due to the DNA damage after the treatment with MCYR. Both the kidney and liver cells are affected

Exposure of cells to low doses of MCLR causes depletion of intracellular glutathione (GSH) followed by a transient DNA damage. Cells survive the treatment and after a few hours the cytoplasmic concentration of GSH reaches normal values. The transient DNA strand breaks are present only during the DNA repair processes (Fig. 4). Acutely induced DNA damage can be, at least partially, repaired (Zegura et al. 2003), but chronic and repeated intoxication may lead to permanent DNA damage.

Microcystin and Nodularin Intoxication

Microcystins comprise a group of roughly a hundred cyclic heptapeptides consisting of up to five non-proteinogenic amino acids in their structure. MCLR is the most studied member of the family, and the provisional guidelines for water safety are based on toxicity data from studies using this toxin. Nodularin, the hepatotoxic pentapeptide, exerts identical toxic effects in vivo.

Acute intoxication by microcystins is very common in animals, and a number of bird fatalities have been reported, but oral intoxication is less likely to occur in man. A thorough study of the effects of feeding rabbits, mice, guinea pigs, lamb, chickens, and duck with lyophilized *Microcystis aeruginosa* revealed congestion of the liver with focal necroses and animal death due to intrahepatic hemorrhage (Konst et al. 1965). Hemorrhage can be explained by disorganization of cytoskeletal microfilaments and membrane blebbing of hepatocytes, leading to the disruption of sinusoids. The lung and kidney are also affected in acute intoxication, and early reports considered those changes to be “hepatocellular microembolisms” of the kidney cortex and lungs.

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Acute intoxications with cyanobacterial hepatotoxins have also occurred in humans. Mild intoxications presented as gastrointestinal problems after swallowing the contaminated water, but a case of pneumonia and even fatal outcome has also been described (Turner et al. 1990; Carmichael et al. 2001). Acute fatal intoxications are exceptional – the most known fatal intoxications occurred in Brazil due to the presence of microcystins in the water used in a hemodialysis unit (Pouria et al. 1998). Initially, the patients complained of gastrointestinal problems and many of them died due to renal and hepatic failure (Jochimsen et al. 1998; Yuan et al. 2006). Tinnitus, intermittent blindness, deafness, and confusion that the patients experienced suggest that microcystins may also be neurotoxic (Feurstein et al. 2011).

Chronic exposure to microcystins is more common but less understood. Feeding mice with water containing cell-free cyanobacterial extract containing microcystins for a period of 1 year caused development of hepatic tumors. Reproductive function was not affected, but 10 % of neonates from animals treated with the cyanobacterial extract showed reduced brain size (Falconer et al. 1988). Bronchopneumonia was also more commonly observed in intoxicated animals. This may be explained by the fact that purified microcystins reduce cytokine production, increase apoptosis and necrosis of human and chicken lymphocytes, and impair the function of neutrophils (Lankoff et al. 2004; Kujbida et al. 2009). There is an unresolved discrepancy between the finding that reproductive function of animals fed by cyanobacterial extract was unaffected and other data showing that male reproductive system is severely affected after chronic intoxication of mice with purified microcystins. Microcystins decrease sperm mobility and viability, increase sperm abnormality, and decrease the level of serum testosterone (Ding et al. 2006; Li et al. 2008; Liu et al. 2010; Chen et al. 2011).

Chronic exposure of experimental animals to known doses of purified microcystins affects several organs. The kidney, intestinal epithelial cells, lungs, heart, brain, immune, endocrine, and reproductive systems are most severely affected (Frangez et al. 2003; Milutinovic et al. 2003; Filipič et al. 2007; Feurstein et al. 2010; Suput et al. 2010). Oxidative stress is an important mechanism for the damage of these organs. In the kidney, microcystins cause chronic inflammation with collapsed glomeruli and shedding of tubular cells. Tubules in the medulla are filled with eosinophilic material. Epithelial cells show “ballooning” degeneration and apoptosis. Microcystin and nodularin-induced oxidative stress causes DNA damage in hepatocytes and in several other organs such as the kidney, brain, and lung. Genotoxic effects of microcystin are one of the mechanisms involved in tumor promotion, carcinogenesis, and apoptosis.

Epidemiological studies have revealed that a long-term ingestion of microcystins in drinking water may be related to liver tumor promotion (Yu 1995). Low doses of microcystins ingested over a prolonged period of time are genotoxic, teratogenic, and possibly carcinogenic or tumor promoting (Ito et al. 1997; Zhou et al. 2002; Falconer and Humpage 2005). The presence of these toxins in drinking water and food is a serious concern in many countries, especially in Asia (Nishiwaki-Matsushima et al. 1992; Ling 2000). A known tumor promoter, okadaic acid, is also a potent protein phosphatase inhibitor, and inhibition of this enzyme is probably responsible for the carcinogenic or tumor-promoting activity of nodularin and microcystins. An important step in the MCLR-induced tumorigenesis may be the hyperphosphorylation of cytokeratins 8 and 18 that is more pronounced than hyperphosphorylation of any other cellular protein. Other metabolites present in cyanobacteria also possess tumor-promoting activity as they inhibit gap junctional communication and activate pro-proliferative and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and mitogen-activated protein kinase (MAPK) pathways, which further supports the view that cyanotoxins are a serious threat to human health.

Inhalation of cyanobacterial toxins may present an important intoxication route (Ito et al. 2001). Lung inflammation and atelectases, the collapse of lung alveoli, occur after aerosol inhalation and after intraperitoneal injection of MCLR. Lung compliance rapidly decreases due to lung congestion and inflammation, presenting as inflammatory infiltrations of lung parenchyma and interstitial edema.

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Cardiotoxic effects of microcystins are important in acute and in chronic intoxications. Rats exposed for 8 months to sublethal doses of microcystins developed atrophy and fibrosis of the heart muscle characterized by enlarged cardiomyocytes with decreased myofibril concentration. The heart is also affected during acute intoxication with microcystins. Initially, the observed cardiovascular effects (Theiss et al. 1988) were explained solely by decreased volume of the circulating blood due to intrahepatic hemorrhage. Now it is known that ROS-induced cardiotoxicity is an important mechanism in the lethal effects of microcystins (Qiu et al. 2009).

Microcystins can reach the central nervous system using brain-specific OATP1A2 present in the brain and in blood-brain barrier. Acute exposure of experimental animals to microcystins causes memory impairment seen as spatial learning and memory retrieval defects. Similar signs and symptoms were observed in patients after acute intoxication with microcystins (Feurstein et al. 2011). Microcystin-induced lipid peroxidation and DNA damage in hippocampus is an important mechanism for memory impairment. Besides that, the subchronic exposure to low doses of MCLR produces genotoxic effects in the central nervous system. These observations and the finding that MCLR induces hyperphosphorylation of tau protein and neuronal apoptosis in the hippocampus of rats (Li et al. 2012) indicate the possibility that MCLR may be involved in the development of neurodegenerative diseases.

A provisional guideline level for drinking water has been determined at a 1 μg/L of microcystin-LR. Data on chronic effects of microcystins on the liver and other organs, including teratogenicity, reproductive toxicity, and carcinogenicity, calls for a reevaluation of this value (Falconer and Humpage 2005).

Effects of Microcystins and Nodularin on Mammalian Cells

Microcystins and nodularin cause cell morphology changes typical of apoptosis. Rounding and blebbing of hepatocytes disrupts liver ultrastructure leading to dilatation of sinusoids and intrahepatic hemorrhage. The ensuing hypovolemic shock and acute liver failure are the leading cause of death after microcystin or nodularin intoxication.

Inhibition of protein phosphatases and adenosine triphosphate (ATP) synthase seems to be the initial step in induction of apoptosis (Mikhailov et al. 2003) and in morphology changes in a variety of cells. Increased expression of tumor suppressor gene p53, phosphorylation of the nuclear protein p53, and externalization of cell membrane phosphatidylserine occur in the early stage in the process of apoptosis (Guzman et al. 2003) accompanied with prominent changes in cell morphology. Besides apoptosis, in renal cell line Vero-E6, MCLR induced autophagy as well. Apoptosis can be initiated by various routes. Mitochondria, intracellular Ca^{2+}, and oxidative stress have an important role in the execution of cell death. Mitochondria are involved in ROS formation and in the release of cytochrome c. In a cell, microcystin-induced mitochondrial Ca^{2+} increases mitochondrial permeability releasing cytochrome c from mitochondria which triggers apoptosis (Ding et al. 2001). Another apoptotic pathway leads through an increase of cytosolic Ca^{2+} that directly activates calcium-binding protease calpain. Microcystin-LR-induced phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and phosphorylation of myosin light chain may be responsible for the formation of apoptotic blebs and for the microcystin-induced apoptosis, although the role of activation of caspases is also important (Ding et al. 2002; Fladmark et al. 2002; Krakstad et al. 2006).

Most studies of the effects of microcystins on cell morphology were performed on hepatocytes, and the observed changes are typical for apoptosis. Membrane defects and blebbing, collapse of cytoskeletal filaments, disruption of cell-to-cell contacts, condensation of chromatin, and loss of nuclei are typical changes observed after exposure of rat or human hepatocytes to microcystins (Batista et al. 2003; Milutinovic et al. 2003) or nodularin. In rat hepatocytes, hyperphosphorylation of keratin intermediate microfilaments is an important event underlying the changes in liver ultrastructure and cell morphology. Typical cell shape changes are caused by polymerization and condensation of actin filaments forming a

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starlike structure radiating from the center of the cell toward the cell membrane. This change in morphology takes place in several cells such as hepatocytes, fibroblasts, and epithelial kidney cells (Khan et al. 1996). In rat kidney cells and fibroblasts, hyperphosphorylation of microtubules, intermediate filaments, and microfilaments contribute to the disruption of cytoskeleton and its condensation at the cell nucleus.

MCLR causes phosphorylation and activation of several subfamilies of MAPK (Zhang et al. 2010) such as p38MAPK, ERK1/2, and stress-activated mitogen kinase/jun amino-terminal kinases (JNK). It seems that especially P38 and ERK1/2 are responsible for the MCLR-induced hyperphosphorylation of intermediate filaments and their condensation in the form of centrally located dense bundles (Chen et al. 2012). Jun plays a critical role in extrinsic and in mitochondrial apoptotic pathways, and it may be a link between the microcystin-induced protein phosphatase inhibition, cytoskeleton disruption, and apoptosis pathways. P38 and Jun transmit stress signals and their activation may lead either to apoptosis, cell differentiation, or tumorigenesis. ERK1/2 mostly transmits mitogenic signaling, and it may be involved both in acute and long-term effects of microcystins and nodularins including tumorigenesis or tumor-promoting activity of microcystins. MCLR inhibits glycogen synthase kinase β, an important enzyme that phosphorylates β-catenin leading to its degradation in proteasomes. β-catenin is involved in hyperplasia and tumor progression (Mucenski et al. 2005; Wagh et al. 2012; Jarde et al. 2013). Since MCLR suppresses glycogen synthase kinase β, the levels of β-catenin increase and promote cell survival. On the other hand, microcystins activate MAPKs, which in turn phosphorylates several proteins including p53. Phosphorylated p53 induces apoptosis by activating downstream events and by induction of proteasomal degradation of β-catenin. An example of multi-potency of microcystins to either activate apoptosis or to promote cell survival is simultaneous activation of p53 and serine/threonine-specific protein kinase Akt/protein kinase B (Takumi et al. 2010). Akt inhibits apoptotic processes and promotes cell survival. Under conditions of reduced p53 activation, a chronic exposure may lead to cell proliferation and tumor promotion due to the increased Akt activity. Exposure of cells to microcystin-RR (MCRR) and MCLR also changes expression of several proteins involved in regulation of cell growth and death, which further expands the variety of cell responses to chronic intoxications with microcystins and nodularin. Interaction between the activated proapoptotic, mitogenic, and cell survival-promoting proteins determines the final effect of microcystins on cells.

Relevance of Experimental Data for Providing Guidelines for Food and Drinking Water Safety

Microcystins and nodularin have caused wildlife and domestic animal intoxications and even human fatalities. Increasing data show that chronic intoxications worldwide may cause degenerative diseases and cancer. All the collected data show that it is important to provide accurate guidelines for safe food and drinking water consumption and for water quality in recreational activities. Experimental data show that interspecies variability is significant; therefore, we must keep in mind that humans may be either more or less susceptible to cyanobacterial toxins than experimental animals. Data were also obtained on cells from different animals, and researchers also used different cell lines, which makes the results difficult to compare. Rat hepatocytes are often used as a model to study hepatotoxic effects of cyanobacterial toxins. They can be readily isolated from rat liver and they are easy to culture. When cell cultures are used for experiments with microcystins, both the origin of the cells and days in culture greatly affect their response. The use of cell lines is even more attractive since the results are more repeatable and comparable, but we must keep in mind that cell lines usually lack several characteristics of normal cells. The HepG2 cell line is derived from human hepatoma and it is very popular in studies of hepatotoxicity, as the cells preserve many original physiological and biochemical characteristics of hepatocytes. Nevertheless, the results obtained on these cells and cells of similar origin must be interpreted with caution and cannot be simply

extrapolated to human hepatocytes from healthy liver. Despite the fact that microcystins affect human health and that interspecies variations are significant, very few studies have been done on human primary isolated cells (Batista et al. 2003; Fischer et al. 2010).

Comparative data on the effects of MCLR on rat and human hepatocytes show that human hepatocytes obtained during major liver resection in patients with tumors are more susceptible to the effects of MCLR than rat hepatocytes. Rat and human hepatocytes treated with MCLR in different concentrations show similar changes in morphology, but the dose/response curve was steeper for human hepatocytes than for rat hepatocytes. Additionally, the variability of dose/response curves between hepatocytes from individual patients was much larger than the variability between rat hepatocytes from different animals (Batista et al. 2003). Comparison of the effects of microcystins on primary human hepatocytes and human embryonic kidney 293 (HEK293) cells confirmed the high susceptibility of human hepatocytes for MCLR (Fischer et al. 2010). This indicates that the water quality guidelines proposed by the World Health Organization (WHO) and adopted by many countries must be carefully observed and possibly reconsidered.

Conclusions

Cyanobacteria are organisms with a great positive and negative ecological impact. Cyanotoxins have caused acute and chronic human illnesses, hepatocellular tumors, and even acute fatalities worldwide. It is surprising that guidelines for water quality monitoring are limited to microcystins and to very few neurotoxins. Official regulations for other cyanotoxins have not been established. Provisional guideline values have been provided by WHO for microcystin-LR in drinking water. Several countries have adopted this regulation, but sound data are not provided for other cyanotoxins. There is an urgent need to determine safe values for the presence of other cyanobacterial substances in food and in drinking water that are involved in the development of neurodegenerative disorders, kidney failure, and other organ dysfunctions.

Many biologically active compounds from cyanobacteria still have to be discovered. Some of them may be involved in initiation and/or progress of chronic diseases, and others, such as nostocyclopeptide M1, may prevent the uptake or toxic actions of cyanotoxins. On the positive side, several cyanobacterial toxins and other compounds may become important leading substances for the development of novel and tissue-specific anticancer drugs.

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Marine and Freshwater Toxins
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Miscellaneous Marine Toxins of Medical Significance

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Abstract

Subaquatic life is in a state of constant mechanical and chemical warfare in which the best adapted will survive. In these complex interactions, some animals can present structures that will inflict trauma, such as teeth, spines, and stings, which can be venomous or not.

Studies on toxins of dinoflagellates and cyanobacteria are expanding, and disease outbreaks associated with these toxins are increasing globally. These poisonings can occur in an individual (as the paralysis for seafood) and in small or large groups (such as the red tides). Various marine animals used as food may be contaminated, which broadens the spectrum of these poisonings and increases the need for additional studies on the activity of these toxins and their mechanisms of action. Studies on therapeutic measures and approaches to reduce or eliminate the risk in the environment are also needed.

Injuries caused by sea urchins are common around the world. Some species cause severe envenomations, but the majority of these injuries are traumatic and not serious. Sea cucumbers can be toxic, and the crown-of-thorns starfish is venomous. Additionally, sea worms present toxins and bristles that cause skin inflammation and are also responsible for painful bites. They are common on the coasts, and the clinical manifestations caused by marine worms present similarity with injuries caused by marine sponges. Leeches do not cause envenomations and rarely are associated with marked manifestation in humans.

Introduction

Subaquatic life is in a state of constant mechanical and chemical warfare in which the best adapted will survive. In these complex interactions, some animals can present structures that will inflict trauma, such as teeth, spines, and stings, which can be venomous or not (Figs. 1 and 2).

Various aquatic animals use toxic chemicals as a defense against aggressors or as a means to capture food. The presence of toxins in poisonous and venomous animals is manifest in many different ways. Thus, we see extremely sophisticated apparatus of inoculation in phyla of simple animals, such as the nematocysts of cnidarians. Innocent shells can kill a human, as seen in envenomations caused by the Conidae family. Certain organisms, such as the puffer fish, some crustaceans, and the blue-ringed octopus, can store potent toxins of microorganisms. Some fish can produce venom in glands on stingers, as observed in stingrays, catfish, scorpionfish, and others. There are fish that can inject venom as snakes or spiders do by using hollow spines linked to an isolated gland, as seen in the envenomation caused by some toadfish (*Thalassophryne* spp.). There is a genus of fish in the Red Sea (blennies of the *Meiacanthus* spp.) that can inject venom through grooved fangs. Such diversity exemplifies the aforementioned chemical warfare and alerts us to the fact that small and seemingly harmless animals can cause serious damage and that much has yet to be studied in this area of toxicology.

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Classification and Distribution of Aquatic Venomous and Poisonous Animals

The presence of venoms and poisons is very common in aquatic environments. Among the various aquatic phyla, toxins are present in all the animals, including the most simple. The marine sponges (phylum Porifera) are tubes of cells without movement but contain mechanical defenses (spikes in the body) and irritant toxins (in the lime that covers their bodies), causing inflammatory processes in victims that touch the animals.

The cnidarians (phylum Cnidaria) have cnidocytes, defense cells carrying neurotoxic, cardiotoxic, and allergenic substances. Some species of sea urchins, sea cucumbers, and starfish can present toxins and cause severe envenomation in humans. Marine worms can also be venomous. Some mollusks can present potent neurotoxins, especially Gastropoda shells of the *Conus* genus and octopuses of the *Hapalochlaena* genus (blue-ringed octopuses).

There are more venomous fish than venomous reptiles, and venomous fish can cause injuries with different levels of toxicity. Though injuries caused by marine and freshwater catfish are the commonest, envenomations caused by stingrays and fish of the Scorpaenidae family are serious and can even cause human death. However, a great number of other fish also carry toxins; every wound caused by stingers, fins, and preopercular spines that presents persistent pain should receive first-aid measures specifically recommended for such envenomation (Cardoso et al. 2009; Haddad 2000, 2003; Haddad et al. 2003, 2004, 2010).

Unlike terrestrial animals, venomous aquatic animals most often have a global distribution, especially marine animals. Marine sponges, jellyfish, Portuguese man-of-wars, sea urchins, and marine worms are found in all seas (especially in waters of higher temperatures); more restricted species usually occur in freshwater environments.

An interesting aspect of injuries caused by aquatic animals is the epidemiology of envenomation. In a study of the entire Brazilian coast, which ran for about 20 years, the author observed more than 1500 patients and found that 50 % of injuries were caused by sea urchins (without envenomations), 25 % caused cnidarians, and 25 % by venomous fish – catfish in particular, but also stingrays and scorpionfish (Haddad 2009a).

What differentiates such accidents are the species present at specific locations. The jellyfish of the Cubomedusae class exist in all tropical and subtropical waters, but species with truly dangerous venom that can cause human death are found in the Indo-Pacific. The only fish with venom that is truly associated with human death is the stonefish, also an inhabitant of the Indo-Pacific area. However, serious accidents caused by jellyfish, Portuguese man-of-war and other venomous fish are found everywhere on the planet, and what must be taken into account is the high morbidity rate rather than the mortality rate, which is relatively low. This question of the duality of morbidity/mortality is extremely important: fishermen injured by venomous fish that provoke necrosis can develop deep ulcers and infections that will not allow them to work for weeks or months. This is a serious problem because a great number of these fishermen live in distant regions, without recourses or associations that can help them. Examples of such injuries are those caused by freshwater stingrays, which are common in certain places in South America. The sting of a freshwater stingray causes extensive and deep local necrosis and long-term disability.

In reality, injuries by aquatic animals can be expected in any area of large freshwater flows or seas. All seas have several species of cnidarians, echinoderms, marine sponges, and venomous fish. Catfish, as well as stingrays, are common in rivers, lakes, and seas. Fishes of the Scorpaenidae family (stonefish, lionfish, and scorpionfish) are common in all marine environments. With globalized travel and trade, there is the possibility of introducing exotic species into local waters via a ship's ballast or the release of pets, as occurred with the lionfish, which originated from the Indo-Pacific and is now widespread throughout North, Central, and northern South America. Some species of jellyfish were introduced into other

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Fig. 1 Recreation is the main reason for increased tourism in aquatic environments. Various animals have defense mechanisms (including poisoning) that can provoke serious injuries in fishermen, bathers, and swimmers (Photograph: Vidal Haddad Junior)

Fig. 2 In freshwater environments, it is possible to see blooms of algae and other microorganisms that can produce outbreaks of poisonings; however, the majority of injuries are caused by venomous fish, such as catfish and stingrays (Photograph: Vidal Haddad Junior)

countries and successfully adapted to their new environments. Incidents such as these are a warning: unexpected accidents – as occurred in certain areas a few decades ago – can still occur, and the problem could increase in coming years (Haddad 2009a).

The reporting of human injuries by aquatic animals around the world is sporadic and without sequence. The clinical characteristics of envenomations and the main causers of injuries have only recently received

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Fig. 3 Shellfish are an important part of the human diet. In a South Korea market, it is possible to see the great variety of edible species (Photograph: Vidal Haddad Junior)

more attention, which will improve the possibility of establishing more effective therapeutic and preventive measures for accidental human envenomation by toxic fish.

Saxitoxin, Shellfish, and Other Neurotoxic Aquatic Animals

The ingestion of certain species of fish and shellfish can cause poisoning. Most poisonings are associated with genera and species of animals (such as crabs, clams, sea turtles, and some fish) in the Indian and Pacific oceans. These animals are also present in other oceans, which makes us assume that all types of intoxication described may occur throughout the world. Dinoflagellates and other microorganisms that produce toxins, which accumulate in these animals, are also found in all marine environments. However, the signs and symptoms of poisoning can be nonspecific and may go unnoticed in emergency rooms. As a result, they require an active search to detect them. It should be borne in mind that most diseases studied in this chapter cause nonspecific gastrointestinal symptoms, which can lead to confusion with bacterial toxins that are common in deteriorated seafood and fish. For best observing full neurological symptoms that identify poisoning by neurotoxins, it is necessary to monitor the patient for a minimum time of 24 h, which is difficult in most emergency services located on the coast. It is important to know that cyanobacteria (especially blooms in lakes of *Anabaena* sp.) can produce neurotoxins, such as anatoxin and saxitoxin, in freshwater environments. These neurotoxins cause death via muscular effects in livestock but rarely are ingested by humans in an amount sufficient to cause death (WHO 2013).

Diseases Caused by Seafood Toxins

Paralysis by seafood toxins is caused by saxitoxin and some derivatives, mainly neurotoxins, produced by dinoflagellates of the genus of *Alexandrium* (formerly *Gonyaulax*) and *Pyrodinium* spp., which are also

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Fig. 4 Fish of the order Clupeiformes are commonly used as human food. The image shows sardines (one of the most consumed fish around the world) (Photograph: Vidal Haddad Junior)

Fig. 5 Sharks are responsible for human attacks around the world. There are also poisonous species, including the bull shark (Photograph: Vidal Haddad Junior)

responsible for the phenomenon of red tides. Saxitoxin and derivatives accumulate in shellfish after filtering seawater (Fig. 3). The ingestion of contaminated seafood causes the paralysis (Meier and White 1995; Williamson et al. 1997). Saxitoxin and its derivatives are potent neurotoxins that block the propagation of action potentials acting on sodium channels. Other conditions caused by seafood toxins are neurotoxicity (associated with the dinoflagellate *Gymnodinium brevis*, producer of brevetoxins), diarrhea (linked to the dinoflagellate *Dinophysis* sp.), and amnesia that simulates clinical manifestations of Alzheimer's disease and causes permanent loss of recent memory. These indications manifest following the intake of seafood contaminated with domoic acid of the diatom species, *Nitzschia pungens*.

In paralysis caused by seafood toxins, symptoms begin to appear about 20 min after ingestion of the contaminated animal, which may be a clam or mussel. Paresthesias in the hands and mouth, progressive muscle weakness, and the sensation of "floating" occur. Manifestations include vomiting, headache, ataxia, and progressive muscle paralysis, including respiratory muscles. Death can occur around 12 h; when not fatal, the duration of manifestations is 2–5 days. In a series of 2334 such poisoning incidents, the death rate was 5.9 %, with the majority occurring in children (Meier and White 1995). There is no tests to confirm the diagnosis of the disease. Gastric lavage and/or vomiting (within 4 h, preferably) may be an

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effective treatment. The poison is an acid; and the use of baking soda, as well as activated charcoal, can help inactivate the effects. Severe cases require mechanical ventilatory support.

Clupeotoxicity

Fish of the order Clupeiformes are commonly used as human food (Fig. 4). The order includes sardines (one of the most widely consumed fish around the world), anchovies, herring, and tarpons. The toxin in such fish has not been identified, but it is suspected that there is an accumulation of some substance existing in dinoflagellates. The toxin is not similar to ciguatoxin because the involved fish feed on plankton, unlike carnivorous fish that are associated with ciguatera (Thomas and Scott 1997). Toxicity in humans is rare but does occur in such fish from the Indian and Pacific oceans and the Caribbean. Symptoms begin after 15–30 min of ingestion, with dryness and a metallic taste in the mouth (characteristic of the disease). These symptoms are followed by nausea, vomiting, and diarrhea. Severe cases have impaired the central nervous system, manifested by dilated pupils, headache, paresthesia, tingling, salivation, muscle cramps, difficulty breathing, paralysis, convulsions, coma, and death. The mortality rate reaches 45 %, and some deaths occurred so quickly that the fish was found in the victim's mouth at the time of death (Thomas and Scott 1997).

Poisonous Sharks

Under certain conditions, the flesh and liver of some sharks, such as the bull shark (*Carcharhinus leucas*) and the tiger shark (*Galeocerdo cuvier*), can become toxic, causing intoxication manifested by neurological symptoms such as seizures, breathing difficulties, coma, and death (Fig. 5). In a case observed in Madagascar, 68 patients died after eating the flesh of a single bull shark (Boisier et al. 1995). The mortality rate reaches 30 %. Toxins are called carchatoxins, but there is no more information about the toxins' composition and origin.

Scombroid Fish Poisoning

Saurin is the toxin involved in this type of poisoning. It is formed by the action of bacteria (especially *Proteus morganii*) after poor conservation of the meat of fish with high blood flow, such as tuna, mackerel, and beautiful (gender *Scombroides*). The toxin develops from histidine, which in itself is harmless to humans. However, the toxin provokes intense histamine phenomena (Meier and White 1995) resulting in histamine poisoning. Within 30–60 min, symptoms appear, including pulsatile headache, vomiting, diarrhea, tachycardia, dry mouth, edema and erythema of the face, conjunctivitis, blisters on the trunk and face, and difficulty breathing. Death is rare. Recovery starts at 24 h. The use of antihistamines effectively treats the poisoning.

Minamata Disease

Minamata disease results from the progressive accumulation of mercury and is potentially fatal in people who eat contaminated fish. It was first described in Minamata Bay, Japan, in 1953. The disease causes progressive deterioration of nerve function: hearing and vision loss, muscle paralysis, and dementia; fetal anencephaly sometimes occurs. In the initial group of 111 patients, 43 died within 7 years (Williamson et al. 1997). It is only possible to prevent Minamata disease by analyzing suspicious fishing areas.

Poisoning by Other Fish

Crinotoxic fish exhibit toxicity secretions on the skin. Poisoning in humans is extremely rare, and these fish use this toxin as a defense against predators. Crinotoxic fish are of the family Ostraciidae (boxfish and trunkfish), which produce pahutoxin; and some flounder, which have pardaxin and pavonins (Mebs 2002).

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Fig. 6 Marine turtles can cause severe poisonings in humans, and the toxins were not known until recently (Photograph: Vidal Haddad Junior)

Pahutoxin is similar to saponins produced by echinoderms and is released into the water, affecting other fish. Pavonins are used as shark repellents.

Palytoxin is one of the most potent toxins in nature, causing intense vasoconstriction, neurologic manifestations, and rhabdomyolysis. Intoxication occurs through ingestion of fish from the Balistidae family (triggerfish). These fish feed on coral zoanthids (genus *Palythoa*). Palytoxin was originally isolated in corals. It appears to be produced by bacteria and to accumulate in some animals in a mechanism supposed similar to the accumulation of tetrodotoxin (Gleibs et al. 2002).

Haff disease, first reported along the Baltic coast in 1924, is an unexplained rhabdomyolysis in people who ingested freshwater fish within 24 h before illness onset. Outbreaks resembling Haff disease were described in Sweden, the Soviet Union, and the United States between 1934 and 1984. Haff disease is rare and probably associated with freshwater fish toxins. A recent outbreak in Manaus, Amazon State, Brazil was associated with the *Piaractus* genus (pirapitinga) (Dos Santos et al. 2009).

Poisoning by Consumption of Turtles

One of the lesser-known poisonings caused by marine animals occurs by ingestion of turtle meat (Fig. 6). Several outbreaks have been reported in the waters of the Indian and Pacific oceans, especially in the Malay Archipelago, Sri Lanka, Cambodia, and southern India. From the first communications on poisoning by eating turtles, it was suspected that turtles accumulate toxic substances in their bodies, such as algae or dinoflagellates, and more likely the former. This hypothesis is reinforced by the restricted area in which such poisoning occurs, which could be linked to a certain species of algae in turtles' diets.

The main species involved in outbreaks is *Eretmochelys imbricata*, the hawksbill sea turtle. There are also reports of poisonings by eating *Chelonia mydas* (green turtle) and *Dermochelys coriacea* (leatherback turtle). Since 1987, 152 deaths were recorded in the Indo-Pacific (155 with an outbreak reported in Cambodia in December 2002). Approximately 30 % of people who eat contaminated turtle meat die. There are descriptions of five deaths in children fed on breast milk of mothers intoxicated by the poison, which shows its power (Thomas and Scott 1997). There is proofs of the envenomation and the clinical manifestations after the ingestion of the meat, but the toxin (called chelonotoxin) is not identified. The toxin poisons the nervous system and causes massive necrosis in the liver. Patients have nausea, vomiting, tachycardia, pallor, stomach pain, cold sweats, dizziness, coldness in extremities, and tingling around the mouth. Sores on the tongue, mouth, and palate are most important signs of the intoxication.

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Fig. 7 Leech: note the sucker mouth designed to ingest blood (Image: Vidal Haddad Junior)

In more severe cases, convulsions and death can occur. Only symptomatic treatments are available. Fishermen rub the turtle blood on the skin before consuming the meat. If irritation develops, they avoid consumption.

Conclusion and Directions

Studies on toxins of dinoflagellates and cyanobacteria are expanding, and disease outbreaks associated with these toxins are increasing globally. These poisonings can occur on an individual basis and in small or large groups. Various marine animals used as food may be contaminated, which broadens the spectrum of these poisonings and increases the need for additional studies on the activity of these toxins and their mechanisms of action. Studies on therapeutic measures and approaches to reduce or eliminate the risk in the environment are also needed.

Phylum Echinodermata and Annelida: Sea Urchins, Starfish, Sea Cucumbers, and Marine Worms

Injuries caused by sea urchins are common around the world. Some species cause severe envenomations, but the majority of these injuries are traumatic and not serious (Haddad 2008a; Haddad et al. 2009). Sea cucumbers can be toxic, and the crown-of-thorns starfish is venomous. Additionally, sea worms present toxins and bristles that cause skin inflammation and are also responsible for painful bites. Marine worms are common on the coasts; and gatherers of mussels are often in contact with these animals, as are researchers of marine fauna and unsuspecting divers. The clinical manifestations have similarity with injuries caused by marine sponges. Leeches do not cause envenomations and rarely are associated with important bleeding. Leeches must be removed from the human skin with alcohol or by applying heat from a flame. Topical antibiotic should be applied for about 7 days. Treatment for bites from marine worms comprises intense washing and topical antibiotics.

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Fig. 8 Sea worms (Polichaete): bristles responsible for skin inflammation are clearly visible (Image: Vidal Haddad Junior)

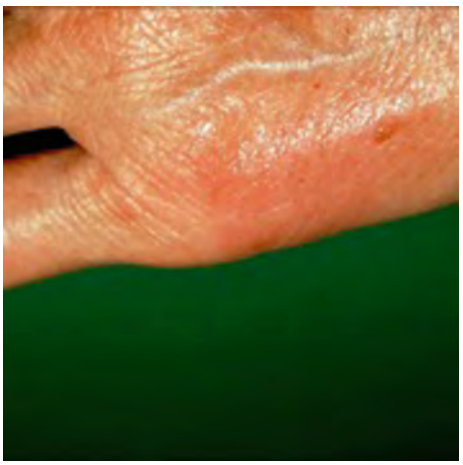

Fig. 9 Bristle worms: erythema and edema in a researcher caused by contact with these animals (Image: Vidal Haddad Junior)

Phylum Annelida: Marine Worms and Leeches

Characteristics of the Phylum

(1.a) Leeches: The phylum Annelida includes leeches, which can cause bleeding in the skin while feeding and releasing an anticoagulant substance called hirudin (Fig. 7). Allergic manifestations and infections may also occur. Leeches, including arboreal leeches, are present in various environments; but the majority is found in freshwater pools. Leeches must be removed by dousing them with alcohol or applying heat from a flame. Mechanical removal can cause injuries. Topical antibiotic should be applied to the lesions for about 7 days.

(1.b) Mechanisms of envenomation: Leeches do not cause envenomation; lesions are of traumatic origin (Haddad 2008).

(1.c) Marine worms: Other important representatives of the phylum are marine worms, Polichaetes, or brush worms of the Amphinomidae family (Fig. 8). Marine worms are similar to terrestrial worms. Their harmful action is basically irritating, but there are reports of victims who presented partial

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Fig. 10 Black sea urchin (*Echinometra lucunter*): common in the Americas, this animal can cause traumatic injuries (Image: Vidal Haddad Junior)

Fig. 11 Crown-of-thorns starfish: a venomous animal responsible for painful injuries in humans (Image: Vidal Haddad Junior)

paresthesia of the affected limb and local necrosis, an indicator of the presence of toxins (Haddad 2008; Haddad et al. 2009).

(1.d) Mechanisms of envenomation: These worms can hurt unsuspecting divers due to powerful bites from their chitinous jaws or envenomation by spikes (bristles) on the body, causing edema, papules, pain, itching, and – more rarely – skin necrosis (Fig. 9). Venom apparatus of marine worms are bristles, which can cause both traumatic lesions and possibly inject toxins. In some species, the irritating aspects are severe and persistent, showing the presence of toxins; there is no information on the composition of these toxins and their mechanism of action. Some injuries present intense pain that is probably caused by toxins, but there are no conclusive studies, and reports of venom in lesions caused by marine worms are anecdotal (Haddad 2008, 2009).

The bites of marine worms are treated by intense washing and topical antibiotics. Skin lesions caused by bristles must be explored with tweezers and adhesive tape in order to remove the bristles. Other measures recommended include applying compresses of vinegar or diluted ammonia. Systemic phenomena are rare

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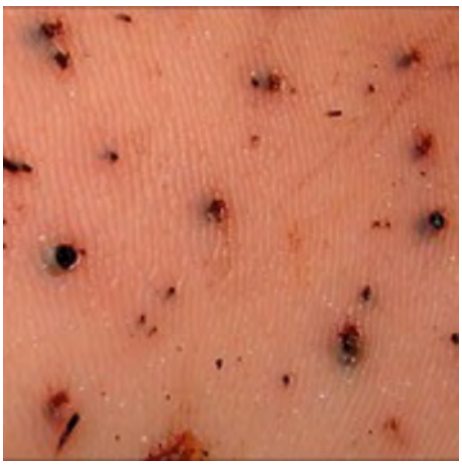

Fig. 12 Black sea urchin: spines entering the human skin cause lesions that can present fragments of various centimeters in the skin. (Image: Vidal Haddad Junior)

and should be treated symptomatically. Occasional necrosis should be treated by antibiotics and intense washing of the site.

Marine worms are common on coasts; gatherers of mussels are often in contact with these animals but usually without the occurrence of injuries. Other people likely to be exposed to contact are researchers of marine fauna, unsuspecting divers, and zoologists conducting field research. The clinical manifestations are similar to injuries caused by marine sponges.

Phylum Echinodermata: Sea Urchins, Starfish, and Sea Cucumbers

Echinoderms are animals with a rounded (radial) body and a mouth seen when the animal is in the prone position.

(1.a) Sea urchins: Sea urchins are animals of the phylum Echinodermata, which are common on shores worldwide. Their body is covered by spines of calcium carbonate, which can measure some centimeters in length; and the pedicellariae, soft tentacles in the spaces among the spines, can produce venom. Bathers and swimmers are injured when they step on the animals, which live in colonies among coastal rocks. Such injuries are frequent, about 50 % of occurrences are caused by marine animals in Brazil (Haddad et al. 2002a). Pain is the more important symptom. The persistence of spines in the plantar region can provoke secondary infections and foreign-body granulomas (Cracchiolo and Goldberg 1977; Mcwilliam et al. 1991; Liram et al. 2000; Rossetto et al. 2006).

(1.b) Mechanisms of envenomation: Sea urchins exhibit hollow spikes of calcium carbonate on their surfaces, which can cause serious trauma (Fig. 10). The pedicellariae are defense organs with three distal jaws through which they inoculate toxins; in some, however, toxin is distributed through body spines. These animals belong to the class Echinoidea. Some species causes hypotension and hemolytic, neurotoxic, and cardiotoxic effects, which are credited to toxins present in the pedicellariae, small tentacles located between the spicules (especially the *Diadema* genus).

(1.c) Starfish: The crown-of-thorns (*Acanthaster planci*) is the only venomous starfish. Toxins contained in the glandular epithelium can cause edema and skin necrosis (Fig. 11). Experiments on laboratory

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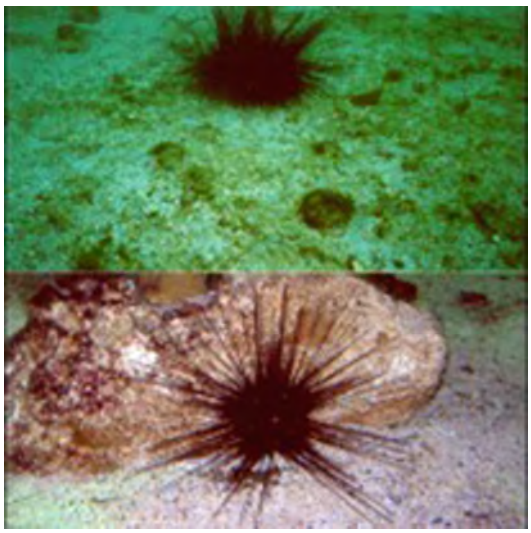

Fig. 13 Sea urchin *Diadema* sp.: the most dangerous genus; envenomations can be severe (Image: Vidal Haddad Junior)

Table 1 Segment of the body involved in 71 sea urchins injuries (Haddad 2008)

Body part	Body side	
Feet (85)	Left 40	Right 45
Hands (19)	Left 10	Right 9
Legs	2	
Palmoplantar involvement	69	

animals showed myotoxicity caused by phospholipase A2. All starfish belong to the Asteroidea class.

(1.d) Sea cucumbers: Sea cucumbers (*Holothuria*) produce holothurin, which irritates the skin and mucous membranes and can cause envenomations when eaten as seafood. Sea cucumbers are in the class Holothuroidea.

Injuries by venomous sea urchins, sea cucumbers, and starfish (*A. planci*) cause skin inflammation manifested by erythema, edema, papules, vesicles, and occasional necrosis. Systemic toxic effects (cardiotoxicity and neurotoxicity) are also described in accidents with venomous sea urchins.

The primary injury caused by sea urchins is due to skin penetration by their spines. It is possible to see the extremity of the spine in the skin and fragments measuring up to 5 cm have been extracted. The most commonly affected areas are the feet and ankles (mainly the plantar regions, when victims step on one),

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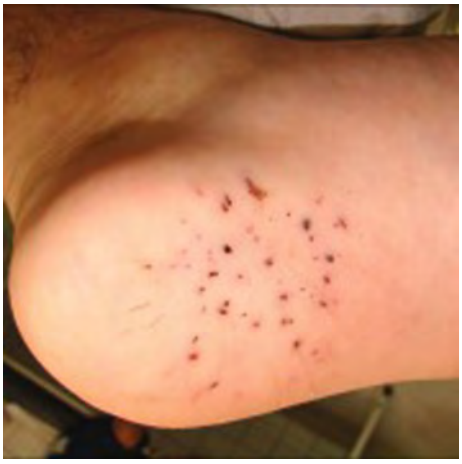

Fig. 14 Foot of a bather who stepped on a colony of black sea urchins (Image: Vidal Haddad Junior)

and hands. Spine penetration causes pain, bleeding, and, in later phases, erythema and edema (Fig. 12). When joints are penetrated, synovitis can occur. Systemic symptoms are especially common when species inject toxin from 15 or more spines, but only from species capable of causing envenomation (such as *Diadema*, *Toxopneustes,* and *Tripneustes* genera). These symptoms include paresthesia, radiating pain, hypotension, muscular weakness, dyspnea, aphony, deafness, and even death. Sea urchins are also responsible for traumatic injuries. When spicules break off in the skin, they cause moderate pain, and their points of penetration serve as gateways to secondary infections, including tetanus. Most spines can be removed, but there may be formation of erythematous nodules (foreign-body granulomas) around the entry point that are difficult to resolve.

All venomous animals of this phylum have labile venom, and immersion of the affected area in warm or hot water (around 50 °C for 30–90 min) is encouraged. Tetanus should be prevented, as well as other secondary infections. In any such injury, removing the spicules should be attempted under local anesthesia. It is necessary to induce superficial scarification with a hypodermic needle and then use to needle for spine withdrawal. Spines are brittle and can be difficult to remove (Haddad 2002, 2012) (Figs. 13 and 14) (Table 1).

Conclusion and Future Directions

Lesions caused by starfish or sea cucumbers are rare, but sea urchins are probably the most common marine animals associated with injuries (trauma and/or envenomation) in bathers or people who live near beaches around the world. It is necessary to understand the effects of toxins and ecological mechanisms linked to stings or bites in humans. The mechanism of toxin injection can be through spines, or *pedicellariae;* however, not all sea urchins that carry toxins are capable of causing envenomation in humans. Sea urchins (Haddad et al. 2012) present toxins with different effects, and a series of studies shows that some toxins have pharmacological importance and potential therapeutic properties (Nakagawa et al. 1991; Sciani et al. 2011).

Cross-References

► Ciguatoxin and Ciguatera
► Classification and Distribution of the Aquatic Venomous and Poisonous Animals
► Equinatoxin: A Review

▶ Immunomodulatory Properties of Sea Cucumber Triterpene Glycosides
▶ Intoxications Caused by the Ingestion of Seafood and Fish
▶ Other Marine and Freshwater Toxins
▶ Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Review
▶ Toxins Produced by Marine Microorganisms: A Review

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Palytoxins: Toxicological Profile

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Abstract

Palytoxin (PLTX), identified in *Palythoa* corals, *Ostreopsis* dinoflagellates, and *Trichodesmium* cyanobacteria, is considered one of the most complicate and toxic molecules found in nature. The recent appearance of *Ostreopsis* spp. in the Mediterranean Sea as well as the uncontrolled diffusion of *Palythoa* corals, widely used as aquarium decorative elements, pose serious concerns for human health. The concomitant detection of putative PLTX and of PLTX analogues poses new problems regarding the toxicological potential of PLTXs, already known for their high toxicity since the 1970s.

In general, adverse effects in humans can occur through different exposure routes: ingestion, dermal, inhalational, or ocular exposure. Among them, oral exposure after ingestion of contaminated seafood is the most dangerous one for human health, although it seems to be limited to tropical and subtropical regions. On the contrary, in temperate areas, such as the Mediterranean Sea, human intoxications are frequently associated with inhalational exposure to aerosolized seawater during *Ostreopsis* blooms.

In vivo toxicity studies indicate that the toxicity of PLTX by oral route is lower than that after parenteral injection, but the European Food Safety Authority suggested an admissible limit of 30 μg PLTX/kg shellfish meat. Also the potential toxicity of PLTXs after aerosol exposure needs to be urgently addressed due to increasing blooms of *Ostreopsis* associated with respiratory and ocular problems as well as dermotoxicity in people exposed to seawater both for recreational and working activities.

Introduction

The history of palytoxin (PLTX) began many decades ago, when a group of researchers decided to verify the Hawaiian legend limu-make-o-Hana, which literally means “The toxic seaweed of Hana”. This legend tells of a man carrying a shark mouth on his back using it to kill fishermen entering in its fishing area. The fishermen, however, killed him and, after burning his body, they scattered his ashes in a bay, called Hana, where grew a toxic algae. The legend tells that the warriors used to dip the tips of their spears in this bay to make them fatal. At the beginning of the 1960s, Prof. Helfrich discovered the exact location of the bay as well as the “algae” of the legend that was found to be a coral belonging to the genus *Palythoa*, species *P. toxica* (Moore et al. 1982). For this reason, the molecule that was discovered 10 years later by Prof. Scheuer was called palytoxin. Since then, PLTX has fascinated researchers for the complexity of its structure, its extreme potency, and its intriguing mechanism of action.

Chemical Structure

The chemical structure of PLTX was elucidated in 1981 by two independent groups, one led by Prof. Hirata in Nagoya (Uemura et al. 1981), the other by Prof. Moore in Honolulu (Moore and Bartolini 1981). The exact chemical formula of PLTX is $C_{129}H_{223}N_3O_{54}$, with a molecular weight of 2680.13 Da. PLTX is considered one of the most complicate and large molecules found in nature. Its structure contains

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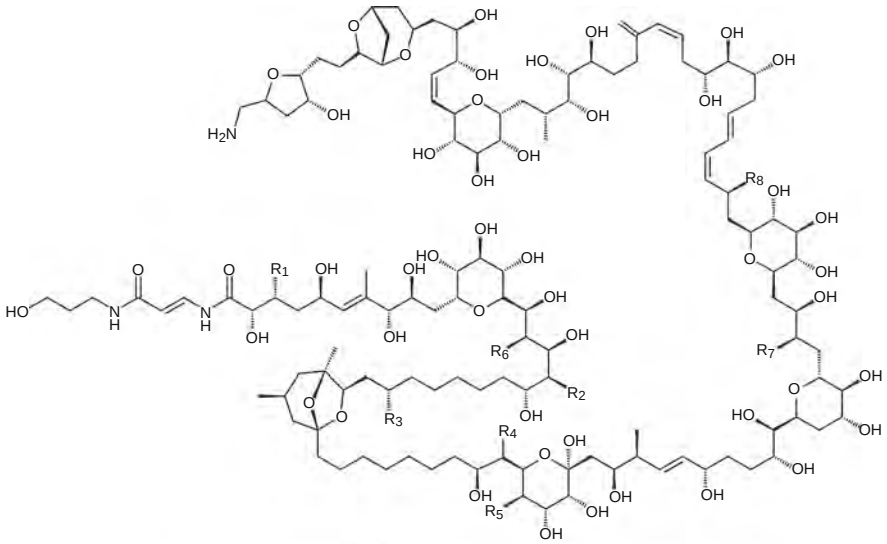

	n	R1	R2	R3	R4	R5	R6	R7	R8
Palytoxin	1	Me	OH	Me	H	OH	OH	OH	OH
Omopalytoxin	2	Me	OH	Me	H	OH	OH	OH	OH
Bisomopalytoxin	3	Me	OH	Me	H	OH	OH	OH	OH
Neopalytoxin	1	-	OH	Me	H	OH	OH	OH	OH
Deoxypalytoxin	1	Me	OH	Me	H	OH	OH	OH	H
42-hydroxypalytoxin	1	Me	OH	Me	OH	OH	OH	OH	OH
Ostreocin-D	1	H	H	H	OH	H	OH	OH	OH
Ovatoxin-a	1	Me	OH	Me	OH	H	H	H	OH

Fig. 1 Differences on the molecular structures between PLTX analogues

129 aliphatic carbon atoms, 40 secondary hydroxy groups, two diene motifs, a conjugate acrylamide-enamide system, three unsaturations, two hydrophobic hydrocarbon chains, cyclic ethers systems, and bicyclic acetals along its backbone (Fig. 1). The structure contains 64 chiral centers and two amino groups, whereas the third nitrogen atom is on C115 in the form of primary amino group (Moore and Bartolini 1981). The conformation of the toxin in aqueous solution has been investigated using X-ray crystallography (SAXS, small-angle X-ray scattering) and nuclear magnetic resonance (NMR). In this study, the structure of PLTX has been compared to that of *N*-acetyl-PLTX, which presents an acetyl on the primary amino group in C115. In particular, in aqueous solution, *N*-acetyl-PLTX shows a monomeric form, assuming the shape of a horseshoe, which measures 30.6 × 23.4 × 13.0 Å. PLTX, instead, assumes a dimeric form in aqueous solution, acquiring the form of ∞ which measures 52.3 × 22.0 × 15.1 Å (Inuzuka et al. 2008). Presently, the parts of PLTX involved in the dimer formation have not been identified, although it is thought that the hydrophobic region (C21–C40) and the region around the conjugated double bonds (C60–C84) are tentatively involved. Moreover, the terminal amino group is probably involved in the interaction of two molecules of PLTX: in the presence of an acetyl group on the terminal amino group (*N*-acetyl-PLTX), dimerization is prevented and the biological activity in vitro is about 100 times lower than that of the reference compound PLTX (Inuzuka et al. 2008).

Depending on the natural source, there are several structural analogues of PLTX that are summarized in Fig. 1. Among them, four are the most studied and characterized under a chemical and/or biological point of view: (i) 42-hydroxy-palytoxin (42-OH-PLTX), the main compound of *Palythoa toxica* (Ciminiello et al. 2009), whose acute toxicity in mice after oral exposure is comparable to that of PLTX (Tubaro et al. 2011a); (ii) a stereoisomer of 42-OH-PLTX extracted from *Palythoa tuberculosa*, whose cytotoxicity is approximately 100 times lower than that of PLTX (Ciminiello et al. 2014); (iii) ostreocin-D (Ost-D), produced by *Ostreopsis siamensis*, which toxicity appears to be lower than that of PLTX (Ito and Yasumoto 2009); and (iv) ovatoxin-a (Ova-a), the major toxin produced by *Ostreopsis* cf. *ovata* in the Mediterranean Sea (Ciminiello et al. 2012), which is still under investigation for its biological effects.

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Producing Organisms

PLTX has been identified in a variety of sources found in tropical, subtropical, and temperate regions. As noted above, the original source of PLTX was a soft coral, *Palythoa toxica*, collected in Hawaii. Subsequently, it has been also identified in other species of *Palythoa*, including *P. tuberculosa*, *P.* aff. *margaritae*, *P. caribaeorum*, and *P. mammillosa*; the latter two were collected from coral reefs of the Caribbean. PLTX and its analogue 42-OH-PLTX have been isolated from species of *P. heliodiscus*, *P. mutuki*, *P. toxica*, and *P. tuberculosa*. Moreover, PLTX has also been isolated from other zoanthid species, such as *Zoanthus solanderi* and *Z. sociatus*, competitors of *Palythoa* species in the coral reef. Zoanthids *Anthozoa* and *Hexacorallia* were found to contain considerable amounts of PLTX (0.5–3.5 mg of crude toxin/g zoanthid), as well. Other organisms such as sea anemones (*Radianthus macrodactylus*) may contain PLTX-like substances. Finally, PLTX and an array of analogues have been identified in various coral reef animals, including fish (Aligizaki et al. 2011; Ciminiello et al. 2009, 2014).

In 1995, the presence of PLTX-like molecules was discovered in benthic dinoflagellates of the genus *Ostreopsis*. This compound was named Ost-D and was isolated from *O. siamensis* (Usami et al. 1995). Interestingly, only *O. siamensis* of the Japanese strain is able to produce Ost-D, while, on the contrary, despite its presence in the Mediterranean Sea, no production of Ost-D by *O. siamensis* has been recorded in this area, so far (Ciminiello et al. 2013). Other strains of *Ostreopsis* were later found to contain PLTX-like molecules: *O. mascareniensis* in which mascarenotoxins were isolated (Lenoir et al. 2004) and *O. ovata* in which ovatoxins were isolated. Interestingly, in *O. ovata* of the Mediterranean strain, PLTX is produced only in traces, while Ova-a is very often the major toxin present (Ciminiello et al. 2012).

To explain the presence of PLTXs in species phylogenetically so different, some authors proposed bacteria as a possible common source for toxin production. In 2000, Frolova et al. (2000), using anti-PLTX antibodies, detected PLTX-like substances in bacteria. Further support to this hypothesis came in 2009 when it was demonstrated that bacteria isolated from *Palythoa caribaeorum* exerted PLTX-like hemolytic activity (Seemann et al. 2009). In addition, PLTX and 42-OH-PLTX were isolated from the marine cyanobacterium *Trichodesmium* (Kerbrat et al. 2011). However, a clear conclusion on the actual source of PLTXs is still a matter of debate.

Vectors of Seafood Poisoning

The presence of toxin-producing species into the ecosystem is related to the entrance of their toxins into the food web. The accumulation of biotoxins in the food web is a common, naturally occurring phenomenon, but it may lead to significant concentrations of toxic compounds in edible organisms and represent a potential threat to human health. PLTXs are no exception. In addition to those that produce it, PLTXs have been found in several other organisms, including crustaceans, fish, gastropods, bivalve mollusks, and echinoderms (Aligizaki et al. 2011). They are found in a variety of habitats, either around coral reefs near *Palythoa* or in habitats supporting microalgae preferred by *Ostreopsis* (Aligizaki et al. 2011).

PLTX has often been reported in crustaceans. Specimens of crabs (*Demania alcalai, D. reynaudii*, and *Lophozozymus pictor*) collected in the Philippines were found to contain PLTX in all tissues tested. Polychaete worms, fishes, and predators that eat zoanthid corals appear to accumulate the toxins. Moreover, a similar toxin bioaccumulation probably occurs in small crustaceans (*Platypodiella spectabilis*) living in close contact with *Palythoa* corals. In addition, PLTX accumulation in the food web can occur via *Ostreopsis*, as well (Tubaro et al. 2011b). The presence of bottom sediments in the viscera of sardines involved in a lethal case of intoxication has been considered key in the hypothesis that benthic microalgae could be related to the accumulation of the toxin in fish. The possible mechanisms of toxin uptake by mollusks were explored by Rhodes et al. (2002). Shellfish actively fed *Ostreopsis siamensis* and some oyster and scallop hepatopancreas contained detectable amounts of toxin. Moreover,

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wild shellfish collected during *Ostreopsis* blooms in Mediterranean Sea were found to contain PLTXs (Aligizaki et al. 2011), suggesting that toxin uptake could occur even in the wild.

Epidemiological Data

A primary concern related to the entrance of biotoxins into the ecosystem is the potential human exposure via ingestion of contaminated seafood. The presence of PLTXs has been associated with several cases of seafood intoxications. However, in all cases, confirmation or quantification of the toxin was rarely possible. In the majority of cases, PLTX was hypothesized as a causative agent on the basis of clinical symptoms and case history (Tubaro et al. 2011b).

In general, exposure routes to PLTX-containing organisms may be by (*i*) ingestion, (*ii*) dermal exposure, (*iii*) inhalational exposure, or (*iv*) ocular exposure. Among them, oral intoxications after ingestion of contaminated fish and crustaceans are the most harmful for human health, although human poisonings seem to be limited to tropical and subtropical regions, so far (Alcala et al. 1988; Noguchi et al. 1987; Onuma et al. 1999; Taniyama et al. 2002). These intoxications can be characterized by initial gastrointestinal symptoms, typically with nausea, vomiting, and diarrhea (Alcala et al. 1988; Onuma et al. 1999). In addition, myalgia and spasms (Noguchi et al. 1987; Taniyama et al. 2002) and, in lethal cases, cardiac alterations, convulsions, and dyspnea followed prior to death (Alcala et al. 1988; Onuma et al. 1999).

In temperate areas, human poisonings were often associated with inhalation and/or cutaneous/systemic exposures after direct contact with aerosolized seawater during *Ostreopsis* blooms or through maintaining aquaria containing zoanthids, widely used as decorative elements. In these cases, the most common symptoms were respiratory distress, rhinorrhea, cough, and fever. Dermatitis was also reported. Toxin identification and/or quantification is often incomplete or missing, and cases of poisoning are frequently ascribed to PLTXs on the basis of symptoms, anamnesis, and environmental data (Tubaro et al. 2011b). Moreover, although ocular exposure is one of the less predictable exposure routes for PLTX, cases of ocular irritation/conjunctivitis have been also described after aerosol exposure to *Ostreopsis* blooms (Durando et al. 2007; Kermarec et al. 2008; Tichadou et al. 2010).

Toxicokinetics

So far, no toxicokinetics studies have been performed. However, two in vitro studies have been carried out on the intestinal Caco-2 cells to predict PLTX intestinal permeability. After the first attempt that was hindered by the high PLTX cytotoxicity (Pelin et al. 2012), a subsequent study showed that PLTX could not significantly pass the Caco-2 monolayer, suggesting that PLTX would be poorly transported to blood (Fernández et al. 2014). These results are in agreement with the lethal oral dose of the toxin which is several times higher than the intraperitoneal lethal dose.

Mechanism of Action

The commonly accepted molecular target of PLTX is the Na^+/K^+ ATPase. It is a transmembrane pump belonging to the family of P-type ATPases, which are essential for maintaining cellular homeostasis: it transfers three Na^+ ions out of the cell in trade for two K^+ ions exploiting the hydrolysis of ATP. The pump alternates in two principal conformational states E_1 and E_2 that, during the transport cycle, could be phosphorylated into the states E_1P and E_2P.

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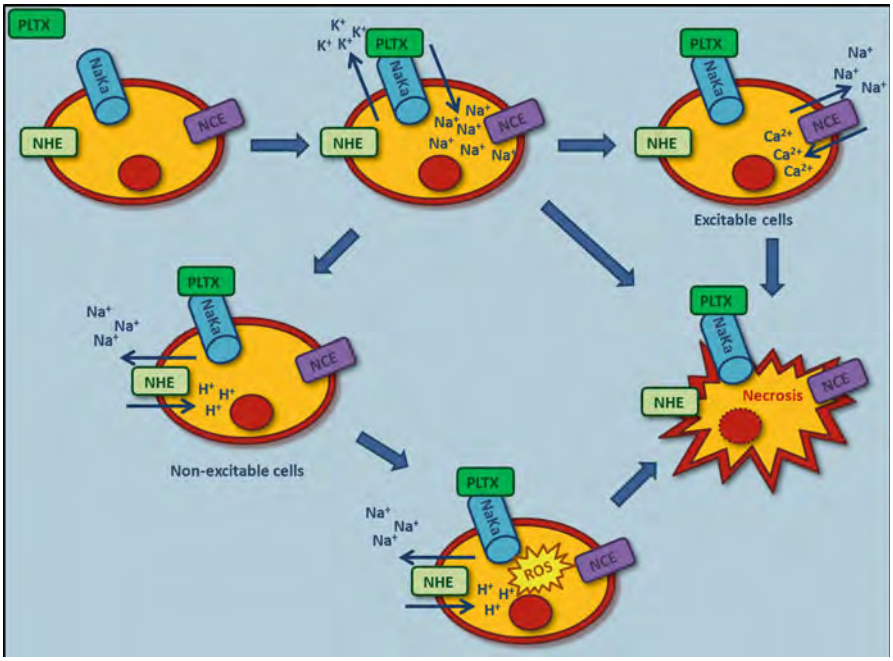

Fig. 2 Mechanism of action of PLTX. The binding of PLTX to the Na^+/K^+-ATPase (*NaKa*) changes it from a transmembrane pump to a nonspecific cationic channel, which induces a consistent ionic imbalance at the cellular level. The first event consists of an intracellular overload of Na^+ followed by secondary Ca^{2+} and H^+ influx depending on the cellular model (i.e., excitable or non-excitable cells, respectively). Ca^{2+} influx seems to be mediated by reverse functioning of the Na^+/Ca^{2+} exchanger (*NCE*) caused by increased intracellular Na^+ concentration and, depending on the cellular model, by voltage-dependent L-type Ca^{2+}-channels. Proton (H^+) influx seems to be mediated by the reverse functioning of the Na^+/H^+ exchanger (*NHE*). It is widely accepted that the cytotoxic effects of PLTX are strictly dependent on this ionic imbalance. Depending on the cellular model, Na^+- or Ca^{2+}-dependent cytotoxic effects have been reported. In non-excitable cells, the intracellular H^+ increase that follows the abnormal influx of Na^+ induced by the toxin seems to be the driving force for ROS production, ultimately leading to an irreversible necrotic cell death. In excitable cell models, on the contrary, Ca^{2+}-dependent cytotoxic effects up to cell death are reported.

It has been well documented that binding of PLTX to the α-β heterodimer of the ATPase changes it from a transmembrane pump to a nonspecific cationic channel, which induces a consistent ionic imbalance at the cellular level. The toxin binding is allowed in the E_2P conformational state of the pump, due to the higher PLTX affinity for the ATPase (Artigas and Gadsby 2004; Rodrigues et al. 2008). This binding results in substitution of cysteines for several residues in the putative fifth and sixth transmembrane helices. As a consequence, the α-helices in the membrane domain are rearranged, allowing the opening of the cytoplasmic gate of the pump. Indeed, the channels that are formed by PLTX might arise as a consequence of a perturbation in the ATPase structure, leading to the loss of control of the gates of the enzyme and, hence, to uncoupling of the ion transports. Moreover, since in the E_2P conformational state the extracellular gate is physiologically open, the contemporary opening of the intracellular gate induces the opening of the channel as long as the pump is phosphorylated. The dephosphorylation of the pump allows its conformational change into the E_1 state. However, PLTX binding also reduces the rate of pump dephosphorylation, protracting the opening of the channel (Artigas and Gadsby 2004; Rodrigues et al. 2008). There is no agreement in literature on whether the dephosphorylation event causes PLTX dissociation. However, computational simulations suggest a crucial role for Na^+/K^+-ATPase phosphorylation in increasing PLTX affinity for the pump. These data are supported by the fact that even sub-micromolar concentrations of ATP could enhance the apparent affinity of PLTX for mammalian Na^+/K^+-ATPase, probably by supporting pump phosphorylation. On the other hand, K^+ occlusion reduces

the pump affinity for PLTX, blocks the induced channels, and impedes the pump phosphorylation. These data are in line with the idea that K^+ is able to reduce Na^+/K^+-ATPase affinity to PLTX by reducing the toxin-binding stability and, therefore, to increase its dissociation rate (Rodrigues et al. 2008).

Many studies report the ability of the cardioactive glycoside ouabain (OUA) to inhibit PLTX effects in vitro (Habermann and Chhatwal 1982; Pelin et al. 2011, 2012; Schilling et al. 2006; Vale-Gonzáles et al. 2007). However, incomplete abolishment of PLTX biological activity in the presence of ouabain suggests that OUA does not completely compete with PLTX on the binding sites (Pelin et al. 2013a). In fact, Artigas and Gadsby (2004) demonstrated that PLTX and ouabain can simultaneously bind to Na^+/K^+-ATPase, suggesting the possibility of two different binding sites on the pump. Indeed, the presence on intact HaCaT cells of a high affinity binding site for PLTX located on the cell surface that appears to be partially insensitive to OUA and partially modulated by OUA in a complex manner was demonstrated, as a negative allosteric modulator against high PLTX concentrations ($0.3–1.0 \times 10^{-7}$ M) and as a noncompetitive antagonist against low PLTX concentrations ($0.1–3.0 \times 10^{-9}$ M). This hypothesis could explain the inability of OUA to totally prevent PLTX-induced cytotoxic effects (Pelin et al. 2013a).

The transformation of Na^+/K^+-ATPase into a nonselective cationic channel results in a sustained cellular ion homeostasis imbalance (Fig. 2). The first event consists of an intracellular overload of Na^+ causing a depolarization of the cellular membrane, which is increased by the massive efflux of K^+ and influx of Ca^{2+}. Ca^{2+} influx seems to be mediated by reverse functioning of the Na^+/Ca^{2+} exchanger (NCE) caused by increased Na^+ concentration and, depending on the cellular model, by voltage-dependent L-type Ca^{2+} channels. Although not yet completely clear, the increased intracellular Ca^{2+} levels may induce opening of K^+ or Cl^- channels, further impairing cell ionic balance. Moreover, the intracellular increase in Na^+ appears to induce an acidification of the cytoplasm, probably due to the reverse functioning of the Na^+/H^+ exchanger (NHE) (Rossini and Bigiani 2011). It is widely accepted that the cytotoxic effects of PLTX are strictly dependent on this ionic imbalance. Depending on the cellular model (i.e., non-excitable or excitable cells), Na^+- or Ca^{2+}-dependent cytotoxic effects have been reported. Indeed, Na^+ overload seems to be the first and crucial step in mediating PLTX-induced early cell damage, as recently demonstrated on human HaCaT keratinocytes. In a Ca^{2+}-free/Na^+-containing medium, a partial reversal of PLTX activity was observed, indicating that the initial Na^+ overload is required as an essential condition for the contribution of Ca^{2+} in mediating PLTX effects (Pelin et al. 2011). Moreover, the H^+ intracellular increase that follows the abnormal intracellular concentration of Na^+ induced by the toxin seems to be the driving force for O_2^- production by reversing the mitochondrial electron transport (Pelin et al. 2013b), ultimately leading to an irreversible necrotic cell death (Pelin et al. 2014). This finding is consistent with previous observations supporting Na^+ dependency of PLTX effects. In excitable cell models, on the contrary, where intracellular signaling is highly dependent on Ca^{2+} concentrations with respect to non-excitable cells, PLTX effects are strictly dependent on Ca^{2+} ions. Indeed, the increased concentrations of Ca^{2+} induced by the initial Na^+ overload may trigger Ca^{2+}-dependent cytotoxic effects such as neurotransmitter release, uncontrolled muscle cell contraction, and Ca^{2+}-dependent cell death (Ares et al. 2005; Sheridan et al. 2005; Schilling et al. 2006).

Toxicity

Human Toxicity

As reported above, exposure routes to PLTX-containing organisms may be by (*i*) ingestion, (*ii*) dermal exposure, (*iii*) inhalational exposure, or (*iv*) ocular exposure. Among them, oral intoxications after ingestion of contaminated fish and crustaceans are the most harmful to human health (Noguchi et al. 1987; Alcala et al. 1988; Onuma et al. 1999; Taniyama et al. 2002). Among these intoxications,

only few were certainly attributed to PLTX by analytic confirmations of the toxin presence in the leftovers. In particular, a fatal case after ingestion of a crab (*Demania reynaudii*) occurred in the Philippines, where a 49-year-old man reported a metallic taste after consuming the crab and, shortly after, developed nausea, tiredness, diarrhea, and vomiting followed by dizziness, numbness of the extremities, muscle cramps, and restlessness. After hospitalization, he experienced alternating periods of normal heart rate and severe bradycardia (30 beats/min), rapid and shallow breathing, and cyanosis around the mouth and hands. Finally, the patient died 15 h after the crab ingestion. In the same period, Noguchi et al. (1987) described a case of a man (54 years old) and a woman (79 years old) poisoned after parrotfish (*Scarus ovifrons*) consumption. The symptoms, developed within 17 and 49 h after ingestions, respectively, were mainly dyspnea, myalgia, and convulsions. Myoglobinuria and high serum levels of creatine phosphokinase (CPK), alanine aminotransferase (AST), and lactate dehydrogenase (LDH) were recorded. The man recovered within 1 week, whereas the woman died for respiratory failure associated with muscular damage, 4 days later. A case of fatal poisoning due to contaminated tropical sardines (*Herklotsichthys quadrimaculatus*) involved a woman in Madagascar (Onuma et al. 1999). The patient referred an unusual metallic taste of the fish and within 2 h developed general malaise, uncontrollable vomiting, and diarrhea. The patient died 1 day after, whereas her son, who ate a similar fish, did not develop any symptom. More recently, 11 people out of 33 were intoxicated after serranid fish (*Epinephelus* sp.) ingestion. The patients (ages between 27 and 57) suffered muscle pain, followed by discharge of black urine and low back and shoulder pain. The hematological analysis revealed significantly increased serum levels of CPK, up to nearly 24,000 IU/l. All the patients recovered after more than 1 month (Tubaro et al. 2011b). Recently, four families out of seven were intoxicated after consumption of a PLTX-containing goldspot herring (*Herklotsichthys quadrimaculatus*) in Taiwan, and a 67-year-old man died after 21 h after ingestion. On arrival at the hospital, the man experienced dizziness, nausea, tingling and numbness of face and limbs, generalized discomfort, and weakness. After 14 h from the ingestion, he developed severe myalgia, arthralgia, abdominal cramps, restlessness, respiratory distress, and anuria (Wu et al. 2014).

Other cases are described in the literature, and, although quantification of toxin in these cases was not performed in the leftovers, they occurred after ingestion of potentially PLTX-containing seafood. In these cases, symptoms were in good agreement with those already described, thus strengthening that PLTX could be the causative agent (Tubaro et al. 2011b).

Regarding dermal exposure, cutaneous toxicity has been associated with skin contact to PLTX-containing zoanthid corals or seawater containing *Ostreopsis* cells. In the former, intoxications occurred primarily in aquarium hobbyists who accidentally came into contact with *Palythoa* while cleaning aquaria. Intoxications were associated with handling of corals with both intact skin and damaged skin. In addition to local effects at the site of contact, such as edema and erythema, systemic symptoms were also observed. Among these, perioral paresthesia and dysgeusia were the most common ones, and, in the most severe cases, transitory alterations of cardiac functions were noted. Probably more numerous, but less severe, is dermatitis associated with contact with seawater/microalgae during *Ostreopsis* blooms (Tubaro et al. 2011b). Although underestimated, Durando and coworkers suggested 5 % incidence of dermatitis in patients hospitalized during *Ostreopsis* blooms in Genoa, Italy, during the years 2005–2006 (Durando et al. 2007; Tubaro et al. 2011b). Furthermore, observations carried out by the "French Mediterranean Coast *Ostreopsis* Surveillance Network" performed along the French Mediterranean and Monaco coasts from 2006 to 2009 concluded that symptoms observed after direct exposure to *O. ovata* were variable, but skin irritation was the most common sign and might be the only one in the presence of low *Ostreopsis ovata* concentrations in seawater (Tichadou et al. 2010).

Also inhalational toxicity has been associated with PLTX-containing zoanthids and *Ostreopsis* proliferation, mainly in temperate climates. Recently, significant amounts of PLTXs, mainly ovatoxins, have been found in marine aerosol during a small-scale monitoring study carried out along the Tuscan coasts

(Italy) in 2009 and 2010 during *O. ovata* blooms (Ciminiello et al. 2014). Several episodes of respiratory problems have been described during *Ostreopsis* blooms in the last two decades. The Italian coasts have seen several occurrences (Durando et al. 2007; Tubaro et al. 2011b), as have other Mediterranean coasts (Kermarec et al. 2008; Tichadou et al. 2010). Recurrent symptoms were severe rhinorrhea, cough, sore throat, fever occasionally associated with dyspnea, mucosal irritation, and conjunctivitis. In most severe cases, systemic symptoms were also recorded, such as nausea, vomiting, headache, and fever over 38 °C (Durando et al. 2007). In the last years, also respiratory intoxications associated with PLTX-containing zoanthids present in home aquaria have been frequently reported. In general, although no serious outcomes have been recorded and all patients survived without serious consequences, the symptoms do not involve only the respiratory tract, but consist also in myalgias, paresthesias, low-grade fevers, and gastrointestinal symptoms (Deeds and Schwartz 2010; Tubaro et al. 2011b).

Ocular exposure is one of the less predictable exposure routes for algal biotoxins. Nevertheless, cases of ocular irritation/conjunctivitis have been described after aerosol exposure to *Ostreopsis* blooms. In 1982, Moore and colleagues reported anecdotally a severe eye injury with edema of the cornea lasting several weeks, associated with the contact with a mucous secretion of *Palythoa tuberculosa* (Moore et al. 1982). In addition, two cases of keratoconjunctivitis have been described in two patients after handling zoanthid soft corals in home aquaria. Even though the presence of PLTX or congeners was not confirmed, the severity of the lesions could be ascribed to a toxin such as PLTX (Deeds and Schwartz 2010; Tubaro et al. 2011b).

Experimental Toxicity

In Vivo Toxicity After Single Administration

The acute toxicity of PLTX to mammals is strongly dependent on the exposure route. PLTX is most toxic after parenteral administration. A wide variety of animal species and exposure routes appear in literature. Unfortunately, some studies were carried out before structural elucidation of PLTX, using PLTX-containing extracts isolated from several matrices, such as fish, crustaceans, and corals, whose molecular weights are not always available or consistent (i.e., PLTX with reported MW 3300). In addition, the varying degrees of purity make dose–effect comparisons problematic. Hence, only data obtained by experiments carried out with commercial toxins or PLTX extracts with known purity will be discussed.

Regarding oral administration, Munday and coworkers estimated the LD_{50} of PLTX to be 510 μg/kg in mice (data obtained according to OECD 425; Munday 2011). This study was expanded by Sosa and coworkers (calculated LD_{50} 767 μg/kg; 95 % confidence limits: 549–1039 μg/kg). Animals treated with doses higher than 600 μg/kg presented scratching, jumping, dyspnea, and limb paralysis. Hematochemical analysis demonstrated dose-dependent increases in circulating levels of LDH, CPK, AST, and ALT. Histological analysis revealed inflammation of the forestomach, consistent with the irritating properties of PLTX (Sosa et al. 2009). Similarly, Ito and Yasumoto (2009) described erosion and slight fluid accumulation in the stomach after oral administration of doses up to 500 μg/kg PLTX.

Studies on PLTX analogues are even fewer and limited to Ost-D (Ito and Yasumoto 2009) and 42-OH-PLTX (Tubaro et al. 2011a). Ost-D exhibits effects similar to those of PLTX, but somewhat less severe at similar doses. Dose-dependent stomach erosion and minor injuries to the small intestine, lung, and kidney were observed 2 h after oral administration to mice (200 and 500 μg/kg). Acute toxicity studies with 42-OH-PLTX suggested symptoms (jumping, loss of righting reflex, and hind limb paralysis) and an LD_{50} similar to that of PLTX (650 μg/kg; confidence intervals: 384–1018 μg/kg, mice) and increased circulating levels of LDH, CPK, AST, ALT, and K^+ (Tubaro et al. 2011a).

Studies describing parenteral administration of PLTXs represent the majority of the toxicity data available for PLTXs. Rhodes and coworkers (2002) calculated an LD_{50} after intraperitoneal

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(IP) administration of 0.72 μg/kg (confidence intervals: 0.64–0.80 μg/kg), and Riobò and coworkers (2008) estimated an LD_{50} of 294.6 ± 5.38 ng/kg for commercial PLTX. In the first study, animals presented abnormal gait, hind limbs splayed backwards, cessation of spontaneous movement, and slowed rate of respiration with occasional gasps before death (Rhodes et al. 2002), and, similarly, in the second study, stretching of hind limbs and lower backs, curvature of spinal column, possible blindness, convulsion, and gasping before death were observed (Riobò et al. 2008). Studies performed in mice after IP administration of PLTX allowed also the identification of the main pathological effects of the toxin. Administration of 2 μg/kg of PLTX isolated from *L. pictor* resulted in severe cardiac alterations with single-cell necrosis in the ventricular and septal myocardium, rounded mitochondria with increased matrix density, and separation of intracellular organelles. At the renal level, the formation of autophagic vacuoles and lysosomes beneath the brush border of convoluted urinary tubules was observed accompanied by occasional destruction of microvilli. The formation of autophagic vacuoles in the pancreatic acinar cells was also observed. The administration of similar doses (1 μg/kg) of the toxin extracted from a crab of *D. alcalai* showed that survived animals presented decreased body weight, paralysis of hind limbs, and decreased weights of thymus, spleen, and liver. At the histological level, the intestine was severely compromised with reddening, congestion, bleeding in the intestinal lumen and degeneration, and loss of epithelial cells. Congestions of jejunum and duodenum were observed 2–6 h postinjection (Munday 2011).

Very few data describe the toxicity of PLTX analogues: Ost-D purified from *O. siamensis* seems to be lethal after IP administration in mice above a dose of 5 μg/kg with limb paralysis and gastric erosions (Ito and Yasumoto 2009). Ova-a isolated from *O. ovata* at a dose of 7 μg/kg caused limb paralysis and death within 30 min of administration (Ciminiello et al. 2012).

In Vivo Toxicity After Repeated Administration

Studies describing the effects of repeated PLTX administration deserve special consideration because they allow the identification of target organs in a situation mimicking a characteristic type of human exposure. Unfortunately, such studies are rare.

One of the first studies describing the effects of PLTX after repeated administration was carried out to evaluate tumor promotion activity of the toxin; in this case 0.5 μg PLTX was applied on the mice skin two times per week for 30 weeks. PLTX exposure after tumor initiation with 7,12-dimethylbenz[a]anthracene resulted in the development of tumors in 62.5 % of the treated animals (Fujiki et al. 1986).

Few years later Vick and Wiles (1990) described the variation of the toxicity of PLTX when rats were treated with a nonlethal dose of the toxin (5 μg/kg PLTX, either per os or intrarectal) before the lethal one (0.25 μg/kg, intramuscular). The experiment resulted in a decreased mortality fraction if the second treatment took place between 24 and 48 h after the first one. Similarly, the protection was observed when the administration of 10 μg/kg PLTX per os was followed by 0.20 μg/kg IV (after 24 h; Vick and Wiles 1990).

Repeated IP administration (29 injections, 0.25 μg/kg) to mice of PLTX isolated from crabs (*D. alcalai*) induced thymus weight loss, although the animals recovered after cessation of the treatment (Ito et al. 1997). Histological analysis after the fifth and tenth injections revealed necrosis of lymphocytes. Spleen weight increased during the dosing period, associated with an increase in the number of megakaryocytic cells. Lymphocytes with condensed nuclei were also observed in the red pulp, as were fibrinous exudates and inflammatory cells such as granulocytes and monocytes. At the end of the treatment regimen, decreased numbers of circulating T-cells and B-cells and increased numbers of circulating granulocytes and monocytes were observed. At the end of the dosing period, animals presented with ascites and ventral organ adhesions, which were still observable at the end of the recovery period (Ito et al. 1997).

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Repeated sublingual administration of PLTX (up to 3 days of treatment, cumulative dosage up to 495 μg/kg) and Ost-D (up to 5 days of treatment, cumulative dosage up to 1000 μg/kg) caused pulmonary congestion and mild alveolar destruction accompanied by stomach ulcers and erosion of the gut. These effects were more severe in mice treated with PLTX than those treated with Ost-D (Ito and Yasumoto 2009).

Very recently, the effects of repeated oral administration of PLTX were investigated in mice. Seven days of PLTX administration caused lethality and toxic effects at doses ≥30 μg/kg/day. Hence, a NOAEL was estimated equal to 3 μg/kg/day, indicating a quite steep dose–response curve. This value, however, is provisional due to the limited number of animal tested. Macroscopic alterations at gastrointestinal level (gastric ulcers and intestinal fluid accumulation) were observed in mice dead during the treatment period. Histological analysis highlighted severe inflammation, locally associated with necrosis, at pulmonary level, as well as hyper-eosinophilia and fiber separation in myocardium (Del Favero et al. 2013).

Mutagenic and Genotoxic Activity

PLTX was negative in the Ames mutagenicity test using strains of *Salmonella typhimurium* with or without microsomal activation. Furthermore, it did not act as an initiator in the in vitro BALB/c 3T3 cell transformation assay or in mouse skin in vivo (Fujiki et al. 1986; Miura et al. 2006).

Tumor-Promoting Activity

PLTX is a tumor promoter, as evidenced by two-stage carcinogenesis studies in mouse skin. However, the mechanism of PLTX tumor promotion is significantly different from other typical tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Indeed, PLTX does not activate protein kinase C (PKC) or increase ornithine decarboxylase (ODC) activity in mouse skin. For these reasons, the toxin has been defined as a non-TPA-type tumor promoter (Fujiki et al. 1986). On the contrary, the mechanism of tumor promotion could be related to prostaglandin E_2 release, because of the ability of PLTX to stimulate the metabolism of arachidonic acid, a common effect of many tumor promoters (Aizu et al. 1990).

In 2006, the ability of PLTX to act as a tumor promoter in mice was confirmed in vitro by the two-stage transformation assay using BALB/c 3T3 cells. Treatment with 1.9×10^{-12} M PLTX was sufficient to increase the number of transformed foci after initiation by 3-methylcholanthrene (Miura et al. 2006).

Treatment

So far, no defined and harmonized pharmacological treatments are adopted in PLTX intoxications. In general, pharmacological treatments are optimized to reduce or limit the symptoms and therefore defined case by case.

Regarding oral intoxications, very few information about pharmacological treatment are given, and when available, they are directed to minimize symptoms, such as gastric lavage, forced diuresis, artificial respiration, fluid therapy, and administrations of atropine, Benadryl, Demerol, and/or adrenaline (Tubaro et al. 2011b). However, in some cases these treatments could not prevent fatalities.

On the contrary, respiratory/dermatological intoxication treatments are more detailed, although no harmonized therapy is present. Indeed, different pharmacological approaches have been described and consist of inhaled and systemic corticosteroids, nebulized β-agonists, or corticosteroids. Alternatively, other pharmacological approaches employed associations of NSAIDs and nebulized β-agonists or associations of corticosteroids and histamine antagonist (Tubaro et al. 2011b).

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Conclusion and Future Directions

The recent appearance of *Ostreopsis* sp. in the Mediterranean Sea as well as the uncontrolled diffusion of *Palythoa* and *Zoanthus* corals widely used as aquarium decorative elements pose serious concerns for human health. The detection of putative PLTX as well as of PLTX analogues in *Ostreopsis* sp., sea urchins, and shellfish poses new problems regarding the toxicological potential of PLTXs, already known for their high toxicity since the 1970s.

Although human poisonings, with some lethalities, were ascribed to the ingestion of PLTXs-contaminated seafood, the lack of accurate PLTX quantitation in the leftovers does not allow to define a NOAEL (no observed adverse effect level) or a LOAEL (lowest observed adverse effect level) for the TDI (tolerable daily intake) or ARfD (acute reference dose) definition. Experimental toxicity studies indicate that the oral acute toxicity of PLTX is lower than its parenteral toxicity. The recent studies after repeated oral administration should be considered to more accurately define the admissible levels of PLTXs contained in seafood. Also the potential toxicity of PLTXs after aerosol exposure needs to be urgently addressed due to increasing blooms of *Ostreopsis* associated with respiratory and eye problems as well as dermotoxicity in people exposed during their recreational and work activities. To this aim, monitoring programs involving chemists, toxicologists, and physicians should be set up to better understand how to manage PLTX intoxications in order to offer harmonized protocols to be used by physicians in these cases.

Cross-References

► Miscellaneous Marine Toxins of Medical Significance
► Other Marine and Freshwater Toxins

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Azaspiracid Toxins: Toxicological Profile

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Abstract

Azaspiracids (AZAs) are a toxin group that originate from marine dinoflagellates of the genera *Azadinium* and *Amphidoma*. After accumulation of these toxins in edible marine organisms and their subsequent consumption, humans develop a gastrointestinal syndrome referred to as azaspiracid shellfish poisoning (AZP). This syndrome is very similar to diarrheic shellfish poisoning (DSP), with main symptoms appearing after a few hours from consumption and including diarrhea, vomiting, and stomach cramps. Due to extensive metabolism in shellfish, more than 30 analogues have been reported to date, and purified compounds for selected analogues have recently been made available for toxicological studies. Currently, only AZA1, AZA2, and AZA3 are regulated in Europe and internationally; however, more recent evidence suggests that AZA6, AZA17, and AZA19 may also be analogues of importance for estimating the full risk of seafood.

Even though animal studies have pointed out target organs (digestive tract, liver, heart, and lung), mechanism of action studies at cellular level are not yet conclusive. While a number of common targets have been excluded (protein phosphatases, kinases, actin depolymerization, G protein-coupled receptors), some evidence points toward ion channel activity of AZAs. Still, in vitro studies do not correlate well with symptoms observed in humans. Also, while some animal studies point toward longer-term effects, no such evidence has been reported from human poisoning events. However, it should be noted that in-depth epidemiological studies are still lacking. Even though all risk assessments have based their evaluation on a single, relatively early poisoning event in 1997, in Arranmore Island, Ireland, producing organisms and toxin occurrences have been reported worldwide, and further occurrence studies should provide a better base for such epidemiological studies.

Introduction

Azaspiracids (AZAs) are marine algal toxins produced by the dinoflagellate genera *Azadinium* (Krock et al. 2009; Tillmann et al. 2009) and *Amphidoma* (Tillmann et al. 2012, 2014). This class of toxins was first identified in the 1990s following an outbreak of human illness in the Netherlands associated with consumption of contaminated mussels from Killary Harbour, Ireland (McMahon and Silke 1996). Although the symptoms were typical of DSP toxins, i.e., okadaic acid (OA) and dinophysistoxins (DTXs), the levels of DSP toxins in these mussels were well below the regulatory level. Subsequently, it was established that the shellfish were contaminated with a unique marine toxin, originally named "Killary Toxin" or KT-3 (Satake et al. 1998a). Shortly thereafter, the toxin was renamed to azaspiracid

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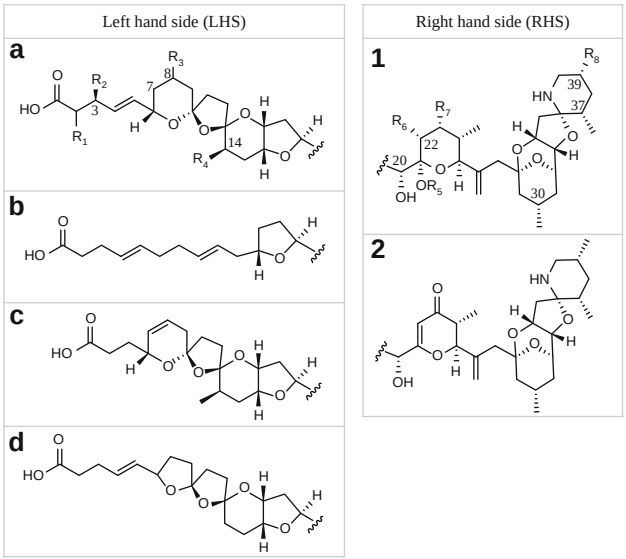
Table 1 Structural variants of AZAs, their protonated masses, origin and toxicity



	Type[§]	R_1	7,8	R_2	R_3	R_4	R_5	R_6	R_7	R_8	$[M+H]^+$	Origin	Status
AZA1	a1	H	Δ	H	H	CH_3	H	CH_3	H	CH_3	842.5	*A. spinosum*	phycotoxin
[#]37-*epi*-AZA1	a1	H	Δ	H	H	CH_3	H	CH_3	H	CH_3	842.5	*A. spinosum*	artefact
AZA2	a1	H	Δ	H	CH_3	CH_3	H	CH_3	H	CH_3	856.5	*A. spinosum*	phycotoxin
AZA3	a1	H	Δ	H	H	CH_3	H	H	H	CH_3	828.5	shellfish	metabolite
AZA4	a1	H	Δ	OH	H	CH_3	H	H	H	CH_3	844.5	shellfish	metabolite
AZA5	a1	H	Δ	H	H	CH_3	H	H	OH	CH_3	844.5	shellfish	metabolite
AZA6	a1	H	Δ	H	CH_3	CH_3	H	H	H	CH_3	842.5	shellfish	metabolite
AZA7	a1	H	Δ	OH	H	CH_3	H	CH_3	H	CH_3	858.5	shellfish	metabolite
AZA8	a1	H	Δ	H	H	CH_3	H	CH_3	OH	CH_3	858.5	shellfish	metabolite
AZA9	a1	H	Δ	OH	CH_3	CH_3	H	H	H	CH_3	858.5	shellfish	metabolite
AZA10	a1	H	Δ	H	CH_3	CH_3	H	H	OH	CH_3	858.5	shellfish	metabolite
AZA11	a1	H	Δ	OH	CH_3	CH_3	H	CH_3	H	CH_3	872.5	shellfish	metabolite
AZA12	a1	H	Δ	H	CH_3	CH_3	H	CH_3	OH	CH_3	872.5	shellfish	metabolite
AZA13	a1	H	Δ	OH	H	CH_3	H	H	OH	CH_3	860.5	shellfish	metabolite
AZA14	a1	H	Δ	OH	H	CH_3	H	CH_3	OH	CH_3	874.5	shellfish	metabolite
AZA15	a1	H	Δ	OH	CH_3	CH_3	H	H	OH	CH_3	874.5	shellfish	metabolite
AZA16	a1	H	Δ	OH	CH_3	CH_3	H	CH_3	OH	CH_3	888.5	shellfish	metabolite
AZA17	a1	H	Δ	H	H	CH_3	H	COOH	H	CH_3	872.5	shellfish	metabolite
AZA19	a1	H	Δ	H	CH_3	CH_3	H	COOH	H	CH_3	886.5	shellfish	metabolite
AZA21	a1	H	Δ	OH	H	CH_3	H	COOH	H	CH_3	888.5	shellfish	metabolite
AZA23	a1	H	Δ	OH	CH_3	CH_3	H	COOH	H	CH_3	902.5	shellfish	metabolite
AZA26	a2	H	Δ	H	H	CH_3	-	-	-	-	824.5	shellfish	metabolite
AZA29	a1	H	Δ	H	H	CH_3	CH_3	H	H	CH_3	842.5	shellfish	artefact
AZA30	a1	H	Δ	H	H	CH_3	CH_3	CH_3	H	CH_3	856.5	*A. spinosum*	artefact
AZA32	a1	H	Δ	H	CH_3	CH_3	CH_3	CH_3	H	CH_3	870.5	*A. spinosum*	artefact
AZA33	b1	H	Δ	-	-	CH_3	H	CH_3	H	CH_3	716.5	*A. spinosum*	phycotoxin
AZA34	c1	H	Δ	-	-	CH_3	H	CH_3	H	CH_3	816.5	*A. spinosum*	phycotoxin
AZA36	a1	H	Δ	OH	CH_3	CH_3	H	CH_3	H	H	858.5	*A. poporum*	phycotoxin
AZA37	a1	H	-	OH	H	CH_3	H	CH_3	H	H	846.5	*A. poporum*	phycotoxin
AZA38	a1	H	-	CH_3	H	H	H	CH_3	H	H	830.5	*A. languida*	phycotoxin
AZA39	d1	CH_3	-	H	-	H	H	CH_3	H	H	816.5	*A. languida*	phycotoxin
AZA40	a1	H	Δ	H	CH_3	CH_3	H	CH_3	H	H	842.5	*A. poporum*	phycotoxin
*AZA41	a1	H	Δ	H	CH_3	CH_3	H	CH_3	H	CH_3	854.5	*A. poporum*	phycotoxin

Compounds highlighted in grey have had their structures confirmed by NMR; [#]orientation of the methyl group at C37. The type refers to variations of the LHS and RHS parts of the molecule.*Double bond in the nitrogen containing ring.

(AZA) to more appropriately reflect its chemical structure: a cyclic amine, or aza group, with a tri-spiro-assembly and carboxylic acid group (Satake et al. 1998a, b).

To date, over 30 AZA analogues have been identified in phytoplankton and shellfish (Hess et al. 2014; James et al. 2003b; Jauffrais et al. 2012b; Lehane et al. 2002; McCarron et al. 2009; Ofuji et al. 1999, 2001; Rehmann et al. 2008; Satake et al. 1998b). Over the last 15 years, AZAs have been reported in shellfish from many coastal regions of Western Europe (Amzil et al. 2008; Magdalena et al. 2003a; Furey et al. 2003; Hess et al. 2007; James et al. 2001, 2002; Twiner et al. 2008), Northern Africa (Elgarch et al. 2008; Taleb et al. 2006), South America (Álvarez et al. 2010), and North America (M. Quilliam,

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pers. comm.; A. Robertson, pers. comm.). In addition, AZAs have been found in Japanese sponges (Ueoka et al. 2009) and Scandinavian crabs (Torgersen et al. 2008). Not surprisingly, the global distribution of AZAs appears to correspond to the apparent widespread occurrence of *Azadinium* (Akselman and Negri 2012; Tillmann et al. 2010, 2011). Empirical evidence is now available that unambiguously demonstrates the accumulation of AZAs in shellfish via direct feeding on AZA-producing *A. spinosum* (Jauffrais et al. 2012c; Salas et al. 2011).

Whereas extensive study of this toxin class has been historically constrained by limited availability of purified material, these restraints are now less of an impediment due to advances in isolation and purification of AZAs from naturally contaminated shellfish (Kilcoyne et al. 2012) and the identification of the toxigenic organism *A. spinosum* coupled with its mass culture in bioreactors (Jauffrais et al. 2012b). As such, certified reference standards of naturally produced AZA1–3 are now commercially available (Perez et al. 2010). Although not yet realized for commercial purposes, limits on toxin supply may be further alleviated by advances in the organic total synthesis of AZA1 (Nicolaou et al. 2004) and AZA3 (C. Forsyth, pers. comm.). Accessibility to purified AZAs has led to rapid progress with respect to understanding AZA toxicology over the last few years.

Toxins

Biosynthesized (Algal) Analogues

AZAs have sporadically been detected in plankton (James et al. 2003a; Krock et al. 2008) and seawater (Fux et al. 2009; Rundberget et al. 2007), but no progenitor of these toxins could be assigned until 2007 (Krock et al. 2009), when a small dinoflagellate, later named *Azadinium spinosum* (Tillmann et al. 2009), was unambiguously identified as an AZA1- and AZA2-producing organism (*ca*. 20 and 7 fg/cell, respectively). Later, Kilcoyne et al. found additional AZAs with molecular masses of 715 (*ca*. 7 fg/cell) and 816 Da (AZA33 and AZA34) in environmental samples and cultures of *A. spinosum* (Kilcoyne et al. 2014b; Table 1).

Recently, two strains of *Azadinium poporum*, a species previously reported to be non-toxigenic (Potvin et al. 2012; Tillmann et al. 2011), were proven to be the producers of two previously unknown AZAs (Krock et al. 2012). AZA37 from a North Sea isolate of *A. poporum* (Tillmann et al. 2011) with a molecular mass of 845 Da (*ca*. 10 fg/cell) was determined as 39-desmethyl-7,8-dihydro-3-hydroxy-AZA-1 by nuclear magnetic resonance (NMR) spectroscopy (Table 1; Kilcoyne et al. 2014c). The other strain of *A. poporum*, from Shiwha Bay, Republic of Korea (Potvin et al. 2012), produced AZA36 with a molecular mass of 857 Da (*ca*. 2 fg/cell), which was determined as 39-desmethyl-3-hydroxy-AZA-2 (Table 1). Both *A. poporum*-derived AZAs have a 3-hydroxy substitution and a 39-desmethyl moiety in common.

Whereas the 3-hydroxy function is also found in shellfish metabolites of AZA1 and AZA2 (e.g., AZA4 and AZA9) (Kilcoyne et al. 2015), the 39-desmethyl moiety is unique to a new class of dinoflagellate AZAs. This new class of 39-desmethyl-AZAs is easily recognized in tandem mass spectrometry by a characteristic *m/z* 348 fragment, whereas all other AZAs have a *m/z* 362 fragment. Two additional AZAs with *m/z* 348 fragment and molecular masses of 815 and 829 Da (*ca*. 11 and 6 fg cell^{-1}, respectively) were also identified in a strain of *Amphidoma languida* (Tillmann et al. 2012).

AZAs were also detected in isolates of *A. poporum* from Chinese coastal waters (Gu et al. 2013). Whereas one strain did not produce any AZAs, three other strains produced exclusively AZA2 at cell quotas ranging from 1.8 to 23 fg/cell. In addition, new AZAs with the *m/z* 348 fragment were detected in a fifth strain, which also produced AZA36 (1.4 fg/cell). In contrast to the Korean strains, this Chinese strain produced AZAs (with the *m/z* 348 fragment) with molecular masses of 919 and 927 Da (*ca*. 0.02 and 0.14 fg/cell, respectively). A sixth strain of *A. poporum* from China produced an AZA with a molecular mass of 871 Da (0.9 to 1.9 fg/cell) that was tentatively identified as AZA11 by comparison of retention

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times and collision induced dissociation (CID) spectra (Gu et al. 2013). Whereas 3-hydroxylated AZAs like AZA36 and AZA37 seem to be biosynthesized by several strains of *A. poporum*, AZA11 may be the first case of an AZA being independently produced by dinoflagellate biosynthesis as well as through shellfish metabolic activity.

It is likely that several more analogues of this toxin group will be reported soon, as there are not yet many strains or species isolated from most regions. For instance, *Azadinium dexteroporum*, a new species recently isolated from the Mediterranean Sea (Italy), has also been reported to produce some novel analogues which have not yet been fully characterized (Percopo et al. 2013).

Shellfish Metabolism

To date, more than 30 AZA structural variants are known (Table 1). As the exact nomenclature of AZAs according to the rules of the International Union of Pure and Applied Chemistry (IUPAC) is long and complicated, AZAs have been named by numbering in the chronological order of their detection or postulation. All AZAs up to AZA23 were originally identified or postulated from shellfish; however, AZA1 and AZA2 are of dinoflagellate origin, whereas AZA4 to AZA23 have not been detected in planktonic samples and have been shown to be shellfish metabolites, with the exception of AZA11 (Gu et al. 2013; Rehmann et al. 2008).

Shellfish are known to transform AZAs by two different types of reactions: (1) hydroxylation at C3 and C23 and (2) carboxylation at C22 and subsequent decarboxylation (James et al. 2003b; Jauffrais et al. 2012c; Kittler et al. 2010; McCarron et al. 2009; O'Driscoll et al. 2011; Rehmann et al. 2008). Recent investigations with feeding experiments (Jauffrais et al. 2012c; Salas et al. 2011) revealed that blue mussels (*Mytilus edulis*) metabolize AZAs quickly. AZA17 and AZA19 were the most abundant metabolites of AZA1 and AZA2, respectively, suggesting that carboxylation of the methyl group at C22 is a preferred metabolic pathway (Jauffrais et al. 2012c). Hydroxylation and decarboxylation seem to be secondary degradation routes (McCarron et al. 2009).

Hydroxylations of AZAs by shellfish metabolism occur at C3 on the carboxylic acid side chain to form 3-hydroxy-AZAs (e.g., AZA7, AZA11) as well as at C23 at the E-ring of the molecule, resulting in 23-hydroxy-AZAs (AZA8, AZA12) (Table 1). Furthermore, the methyl group at C22 can be oxidized to 22-carboxy-AZAs (AZA17, AZA19), which are subsequently decarboxylated to form the 22-desmethyl-AZAs (AZA3, AZA6) (McCarron et al. 2009; O'Driscoll et al. 2011). AZA 22-decarboxylation may be a shellfish metabolic activity; however, this reaction occurs rapidly during heating of shellfish meat and slowly in extracts stored at ambient temperature (McCarron et al. 2009). In addition, combinations of these processes are possible, to produce many of the remaining AZAs (AZA4, AZA5, AZA9, AZA10, AZA13, AZA14, AZA15, AZA16, AZA21, and AZA23). Some of the other AZAs originally detected without structural elucidation were later identified as extraction artifacts (AZA29, AZA30, and AZA32) (Jauffrais et al. 2012a). In contrast to shellfish metabolism, phase I metabolites of AZA1 using rat liver microsomes (S9 mix) included an oxidation of the F-ring of the molecule, which is not observed in shellfish metabolites. Glucuronides were found as the only phase II metabolites of AZA1, and via precursor ion experiments, it could be proven that glucuronic acid is bound to AZA1 at C1 via an ester linkage (Kittler et al. 2010).

One study reported on binding of AZAs to proteins (Nzoughet et al. 2008) found that AZAs in mussel hepatopancreas bind to as yet unidentified proteins with molecular masses of 21.8 and 45.3 kDa.

Chemical Degradation Products and Artifacts

Among the modifications of AZA profiles due to extraction and/or sample processing are (1) epimerization, (2) 22-decarboxylation, and (3) formation of methyl derivatives.

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Isomers of AZAs have previously been reported which were produced as a result of degradation of the main analogues in acidic environments (Alfonso et al. 2008; Rehmann et al. 2008). The development of a neutral method led to the discovery of isomers of AZAs which were not resolved at low pH (McCarron et al. 2011). These isomers recently identified as 37-*epi*-AZAs are spontaneous epimerization products whose formation is accelerated with heat, with proportions ranging from 2 % to 15 % of their parent analogues (Kilcoyne et al. 2014a). As most biosynthetic processes are stereospecific, it can be assumed that only one enantiomer is produced by the organism. Similar occurrences are known from paralytic shellfish poisoning (PSP) toxins, where only the energetically less-favored betamers are produced, which slowly epimerize to the more stable alphamers until equilibrium distribution (Cembella 1998). The formation of the 22-desmethyl-AZAs (AZA3, AZA4, AZA6, and AZA9) following heat treatment was demonstrated, and it was shown that these AZAs result from decarboxylation of their 22-carboxylated progenitors (McCarron et al. 2009). Even though it has been clearly shown that this decarboxylation is a thermally accelerated reaction, the 22-desmethyl-AZAs can be detected in lower amounts in fresh, uncooked shellfish indicating that this reaction proceeds even at lower temperatures (McCarron et al. 2007, 2009).

In contrast, methyl esters and 21-methyl ketals of AZAs are clearly artifacts. The methylation of AZAs easily occurs if methanol is used for extraction of algal biomass or as a sample storage solvent for such extracts (Jauffrais et al. 2012a). The methylation is a relatively slow process at low temperatures but is promoted by high temperatures and alkaline or acidic conditions. For instance, planktonic field samples that were extracted with methanol and stored in the same solvent at −20 °C, after 5 years, displayed more than 50 % of the total AZA content as methyl esters (Krock et al. 2012). However, methyl esters can be detected in relatively fresh methanolic extracts of AZA-containing samples. For example, the reported isomer of AZA2 in *A. spinosum* (Tillmann et al. 2009) was later identified as AZA1 methyl ester. Consequently, the use of methanol as extraction solvent may be avoided and replaced by acetone or aqueous acetonitrile, if analysis for AZAs is of special concern (Kilcoyne et al. 2012).

Producing Organisms

As described above, four of the currently known 12 species of *Amphidomataceae* have toxin-producing strains (Tillmann et al. 2014): *A. spinosum*, *A. poporum*, *A. dexteroporum*, and *A. languida*. This does not mean that further studies will not change this picture as for several species only one strain has been isolated so far. Due to the fact that a number of strains of these species have been found in many areas of the globe (Europe, Australasia, America), ubiquitous distribution of the genus is likely. This is also coherent with increasing reports of toxins identified in many seafood or marine organisms or marine environmental samples.

Vectors of Poisoning

The main vector of AZP is mussels (*Mytilus edulis*), as all reported shellfish poisoning incidents have been related to shellfish from mussels originating from Irish coastal waters (EFSA 2008; Salas et al. 2011; Twiner et al. 2014). However, other shellfish species, including Japanese and flat oysters (*Crassostrea gigas* and *Ostrea edulis*) and razor fish (*Ensis siliqua*), have also been shown to be contaminated with AZAs (Hess 2002). Furthermore, scallops (*Pecten maximus* and *Argopecten purpuratus*) have also been shown to be contaminated with AZAs and cannot, in principle, be excluded as potential vectors (López-Rivera et al. 2010; Magdalena et al. 2003b). Furthermore, it should be noted that crustaceans, such as crabs (*Cancer pagurus*), may accumulate AZAs due to their feeding on contaminated mussels (Torgersen et al. 2008). Although in that particular event the accumulation had not led to human poisoning, such a vector cannot be excluded as it had previously been shown that consumption of contaminated crabs may lead to poisoning with a similar lipophilic toxin group: OA (Torgersen et al. 2005). The occurrence of

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toxins in crustaceans is still an underestimated risk as European and other international legislation do not require monitoring of crustaceans for algal toxins.

Epidemiological Data

The first confirmed AZP event occurred in November 1995. Mussels harvested from Killary Harbour, Ireland, were exported to the Netherlands, resulting in eight people falling ill with DSP-like symptoms of gastrointestinal illness, including nausea, vomiting, severe diarrhea, and stomach cramps (Furey et al. 2010; McMahon and Silke 1996; Satake et al. 1998a, b). The absence of known DSP toxins OA and DTX2 led to the discovery and identification of a novel etiological agent, temporarily called Killary Toxin-3 before being renamed to AZA1 (Satake et al. 1998b). Mussels collected from the same area 5 months after the event were shown to contain (in μg/g whole meat) AZA1 (1.14), AZA2 (0.23), and AZA3 (0.06) (Ofuji et al. 1999; Ryan et al. 2008).

In September–October 1997, as few as 10–12 AZA-contaminated mussels were consumed by individuals in the Arranmore Island region of Donegal, Ireland. At least 20–24 people were believed to have been exposed to AZAs in this event, but only eight sought medical attention. Symptoms included nausea, vomiting, and diarrhea for 2–5 days prior to full recovery. Analysis of the shellfish revealed five AZA analogues, AZA1–5, with most of the toxin concentrated in the digestive glands (Ofuji et al. 1999, 2001) at levels exceeding 30 μg/g (estimated at 6 μg/g whole mussel meat) (Furey et al. 2010). The AZAs persisted in the mussels at elevated levels for at least 8 months (Furey et al. 2010).

In September 1998, mussels exported from Clew Bay, Ireland, to Ravenna, Italy, were consumed, and ten people fell victim to AZP with typical gastrointestinal symptoms. Digestive glands were shown to contain *ca.* 1 μg/g AZA_{total} with three AZA analogues present (in μg/g digestive gland): AZA1 (0.5), AZA2 (0.06), and AZA3 (0.44) (Furey et al. 2010).

Also in September 1998, a large shipment of mussels from Bantry Bay, Ireland, was sent to France, resulting in an estimated 20–30 human illnesses due to AZP. Ironically, these shellfish had been deemed safe according to the DSP mouse bioassay; however, it was later determined that the DSP mouse bioassay is susceptible to false negatives for the AZA toxins. Coincidently, the French government posed an embargo on the import of Irish shellfish for most of 1999. Follow-up analysis of the shellfish by LC/MS determined that high levels of AZA were present (up to 1.5 μg/g whole meat) (Furey et al. 2010).

In August 2000, between 12 and 16 people from various regions (Warrington, Aylesbury, Isle of Wight, Sheffield) of the United Kingdom were intoxicated following the consumption of frozen, precooked mussels that originated from Bantry Bay, Ireland. Symptoms included nausea, diarrhea, abdominal pain, and cramps (Ryan et al. 2008). These mussels were also deemed safe for human consumption based on results from mouse bioassays; however, LC/MS analysis determined the presence of AZA1–3 in an uneaten portion from this same batch. Toxin concentrations were 0.85 μg/g shellfish meat (not including the digestive gland), which likely represented an underestimation of the total concentration (Furey et al. 2010).

No other AZP events occurred until 2008 when there were two events. In April 2008, many people in France fell ill due to AZP following the consumption of contaminated frozen, precooked mussels from Ireland. This event was categorized by the French government as a "large outbreak" that paralleled a separate incident of salmonellosis (RASFF 2008). Unfortunately, these contaminated shellfish were accidently released onto the consumer market after being identified as containing unsafe levels of AZA toxins and stored in quarantine (Furey et al. 2010).

The second 2008 AZP event occurred in the United States in July. Frozen, precooked mussels from Bantry Bay, Ireland, were exported and intoxicated two people. It is estimated that each person ate between 113 and 340 g of shellfish. Within 5 h following the meal, each person experienced abdominal heaviness, vomiting (5–15 times), and diarrhea for up to 30 h. Analysis of similar products with the same

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lot number revealed the presence of AZA1–3 with up to 0.244 μg AZA_{total}/g tissue (Klontz et al. 2009). As a result of this event, over 150 tonnes of commercial product were removed from the market and voluntarily destroyed by the manufacturer (R. Dickey, pers. comm.).

Due to the limited epidemiological data available from the abovementioned human AZP events, nearly all information regarding AZA toxicology has been obtained from controlled in vitro and in vivo experiments. Many of these efforts have been directed toward assessing the risk of AZA consumption in contaminated shellfish and accompanied by intensive, wide-ranging studies aimed at identifying the molecular target(s) of AZA.

Toxicokinetics

Experimental Data

"In Vivo" Experimental Data

After acute oral administration by gavage of sublethal doses of AZA1 to mice (100–300 μg/kg), a dose-dependent increase of the toxin levels was observed in different tissues. At 24 h from the toxin administration, the total amount of AZA1 in the internal organs was only about 2 % of the administered doses, with the highest distribution in the liver, followed by kidneys, lungs, spleen, and heart. Considering the toxin concentrations, the highest levels were detected in the stomach and duodenum, followed by kidneys and spleen, lungs, jejunum, heart, liver, ileum, colon, blood, and only traces in the brain. The renal concentration of AZA1 remained almost unchanged after 7 days, suggesting a toxin transport from the other organs to the kidneys (Aasen et al. 2010, 2011). Following the administration of higher doses of AZA1 (660–1140 μg/kg), the highest toxin levels were found in the stomach, and lower levels were detected, in descending order, in the duodenum, jejunum, ileum, and colon. The highest concentrations in the internal organs were in the spleen, lungs, and kidneys, followed by lower levels in the liver, thymus, and heart, while low concentrations were found in the blood (Aune et al. 2012).

Recently, the comparative distribution of AZA1, AZA2, and AZA3 was evaluated in the main internal organs of mice, 24 h after gavage and after 14 days of toxin withdrawal. A dose dependency in AZA1–3 absorption from the gastrointestinal tract was observed, and the pattern of toxin distribution was very similar among the three toxins. At 24 h, the highest AZA levels were detected in the stomach and, in descending order, in the intestinal content, liver, small intestine, kidneys, lungs, large intestine, heart, and brain. Recoveries were *ca.* 6–23 % for AZA1 and *ca.* 4–19 % for AZA2 and were lower for AZA3 (*ca.* 2–8 %) after 24 h. After 14 days from AZA administration (300 or 500 μg/kg), only AZA1 and AZA2 were still detectable in the internal organs, with a total recovery lower than 3 % (Tubaro et al. 2014).

The influence of other marine algal toxins (yessotoxin or OA) on AZA1 distribution in the main internal organs of mice was also investigated after their single oral co-administration. Oral co-administration of yessotoxin (1 or 5 mg/kg) and AZA1 (200 μg/kg) to mice did not influence the toxin absorption as compared to the absorption of each toxin alone (Aasen et al. 2011). On the contrary, oral co-exposure to AZA1 (570 μg/kg, corresponding to its LD_{10} determined in the same study) and the diarrhetic toxin OA (780 or 880 μg/kg, the toxin LD_{10} and LD_{50} in the same study) resulted in a very low absorption of each toxin from the gastrointestinal tract, even lower than that recorded after the administration of each toxin alone (Aune et al. 2012).

"In Vitro" Experimental Data

To evaluate the liver metabolism of AZA1, an in vitro study was carried out incubating the toxin with a fraction from rat liver homogenate (S9 fraction) and supplemented co-factors. The study demonstrated

that the toxin is converted into oxygenated phase I metabolites, including two dehydrogenated derivatives. In terms of phase II metabolism, only the conjugation of glutathione and glucuronidation were investigated; they observed conjugations to glucuronic acid but not glutathione adduct formation (Kittler et al. 2010).

Human Data

No data on toxicokinetics (i.e., absorption, metabolism, distribution, and elimination) of AZAs in humans are available.

Mechanisms of Action

Since the AZA toxin class has been identified as a unique algal toxin group, many targets and mechanisms have been proposed and tested. Based on the similarities in gastrointestinal symptoms that AZAs have in common with the DSP toxins (OA and DTXs), known protein phosphatase (PP) inhibitors, AZAs were originally classified together with the DSP toxins (Twiner et al. 2008). As such, it was first postulated that the most likely mechanism of action was PP inhibition (Flanagan et al. 1999). PPs are well-described regulators of cell signaling pathways where they act in a manner opposite to that of kinases by removing phosphate groups from proteins. The serine/threonine PPs are known to be inhibited by OA (Cohen 1989). However, the effects of crude blue mussel extracts containing AZAs demonstrated no indication of PP1 enzyme inhibition (Flanagan et al. 2001), and a subsequent study utilizing the same assay format but with PP2A also found no effects of purified AZA1 on enzyme activity (Twiner et al. 2005). Furthermore, the AZAs have been subsequently tested against a non-receptor phosphotyrosine PP (PTP1B) and a T cell protein tyrosine phosphatase (TCPTP) with no change in enzyme activity (M. Twiner, unpublished data). Collectively, these data suggest that the AZA toxins are not PP inhibitors.

Not unlike PPs, cellular kinases play a very important role in cell communication, signaling, metabolism, and cell death. In particular, mitogen-activated protein kinases (MAPKs) present a multitude of different pathways and play crucial roles in the regulation of cell death and survival induced by several mechanisms. Although the AZA cytotoxicity response was not altered by inhibitors of extracellular signal-regulated kinase (ERK) or p38 MAPK, cytotoxicity was reduced in the presence of a c-Jun N-terminal protein kinase (JNK) inhibitor (Vale et al. 2007) that corresponds to JNK activation (Vale et al. 2010). However, this phenomenon may only be restricted to primary cerebellar granular cells (CGCs) as it was not observed in neocortical neurons (Cao et al. 2010). In a similar manner, the cAMP pathway and protein kinase C and phosphatidylinositol 3-kinase were ruled out as possible targets (Vale et al. 2007). Furthermore, AZAs were tested on 40 additional kinases without any indication of inhibition (Twiner et al. 2014) strongly suggesting that AZAs are not kinase inhibitors.

Following the observation that AZAs are potent cytotoxic agents and cause extensive morphological and cytoskeletal changes to cultured cells (Twiner et al. 2005, 2012b), the effects of AZAs on actin were assessed. However, AZAs did not directly alter actin polymerization or depolymerization in vitro (Twiner et al. 2014) suggesting that intracellular cytoskeletal rearrangements mediated by AZA toxins are likely an indirect or downstream effect of a toxicological response.

In cultured neurons, AZA was tested for its effects on neurotransmitter release. AZA1 did not have any effect on cholinergic, purinergic, or ionotropic receptors, i.e., gamma-aminobutyric acid ($GABA_A$) (Vale et al. 2010). However, the effects of AZA1 on bioelectrical activity of spinal cord neurons were enhanced in the presence of a $GABA_A$ inhibitor (Kulagina et al. 2006). AZAs were also tested for its effects on 76 G protein-coupled receptors (GPCRs), but none of these were altered (stimulated or inhibited) (Twiner et al. 2014), essentially ruling out GPCRs as an AZA target.

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There are ample publications available that demonstrate AZA toxins alter ion flux in various cell types. The AZAs have been shown to alter intracellular calcium flux (Roman et al. 2002), proton homeostasis (Alfonso et al. 2006), and membrane hyperpolarization (Vale et al. 2010). In CGCs, anion channel blockers and ouabain greatly ameliorated the cytotoxic effect of AZA1 in immature neurons and completely eliminated it in older cultures (Vale et al. 2010). Furthermore, short exposures of cultured neurons to AZA1 caused a significant decrease in neuronal volume that was reduced by preincubation of the neurons with a chloride channel blocker or amiloride (Na^+-K^+-ATPase blocker) (Vale et al. 2010). In neocortical neurons, voltage-gated sodium channels (VGSCs), *N*-methyl-D-aspartic acid (NMDA), glutamate receptors, and L-type Ca^{2+} channels were not observed to play a role in AZA1-induced neurotoxicity, which is consistent with reports that AZA1 alone does not affect VGSC (Kulagina et al. 2006; Twiner et al. 2014). However, a recent study demonstrated that AZAs in the presence of glutaric acid (also found at significant concentrations in shellfish areas known for AZP events) inhibited sodium current through Na_v 1.6 channels (Chevallier et al. 2015) opening up the possibility that co-occurring secondary compounds may play a synergistic role in AZA toxicity. Nonetheless, AZAs have been shown to inhibit hERG (human *ether-à-go-go*-related gene) channels, a specific type of potassium ion channel (Twiner et al. 2012a). hERG channels are important in the cardiac action potential by mediating the "rapid" delayed rectifier current (I_{Kr}) (Sanguinetti and Tristani-Firouzi 2006) that plays a significant role during the repolarization phase where alternations of this channel can lead to long QT syndrome and sudden cardiac death. Subsequent in vitro exposure studies demonstrated AZA2 caused an increase in the levels of membrane hERG channels without a change in potassium current through the channel (Ferreiro et al. 2014b). Electrocardiogram (ECG) studies in rats showed arrhythmic electrical activity including prolonged PR intervals consistent with second- and third-degree heart block but without a prolonged QT (Ferreiro et al. 2014a) clearly demonstrating cardiotoxicity and the need for follow-up studies.

In summary, AZAs do not appear to be inhibitors of PP, kinases, and GPCRs or inhibit actin polymerization/depolymerization, but they have been shown to be cytotoxic, affect cytoskeleton arrangements, and inhibit potassium channels (hERG) and voltage-gated sodium channels (Na_v 1.6; in the presence of glutaric acid). Inhibition of these ion channels may prove to account for the cardiotoxicity observed in rats. However, at this point in time, there does not yet appear to be a scientific consensus regarding a particular target or mechanism for the AZA toxin class that can jointly account for the wide array of effects that have been experimentally observed and the gastrointestinal symptoms observed in exposed humans.

Toxicity

Human Toxicity

Human consumption of AZA-contaminated seafood induces an acute illness characterized by gastrointestinal symptoms, which occur within hours and include nausea, vomiting, severe diarrhea, and stomach cramps. The symptoms persist for 2–3 days, and, to date, no long-term effects have been reported (James et al. 2004; Twiner et al. 2008). On the basis of information from one incident in humans, the European Food Safety Authority (EFSA) Panel on contaminants in the food chain (CONTAM Panel) estimated 113 g AZA1 equivalents/person as the most probable lowest-observed-adverse-effect level (LOAEL) resulting in AZP, which corresponds to 1.9 μg AZA1 equivalents/kg for a person of 60 kg body weight. These values were used to establish the acute reference dose (ARfD), equal to 0.2 μg AZA1 equivalents/kg body weight (EFSA 2008).

Considering a possible risk of chronic effects due to repeated consumption of seafood contaminated by low levels of AZAs, the scarcity of epidemiological data does not allow for establishing any link between

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AZA exposure and the occurrence of long-term adverse effects, such as cancer or birth defects in humans (Twiner et al. 2008).

Experimental Toxicity

In vivo toxicological studies on AZAs are limited due to the low availability of pure toxins, and the first studies had been carried out using shellfish extracts contaminated by AZA1.

Single Administration

The first evidence of acute AZA toxicity following *intraperitoneal* (*i.p.*) administration in mice was recorded after injection of acetone extracts from contaminated mussels which caused "neurotoxin-like" symptoms, including sluggishness, dyspnea, spasms, progressive paralysis, and death within less than 1–2 h. Subsequently, the lethal *intraperitoneal* dose in mice of purified AZA1 has been estimated at 200 μg/kg (Satake et al. 1998b). AZA1 was less toxic than AZA2 and AZA3 (lethal dose, 110 and 140 μg/kg for AZA2 and AZA3, respectively) and more toxic than AZA4 and AZA5 (lethal dose, *ca.* 470 μg/kg and <1 mg/kg for AZA4 and AZA5, respectively) (Ofuji et al. 1999, 2001). However, due to the scarcity of pure toxins, the lethal doses were determined using a low number of animals, and the information provided by these data should be considered only as indicative (EFSA 2008). Nevertheless, the *i.p.* lethal doses of AZA1, AZA2, and AZA3, the three AZAs regulated at European Union level, have been used to derive their toxic equivalency factors (TEF, 1.0, 1.8, and 1.4 for AZA1, AZA2, and AZA3, respectively), currently adopted to convert the amounts of individual toxins to AZA1 equivalents during the estimation of total AZAs in edible shellfish (EFSA 2008; Twiner et al. 2008). Signs and symptoms observed in mice after *i.p.* injection of purified AZAs included progressive paralysis of the limbs, dyspnea, and convulsions before death (Furey et al. 2010; Twiner et al. 2008).

Recently, AZA2 was investigated for its effects on electrocardiogram (ECG) and cardiac biomarkers, after intravenous injection in rats. The toxin (11 or 55 μg/kg) did not induce QT interval prolongation on rat ECGs, in spite of being an in vitro blocker of the hERG cardiac potassium ion channels, and did not increase the plasma levels of selected biomarker indices of acute myocardial damage. Nevertheless, both the doses of AZA2 altered the cardiac electrical activity in some rats, prolonging PR intervals (4/8 and 1/2 rats at the low and high doses, respectively) and altering the cardiac rhythm (3/8 and 2/2 rats at the low and high doses, respectively). Even though more studies are needed to clarify these effects, the potential cardiac toxicity of AZAs should be considered as possible threat for human health, particularly for subjects with cardiovascular diseases (Ferreiro et al. 2014a).

After oral administration of AZA1 (≥300 μg/kg) to mice by gavage, Aune et al. (2012) recorded lethal effects within 24 h at the dose of 600 μg/kg, estimating an LD_{50} (lethal dose for 50 % of the treated animals) of 775 μg/kg. Other studies recorded lethal effects at doses ranging from 250 to >700 μg/kg (Aasen et al. 2010, 2011; Ito et al. 2000, 2002, 2006), with individual differences in sensitivity to AZA1 and age-dependent lethality (Ito et al. 2002). In a more recent comparative acute oral toxicity study on AZA1–3 in mice, lethality was recorded at a lower dose of AZA1 (230 μg/kg), 500 μg/kg of AZA2, and 650 μg/kg of AZA3, whereas their LD_{50} based on 24 h lethality data were 443, 626, and 875 μg/kg, respectively. Thus, the acute oral toxic potency is AZA1 > AZA2 > AZA3, and, giving a reference value of 1.0 to AZA1, the relevant TEFs based on LD_{50} correspond to 1.0 (AZA1), 0.7 (AZA2), and 0.5 (AZA3) (Tubaro et al. 2014). These values are different from those currently adopted to quantify total AZAs in edible shellfish, derived after *i.p.* AZA injection in mice (EFSA 2008; Twiner et al. 2008).

Before death, common symptoms and signs of toxicity recorded in mice orally treated with AZA1, AZA2, or AZA3 included immobility, laying in sternal recumbence without moving, tremors, abdominal breathing, hypothermia, and cyanosis. Although diarrhea is the main sign of toxicity in humans after

ingestion of AZA-contaminated seafood, oral exposure to AZA1–3 did not cause visible diarrhea in mice (Aune et al. 2012; Tubaro et al. 2014).

Acute oral toxicity studies on AZA1 in mice resulted in multiple organ damage, with three main targets: (i) gastrointestinal tract, with erosions of villi in the duodenum; (ii) liver, with fatty changes and single-hepatocyte necrosis; and (iii) lymphoid tissues, with necrotic lymphocytes in the thymus, spleen, and Peyer's patches. In particular, after sublethal oral dose administration (100–300 μg AZA1/kg), macroscopic or microscopic changes were noted only in the gastrointestinal tract and liver of mice. Within 4–24 h from the administration, the toxin induced stomach constipation, fluid accumulation in the upper small intestine, and tissue alterations in the duodenum (mild cellular detachment in the tips of the villi, expansion of the crypts, necrotic/apoptotic changes in the lamina propria with neutrophilic infiltration), which recovered within 7 days (Aasen et al. 2010, 2011; Ito et al. 2000). Moreover, at 300 μg/kg, Ito et al. (2000) noted the accumulation of fat droplets in the liver of mice after 1 h which became progressively clear, changing the dark red color of the liver to pinkish red after 4 h. At higher oral doses (420–900 μg AZA1/kg), pathological findings were recorded also in lymphoid tissues, in addition to the liver and gastrointestinal tract. Necropsy highlighted a pale liver after 1–24 h, while fatty changes in the hepatocytes were observed by light microscopy. After 10–30 h, material retention in the stomach (up to around four times in weight relative to controls) and watery content in the upper and middle small intestine were macroscopically evident (Aune et al. 2012). Histological analysis of the small intestine after 8 h showed tissue changes (degeneration of epithelial cells, erosions of villi, and necrosis of the lamina propria), even though signs of recovery were noted after 24 h. There were also time- and dose-dependent necrotic T and B lymphocytes in the thymus, spleen, and Peyer's patches of the small intestine. At 600 and 700 μg/kg, a significant decrease in the number of non-granulocytes (lymphocytes, monocytes, and macrophages) was quantified in the spleen (Aune et al. 2012; Ito et al. 2000, 2006).

The acute oral toxicity in mice of eight synthetic AZA1 analogues (6-, 10-, 13-, 14-, 16-, 17-, 19-, and 20-*epi*-AZA1) and twelve AZA1 fragments in comparison to synthetic AZA1 (doses, 0.5–3 mg/kg) had also been evaluated (Ito et al. 2006). Only AZA1 and its diastereoisomer C_1-C_{20}-*epi*-AZA1 exerted significant toxicity, the latter being only one-fourth as toxic as AZA1. This finding suggests that the entire AZA1 molecule and its stereospecific orientation are necessary to exert the toxic effects.

Other toxicity studies evaluated the effects of single oral co-administration of AZA1 and other algal toxins that may co-occur in edible shellfish, with possible interaction between these compounds and consequent influence of the respective toxicities. Thus, the effects of single oral co-exposure to AZA1 and two structurally different marine algal toxins (yessotoxin or OA) were studied in mice. The combined oral administration of AZA1 (200 μg/kg) and yessotoxin (1 or 5 μg/kg), a dinoflagellate polyether previously shown to induce in vivo ultrastructural changes in cardiomyocytes of mice, provoked only the gastrointestinal alterations recorded after AZA1 alone administration. Thus, the oral co-exposure to AZA1 and yessotoxin did not result in enhanced toxic effects with respect to those of the single toxins (Aasen et al. 2011). Similarly, co-administration of AZA1 (570 μg/kg, corresponding to its LD_{10} determined in the same study) and OA (780 or 880 μg/kg, the toxin LD_{10} and LD_{50} in the same study) did not result in additive or synergistic toxic effects, which were restricted to the gastrointestinal tract (Aune et al. 2012).

Repeated Administration

The effects of repeated oral doses of AZA1 were evaluated relatively early in mice, and the animals had been monitored for recovery of tissue injuries for up to 90 days after toxin withdrawal (Ito et al. 2002). In particular, groups of two and three mice were orally administered with 300 or 350 μg AZA1/kg 3 days before a second oral dose of 300 or 250 μg/kg, respectively. Similarly, a group of five out of 16 mice that survived after the treatment with 450 μg/kg were administered after 3 days with a second oral dose of 300, 350, or 450 μg/kg. A mouse treated with 450 μg/kg followed by 350 μg/kg died after the second

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administration, while the other nine surviving mice were sacrificed between 7 and 90 days post treatment to evaluate the recovery of organ injury. Tissue injuries, recorded in the liver, lymphoid tissues, lungs, and gastrointestinal tract, recovered after different times from toxin withdrawal. The fatty changes in the liver recovered after 7 days, but single-cell necrosis, swollen cells, and inflammatory cells were still present. After 10 days, polynuclear giant cells enclosing debris were noted around the central vein, where they were still noted together with small granulomas after 15 days, even at much less extent. A faster recovery was noted in the lymphoid tissues: although signs of atrophy and some inflammatory cells were still noted in the thymus and spleen after 10 days, necrotic cells were absent, while after 10 days the tissue changes became indiscernible. At pulmonary level, infiltration of cells in the alveolar wall was observed up to 8 weeks. Tissue injuries in the gastrointestinal tract were the most persistent. Mild erosions, often accompanied by active mucus secretion and bacterial infection, were sporadically noted in the stomach even after 90 days. In the small intestine, deformed villi with double-triple heads were often noted after 10–20 days from the first administration. The heights of the villi recovered to normal levels after 3 months, even though epithelial cells did not completely cover their tops (Ito et al. 2002).

Ito et al. (2002) also evaluated the effects in mice of low oral doses of AZA1 (1–50 μg/kg), administered *ca*. twice a week, up to 40 times for a period of 145 days. Surviving mice were kept alive up to 3 months after withdrawal to evaluate the progression of the toxin effects. At the doses of 50 and 20 μg/kg, a marked weight loss was recorded mainly after 30 treatments so that mice appearing too weak to survive were sacrificed before the scheduled 40 administrations (9/10 after 18–40 treatments at 50 μg AZA1/kg and 3/10 mice after 32–36 treatments at 20 μg/kg). In these mice, the weight of different organs (heart, liver, kidneys, spleen, and thymus) was reduced in comparison to control mice. The main pathological changes in the weakened mice were recorded in the small intestine (shortened villi and reduced surface area for nutrient absorption) and lungs (interstitial pneumonia). Other pathological changes were recorded at gastric level (swollen stomachs, mild erosions in the mucosa, and sticky gastric juice secretion), spleen, and thymus (narrowed areas of red pulp, decreased lymphocytes, and neutrophil infiltration). On the other hand, no severe injuries were recorded in the liver (only few mice showed focal necrosis, single-cell necrosis, minor inflammation, mitosis, and congestion), contrary to the observations at higher doses, after single or double administration.

Considering also the mice sacrificed after the withdrawal, a low incidence of lung tumor formation and gastric hyperplasia was recorded. Lung tumors were observed in four mice: one at 50 μg/kg (after 32 treatments) and three at 20 μg/kg (one after 36 treatments and the other 2 and 3 months after withdrawal). Hyperplasia in the gastric mucosa was recorded in six mice administered with 20 μg AZA1/kg (one after 32 treatments and five in post withdrawal periods). At the end of 3 months of withdrawal, the lamina propria of the small intestinal villi showed atrophy, and many villi had open tops indicating incomplete recovery (Ito et al. 2002).

No signs of toxicity were noted in mice administered with 1 and 5 μg AZA1/kg. Nevertheless, one out of five mice exposed to 5 μg AZA1/kg showed constipation after more than 2 months of toxin withdrawal, probably due to ulceration and perforation of the digestive tube, while all five mice treated at the same dose had small intestinal erosions attributed to unhealed injuries. In addition, after 3 months of recovery, 1/6 mice dosed with 1 μg AZA1/kg showed a hyperplasic nodule in the liver, surrounded by many mitotic cells (Ito et al. 2002).

Ito et al. (2004) further investigated the potential AZA1 carcinogenicity after repeated oral administration to groups of 10–23 mice, at doses up to 20 μg/kg. Group 1 was administered with 20 μg/kg (twice a week, 40 doses), group 2 received 20 μg/kg (twice a week, 33 doses), group 3 received 5 μg/kg (twice a week, 40 doses), group 4 received 10 μg/kg (once a week, 20 doses), and group 5 was administered with 5 μg/kg (once a week, 20 doses). The doses in groups 1 and 2 were changed depending on the health condition of mice as follows: group 1 (17 treatments at 20 μg/kg and 23 treatments at 10 μg/kg) and group

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2 (13 treatments at 20 μg/kg, five treatments at 15 μg/kg, and five at 10 μg/kg). Then, each mouse that survived up to 20 weeks was dosed as follows: 19.2 μg/kg (group 1), 19.6 μg/kg (group 2), 8.2 μg/kg (group 3), 7.7 μg/kg (group 4), and 4.3 μg AZA1/kg (group 5). Sixty-six mice were sacrificed at 8 months and did not show any tumor. However, multiple lymphatic nodules were observed in the lungs of 10/27 mice at 8 months from groups 1 and 3. In the residual 20 mice (ten mice of group 2, six of group 3, and four of group 4), five tumors were noted within 1 year. They included two malignant lymphomas and three lung tumors (one adenocarcinoma and two epithelial-type tumors), but one lung tumor was noted also in controls (EFSA 2008; Ito 2008; Ito et al. 2004). Since the ICR mice used in the study show a relatively high ratio of spontaneous tumors at 2 years, it has been suggested that the nine recorded tumors at 1 year might be spontaneous. Nevertheless, it has to be considered the possibility that AZA1 could be tumorigenic by itself or a tumor promoter (Ito 2008; Ito et al. 2004).

Mutagenicity and Genotoxicity

No mutagenicity or genotoxicity data are available for AZAs.

Tumor Promotion

Even though tumors were noted in 9/126 (7 %) in a 1-year toxicity study in mice, treated with repeated oral doses of AZA1 (20 or 50 μg/kg), further studies should be carried out to confirm the toxin carcinogenicity or its tumor promotion activity (Ito 2008; Ito et al. 2004).

Treatment

Similarly to DSP, no specific antidotes are currently available for AZP, which treatment is only symptomatic and supportive.

Conclusion and Future Directions

Since the initial isolation of AZAs from shellfish implicated in poisoning events in the mid-1990s, much progress has been made over the last decade, both in terms of chemical and toxicological understanding. Semi-preparative purification allowed for structural clarification for 16 of more than 30 known analogues. Also, metabolism pathways have been clarified in shellfish, in particular mussels. As a consequence, several currently non-regulated analogues have been pointed out to be of importance: AZA6, AZA17, and AZA19, and official control procedures should be revised to take account of these analogues as their occurrence is going to be an important knowledge in future risk assessment exercises.

The discovery of a producing organism, *Azadinium spinosum*, reported in 2009, has clarified why the biological origin remained cryptic for about 12 years: the organism is very small and easily destroyed in standard procedures for phytoplankton analysis. Since then, 11 other species have been identified in this new and one related genus (*Amphidoma*), resulting in four species known to produce AZAs (*Azadinium spinosum, Azadinium poporum, Azadinium dexteroporum, Amphidoma languida*). The recently established worldwide distribution of these species and the reports of toxins accumulating in shellfish from all regions of the globe raise the hope that knowledge will rapidly increase for more complete epidemiological studies.

Complete clarification of the mode of action of AZAs is still lacking and thus impedes development of a medical treatment, other than the current treatment of the gastrointestinal symptoms. Further isolation

of toxins and in-depth toxicological studies are also required to assess the potential effects of low-level repeated exposure or co-exposure with other toxins or contaminants.

Cross-References

► Okadaic Acid and Other Diarrheic Toxins: Toxicological Profile

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Domoic Acid and Other Amnesic Toxins: Toxicological Profile

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Abstract

Domoic acid (DOM) is a marine toxin found in the red alga *Chondria armata* and produced worldwide by a variety of phytoplankton (*Pseudo-nitzschia* spp.) under certain environmental conditions. Planktonically derived DOM bioaccumulates in filter-feeding mollusks, such as mussels, clams, and oysters, as well as in planktivorous fish species, such as anchovies and sardines. These species serve as vectors through which humans and a variety of wildlife species become intoxicated with DOM. A structural analogue of glutamic acid, DOM is a highly potent neurotoxin that produces excitotoxicity both directly and indirectly in the central nervous system by a selective activation of the kainate receptor subtype of glutamate receptors. In the only properly documented case of human toxicity following DOM exposure, affected individuals experienced gastrointestinal toxicity as well as neurological symptoms including headache, amnesia, seizures, coma, and death occurring roughly in a dose-dependent manner. Seizures, neuronal necrosis, and death due to DOM have also been well characterized in piscivorous birds and mammals, notably California sea lions (*Zalophus californianus*). In experimental animals, DOM is known to penetrate the central nervous system poorly and to be eliminated almost exclusively by renal excretion. Consequently, conditions of reduced blood–brain integrity (fetal/neonatal, acute inflammation) and/or impaired renal clearance significantly enhance DOM toxicity in laboratory animals and may confer additional risk in certain populations (e.g., diabetics, neonates, elderly) although this is not yet established. Currently, the only approved therapies for DOM intoxication are anticonvulsant drugs and maintenance therapy, although a number of glutamatergic antagonists are in preclinical development.

Introduction

In certain islands within the Japanese archipelago, the red alga "doumoi" (*Chondria armata*) has for centuries been harvested, dried, pulverized, and used medicinally as an anthelmintic. Indeed, the initial isolation and characterization of the active ingredient "domoic acid" (DOM) (Fig. 1) was in relation to the potential for use as an anti-insecticidal agent (Takemoto and Daigo 1960), and while the structurally similar compounds glutamic acid and kainic acid (Fig. 1) were the subject of intensive scientific research in the late 1960s, 1970s, and 1980s, the literature of the same period is almost devoid of articles on DOM.

That situation changed dramatically in the late 1980s after DOM was identified by scientists at the federal Institute for Marine Biosciences in Halifax, Canada, as the toxic agent responsible for a severe episode of human poisoning linked to consumption of contaminated blue mussels (*Mytilus edulis*) cultured in eastern Prince Edward Island (Wright et al. 1989). Unlike the mainly peripheral symptoms associated with more common shellfish toxins such as those responsible for paralytic shellfish poisoning (PSP) or diarrhetic shellfish poisoning (DSP), the 107 confirmed cases of DOM poisoning in Montreal (Perl et al. 1990) were characterized largely by neurological symptoms including headaches, seizures, and amnesia (Teitelbaum et al. 1990). These symptoms (as well as gastrointestinal disturbances, coma, and

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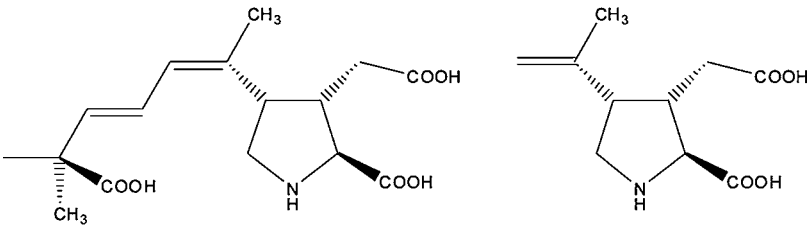

Fig. 1 Structure of domoic and kainic acid

death) appeared dose related (see below) and led to the designation of a new syndrome; amnesic shellfish poisoning (ASP) agreed upon at a 1990 symposium held in Ottawa, Canada.

While it was well established that DOM exists in *Chondria armata* (see above), that species does not exist in the waters of eastern Canada, and consequently the source of the DOM that contaminated shellfish was unknown. Because bivalves, such as blue mussels, are voracious filter feeders of plankton (especially when suspended higher in the water column as they are in aquaculture), a professor of biology at the University of Prince Edward Island, Louis Hanic, proposed that the source could be planktonic. Subsequent investigations confirmed that the DOM contaminating Prince Edward Island mussels was formed by a diatom, *Nitzschia pungens f. multiseries*, although subsequently a variety of planktonic diatoms of *Pseudo-nitzschia* spp. have been identified worldwide as being capable of producing DOM under certain environmental conditions (for review, see Bates and Trainer 2006).

Chemically domoic acid is (2S,3S,4S)-2-carboxy-4-1-methyl-5(R)-carboxyl-l(Z)-3(E)-hexadienyl pyrrolidine-3-acetic acid ($C_{15}H_{21}NO_6$) and has an anhydrous molecular weight of 311.14. The compound is a crystalline, water-soluble molecule with chemical properties similar to other acidic amino acids. In addition to the parent compound, a number of isomers of domoic acid have been identified (for review see Quilliam 2003). Isodomoic acids A, B, and C are present in small amounts in the red alga *Chondria armata* as are isodomoic acids G and H. The isomers isodomoic acids D, E, and F, as well as the 5′ epimer, have also been identified in small amounts from both plankton cells and shellfish tissue although it seems likely that these isomers form on exposure to UV light rather than being metabolic products produced by the plankton (Quilliam 2003).

Because DOM (and DOM isomers) are produced primarily by plankton in many marine ecosystems, there are multiple marine invertebrates and planktivorous fish species that have been identified as the primary vectors for DOM intoxication of higher-order wildlife (see below) and (potentially) humans. These include various species of mussels, clams, oysters, anchovies and sardines (for review see Doucette and Tasker 2008), and most recently cephalopods (Lopez et al. 2013). Largely because of the need to understand and avoid future toxicity outbreaks in humans and wildlife, the toxicological and pharmacological properties of DOM (and more recently DOM isomers) have been extensively characterized and are described in more detail in the ensuing sections.

Pharmacokinetics/Toxicokinetics

Experimental Data

In most in vivo experimental studies, DOM is administered parenterally (either intraperitoneally [i.p.] or subcutaneously [s.c.]), despite the fact that the most common route of exposure for intoxication of wildlife and humans is by oral ingestion. This is primarily for two reasons, namely, (1) the relative ease of parenteral versus enteral administration in rodents and (2) the comparatively poor and erratic absorption

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of the toxin from the gut. The few studies of oral administration that have appeared in the literature indicate that DOM absorption is highly erratic and is approximately 5–10 % whether studied in mice and rats (Iverson et al. 1989) or in cynomolgus monkeys (Tryphonas et al. 1990). Bioavailability following parenteral administration on the other hand appears to be complete in all species studied to date.

Once absorbed, DOM appears to be largely restricted to the vascular compartment with an interpolated apparent steady-state volume of distribution (Vd_{ss}) of approximately 0.20 l/kg (Suzuki and Hierlihy 1993). This is because DOM is a very hydrophilic molecule that crosses membranes poorly. It is the hydrophilic nature of the molecule that also accounts for the comparatively poor blood–brain barrier (BBB) penetration of DOM in normal adult rats, where mean transfer constants ranging from 1.60 to 1.82 ml/g/s $\times$ 10^6 have been reported (Preston and Hynie 1991). These data argue against the existence of a carrier protein (despite DOM being an amino acid structurally similar to proline) and suggest that the highly charged state of DOM at physiological pH results in poor central nervous system (CNS) penetration kinetics. These same data, however, can be interpreted as meaning that once access to the CNS is achieved that DOM is an extremely potent neurotoxin. Studies in which DOM is administered directly to the CNS by intrahippocampal administration confirm this by reporting toxicity in rats at doses 0.0075 times those required to produce toxicity after systemic administration (Sawant et al. 2008). Poor penetrance of the intact BBB as well as the extreme potency of the molecule within the brain also implies that either physiological (e.g., the very young or very old) or pathological (e.g., fever) conditions in which BBB integrity is incomplete or compromised should confer considerably increased risk. Certainly, studies of DOM administered to very young rats prior to blood–brain barrier closure have reported significant toxicity at doses far below those required to produce equivalent toxicity in adult rats (Xi et al. 1997; Doucette et al. 2000).

Also consistent with the very hydrophilic nature of the molecule, DOM appears to be eliminated almost exclusively by renal excretion. Preston and Hynie (1991) reported almost complete elimination of a single intravenous (i.v.) dose within 30 min in intact rats, with clearance substantially reduced following nephrectomy. Subsequently, a more detailed study by Suzuki and Hierlihy (1993) reported that serum clearance of DOM was consistent and almost entirely due to renal excretion over a wide range of dosages (0.5 ng/kg–2.0 mg/kg, i.v.). These authors calculated serum elimination rate constants (k) of 0.025–0.035 min^{-1} (equivalent to an elimination half-life of approximately 13 min) as well as total body clearance values of 7.75–10.82 ml/min/kg and renal clearance values ranging from 8.80 to 12.20 ml/min/kg. Further, renal excretion in this study appeared to be mainly by glomerular filtration because kinetics were not altered by the presence of probenecid.

Human Data

The pharmacokinetics and toxicokinetics of DOM in humans have never been studied experimentally. Consequently, they can only be inferred from the animal data (see above) and the profile of toxicity noted in the original 1987 toxicity event (Perl et al. 1990; Teitelbaum et al. 1990). Thus, although speculative there are several intriguing observations from the human toxicity data. Because the contaminated mussels were served at several busy Montreal restaurants, it is widely assumed that many more people consumed toxic mussels than presented with signs of toxicity. Of the confirmed cases, those patients who were retrospectively presumed to have consumed the lowest quantities of DOM presented mainly with gastrointestinal (GI) symptoms (see below). Accordingly, it is reasonable to assume that other patrons may have had GI distress but not presented at the hospital. However, it is also reasonable to assume that many diners consumed as much DOM as the more severely affected persons but did not experience sufficient toxicity to justify going to the hospital (health care in Canada is freely available so major economic barriers do not apply). If true, then for some reason those persons who were intoxicated and required hospitalization were more sensitive to the toxin, the two most likely causes being increased oral

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bioavailability and/or reduced clearance resulting in more elevated and prolonged serum concentrations. Around the time of the original event, epidemiologists and toxicologists in both academia and the federal Health Protection Branch completed a number of largely unpublished attempts to determine if other components of the meal (sauces, wine, etc.) might have increased oral bioavailability in the affected individuals. No such effect was ever reported. It is of note, however, that all of the individuals who presented with neurological symptoms were middle-aged or older (minimum age 40 with approximately half of the patients over 60 years of age), so impaired kidney function and reduced BBB integrity (see above) are possible explanations for increased sensitivity. The demographics of the patient population are discussed in greater detail in the "human toxicity" section later in the chapter.

Mechanism of Action

Given that DOM is a rigid analogue of glutamic acid and has considerable structural similarity to kainic acid (KA) (Fig. 1), it is not surprising that the primary site of action is the kainate receptor subclass of ionotropic glutamate receptors. Unlike kainic acid, however, DOM binds with greatest affinity to the so-called "low-affinity" kainate receptor subunits designated GluK1 and GluK2 (formerly GluR5 and 6, respectively) allowing for a pharmacological dissociation of the actions of DOM and KA through the use of antagonists selective for these subunits both in vitro and in vivo (Verdoorn et al. 1994; Tasker et al. 1996). The actions of DOM and KA in the brain are not, however, as simple as specific activation of postsynaptic kainate receptors at glutamatergic synapses. Both DOM and KA mediate their toxic effects through the participation and coactivation of both N-methyl-D-aspartate (NMDA) receptors and non-NMDA (AMPA [alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid]/kainate) receptor subtypes. While DOM stimulates AMPA/kainate receptors (AMPA/KARs) directly, NMDA receptor activation appears to be a consequence of either AMPA/KAR-mediated stimulation of EAA efflux (Novelli et al. 1992; Berman and Murray 1997) or reduced tonic inhibition by gamma-aminobutyric acid (GABA) as a result of the activation of presynaptic kainate receptors on GABAergic interneurons (Cunha et al. 1997). Further, DOM in vitro elicits a non-desensitizing response via activation of low-affinity AMPARs (Diaz-Trelles et al. 2003); an effect that does not require coincident activation of KA receptors (Berman and Murray 1997; Diaz-Trelles et al. 2003). DOM has also been shown to evoke the release of both glutamate and aspartate in cerebellar granule cells (CGCs) (Berman and Murray 1997). This appears to occur via a non-vesicular mechanism that is dependent upon reversal of the high-affinity glutamate transporter and cell swelling and, therefore, synergistically potentiates glutamate/aspartate-mediated neurotoxicity (Berman and Murray 1997).

Application of DOM produces a rapid and concentration-dependent increase in intracellular calcium in a variety of neuronal preparations (for review see Perez-Gomez and Tasker 2014). Moreover, some studies have reported that DOM-stimulated increases in cytosolic free calcium are dependent upon the activation of L-type voltage-sensitive calcium channels (VSCC) following activation of non-NMDA receptors, although the contribution of a calcium increase through VSCC to DOM toxicity remains controversial.

In some in vitro preparations, DOM-induced neuronal cell death appears to be primarily necrotic in nature, while in others, both necrosis and apoptosis are observed. No signs of the classical caspase-dependent apoptotic pathway were detected in organotypic hippocampal slices using different concentrations and time of exposures to DOM (Perez-Gomez and Tasker 2012). In mixed hippocampal cell cultures, DOM neurotoxicity was also found to be necrotic when examined both biochemically and morphologically (Roy and Sapolsky 2003), and protection afforded by two virally derived caspase inhibitors (p35 and crmA) was ascribed to their ability to sequester free radicals and to stabilize the

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mitochondrial membrane potential, respectively. However, acute in vitro studies in pure mouse CGCs have shown that DOM can cause either necrotic or apoptotic cell death, depending on its concentration. A high concentration of DOM (1 h, 10 μM) activates both the AMPA/KA and NMDA receptors resulting in a rapid accumulation of intracellular calcium, promoting GSH efflux and a concomitant decrease in intracellular GSH that induces increased lipid peroxidation and leads to necrotic neuronal death (Giordano et al. 2006). On the other hand, AMPA/KA receptors have been shown to mediate low-concentration (<0.1 μM) DOM-induced apoptosis (Giordano et al. 2007) with DOM inducing oxidative stress, leading to mitochondrial dysfunction and activation of caspase-3.

A few studies have been conducted exploring the possibility that DOM affects not only neurons but also other cells of the central nervous system, such as astrocytes or microglia. It has been shown that exposure of astrocytic cultures to DOM did not induce any cell death (Novelli et al. 1992; Giordano et al. 2006) but resulted in ultrastructural changes and altered expression of chemokines/cytokines, tyrosine kinases (Trk), and apoptotic genes (Gill et al. 2008).

The hypothesis that DOM might lead to the activation of microglia has been tested as well, and the results seem to depend on the duration of the exposure as well as on the type of culture used. In organotypic hippocampal slice cultures, low-concentration DOM insult (2 μM, 24 h) induced a significant increase in the number of reactive microglial cells 7 days after treatment (Perez-Gomez and Tasker 2012). On the other hand, DOM treatment (1 mM, 4–24 h) does not appear to activate neonatal rat microglial cultures and the concomitant expression and release of pro- and anti-inflammatory mediators (Mayer et al. 2007).

Toxicity

Human Toxicity

The only fully documented case of human intoxication with DOM occurred in Montreal, Canada, in the late 1987. Following consumption of contaminated blue mussels (*Mytilus edulis)* grown by long-line culture in Prince Edward Island, over 100 persons were treated in the hospital with gastrointestinal (vomiting, abdominal cramps, diarrhea) and/or neurological (severe headache and memory loss) symptoms (Perl et al. 1990; Teitelbaum et al. 1990). Of the 107 persons that met the case definition (47 men and 60 women), 19 were hospitalized from 4 to 101 days, 12 of these were admitted to intensive care, and three died in the hospital at 12–18 days after admission. Another patient died after 3 months. Most of the patients answered a questionnaire, in which they provided information on their age and medical history, the symptoms experienced, and the amount of mussels consumed. Approximately half (49) of these patients were between 40 and 59 years old, and 38 patients were 60 years or older. Symptoms of illness included nausea (77 %), vomiting (76 %), abdominal cramps (51 %), diarrhea (42 %), headache (43 %), and memory loss (25 %). None of the younger patients (20–39 years) suffered memory loss, and their only symptoms were of a gastrointestinal nature. Based on the hospital charts of 16 of the 19 most severely affected individuals, all of the patients less than 65 years old had preexisting illnesses, including diabetes (3), chronic renal disease (2), and hypertension with a history of transient ischemic attacks (1), considered to be predisposing factors; a hypothesis subsequently supported by the experimental data obtained in animals (see below). All of the patients admitted to intensive care had serious neurologic dysfunction, including coma (9), mutism (11), and seizures (8). Seven patients had unstable blood pressure or cardiac arrhythmias. Based on data from the analysis of the unconsumed portion of mussels and recall estimates on portion size, the estimated exposure to DOM could be retrospectively estimated for nine patients and one person who did not become ill. Increasing exposure (dose) correlated with the clinical course of events (Table 1). All patients reported gastrointestinal illness, but only one of six patients who consumed

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Table 1 Estimated exposure and clinical course of patients who ingested DOM in contaminated mussels during the 1987 outbreak of amnesic shellfish poisoning in Canada (Originally published in Perez-Gomez and Tasker (2014))

			Clinical symptoms and treatment			
Patient	Age	Estimated DOM consumed (mg)	GI	Memory loss	Hospitalized	ICU
1	72	60	Y	–	–	–
2	62	70	Y	Y	–	–
3	70	80	Y	–	–	–
4	61	90	Y	–	–	–
5	67	110	Y	–	–	–
6	71	110	Y	–	–	–
7	74	270	Y	Y	Y	–
8	68	290	Y	Y	Y	Y
9	84	290	Y	Y	Y	Y

Data adapted from Perl et al. (1990)
GI gastrointestinal symptoms, *ICU* intensive care unit

between 60 and 110 mg DOM suffered memory loss, and none required hospitalization. All three patients who had consumed 270–290 mg DOM suffered neurological symptoms and were hospitalized. One person who consumed only 20 mg DOM did not become ill. Because of the memory impairments seen in severely affected patients, the condition was subsequently termed "amnesic shellfish poisoning" (ASP).

Neuropathological examination of the four patients who died indicated neuronal necrosis and astrocytosis particularly in the hippocampus and the amygdaloid nucleus, as well as lesions in the claustrum, the secondary olfactory areas, the septal area, and the nucleus accumbens. Two of the four patients had prominent thalamic damage, and the subfrontal cortex was also damaged in three of the patients. Neurofibrillary tangles and senile plaques, typical features of Alzheimer's disease, were not observed in these patients (Teitelbaum et al. 1990).

In addition to the excellent investigations that occurred at the time of the initial incident, the neurologic manifestations of domoic acid intoxication from the 1987 Canadian outbreak were studied over a period of several years in 14 of the patients, all from the Montreal area (Gjedde and Evans 1990). This study included neuropsychological testing, an assessment of motor function and positron emission tomography (PET) to assess glucose metabolism in specific regions of the brain. All 14 patients were confused and disoriented 1.5–48 h postexposure, and their behavior ranged from agitation or somnolence to coma, with maximal deficits between 4 h (least affected) and 72 h (for comatose patients) postexposure. Most improved within 24 h to 12 weeks. Patients characteristically had anterograde memory disorder with relative preservation of other cognitive functions. Those individuals with a moderate memory disturbance were generally able to encode information but had difficulty with delayed recall. More severely affected individuals had some difficulty learning verbal and visuospatial material, and their delayed recall was also very poor. In addition, some of these latter patients also had retrograde amnesia extending several years back. Electroencephalograms (EEGs) in seven patients obtained within 1 week postexposure revealed moderate to severe generalized slowing of background activity. At about 4 months postexposure, the electroencephalograms of 11 patients showed mild to moderate generalized disturbance of background activity, and the other three had normal readings. In addition to central changes, there was also considerable evidence of peripheral nerve damage and changes in sensory thresholds. The authors suggested that domoic acid induced acute nonprogressive neuropathy involving anterior horn cells or diffuse axonopathy predominantly affecting motor axons.

One final interesting report of the 1987 incident involves the initial findings and follow-up of an 84-year-old man who suffered domoic acid intoxication and who subsequently died, 3.25 years after exposure (Cendes et al. 1995). The patient's symptoms during the phase of acute toxicity were

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comparable to those of others (see above), but approximately 1 year post-intoxication, he experienced complex partial seizures consisting of staring, twitching of the left lower part of the face, and then clonic movements of the left arm and leg. Gross examination of the patient's brain postmortem (death due to pneumonia) revealed atrophy of the hippocampi and slight dilatation of the ventricular system and of the Sylvian fissure. Histologically, the hippocampi showed complete neuronal loss in the H1 and H3 (equivalent to CA1 and CA3 in rodents) regions, almost total loss in H4 (equivalent to CA4), and moderate loss in H2 (equivalent to CA2). The amygdala showed patchy neuronal loss in medial and basal portions with neuronal loss and gliosis in the overlying cortex. Mild to moderate neuronal loss and gliosis were seen in the dorsal and ventral septal nuclei, the secondary olfactory areas, and the nucleus accumbens. It was suggested that temporal lobe epilepsy following domoic acid exposure might develop after a "silent period" of 1 year (Cendes et al. 1995). Interestingly, delayed-onset models of epilepsy following DOM administration have subsequently been described in rats (Doucette et al. 2004) and sea lions (Buckmaster et al. 2014) (see below).

Although the 1987 incident in Montreal remains the only confirmed case of human intoxication with DOM, an outbreak of DOM poisoning may have occurred in October/November 1991 in Washington state, USA. Approximately two dozen people became ill after ingesting razor clams harvested along the Washington and Oregon coasts. Although gastrointestinal or neurological symptoms were observed within 36 h of ingestion, no other symptoms consistent with DOM intoxication were reported. A total of 21 possible DOM victims and 43 non-victims were interviewed. Based on the concentrations of DOM measured in razor clams, the mean total DOM consumption by the 21 victims would have been 17 mg (estimated to be 0.28 mg/kg b.w.) and 8 mg by the unaffected individuals (0.13 mg/kg b.w.). These amounts are considerably less than those estimated in the Montreal incident (see Table 1). A total of 13 of the 21 patients developed neurological symptoms, but all recovered. Unfortunately the incident was not well reported and was unsubstantiated (Jeffrey et al. 2004).

Wildlife Toxicity

As stated above, it is well established that DOM produced in planktonic species can bioaccumulate in a wide variety of invertebrate filter feeders such as shellfish, and while these invertebrates appear to not experience toxicity, they do serve as vectors for the intoxication of shellfish-consuming species. Similarly, planktonically derived DOM is known to appear in high concentrations in certain fish species such as the northern anchovy (*Engraulis mordax*) (Lefebvre et al. 2001) that are commonly consumed by both piscivorous sea birds and marine mammals.

There have been numerous anecdotal reports of domoic acid intoxication in piscivorous sea birds; however, the best documented cases are those described by Work and colleagues in 1993 and by Sierra Beltran et al. in 1997. Work et al. (1993) described the epidemiology of a domoic acid epidemic in Monterey Bay, California, in which 95 Brandt's cormorants (*Phalacrocorax penicillatus*) and 43 brown pelicans (*Pelecanus occidentalis*) died. They also noted the deaths of several other species, including double-breasted cormorants, pelagic cormorants, and western gulls in the same incident. The source of domoic acid in these cases appeared to be anchovies which were recovered from the stomachs of many of the dead birds and were shown by HPLC analysis to have high concentrations of domoic acid. In terms of behavioral toxicity, intoxicated cormorants showed few overt signs of CNS toxicity but were generally lethargic or docile. In contrast, affected pelicans displayed many signs of toxicity that were reminiscent of DOM toxicity in other species. These included side-to-side head motions, fine motor tremors, scratching, vomiting, loss of awareness, loss of postural control, convulsions, and death. In a similar incident involving 150 dead sea birds in Cabo San Lucas, Mexico, Sierra-Beltran et al. (1997) reported on the presence of domoic acid in viscera from five dead pelicans which they analyzed by both mouse bioassay and HPLC. The other birds were not tested. In this case the source of domoic acid appeared to be consumption of contaminated mackerel.

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Perhaps the most extensive literature on DOM toxicity in wildlife species is concerned with on-going incidents of toxicity in California sea lions (*Zalophus californianus*) and to a lesser extent in dolphins and whales on the west coast of the USA. For an overview and original citations for these studies the reader is referred to a comprehensive review by Ramsdell (2010). The first such report concerned an event in which many sea lions died, and many others displayed signs of neurological dysfunction along the central California coast during May and June 1998. A bloom of *Pseudo-nitzschia australis* (diatom) was observed in the Monterey Bay region during the same period, and this bloom was associated with the production of domoic acid that was subsequently detected in fish, including the northern anchovy (*Engraulis mordax*) and in sea lion body fluids. Clinical signs in affected sea lions were ataxia, head weaving, muscle tremor, titanic convulsions, rubbing, and lethargy, with 48 (69 %) of the initially affected sea lions either dying due to toxicity or having to be euthanized. Seizures, often progressing to *status epilepticus* for over an hour, were common in the most severely affected animals and ranged in duration from a few minutes to 30 min, with frequency recorded between one and thirty seizures over a 24 h period. In animals that survived, the frequency of seizures gradually decreased over a 1 month period. Histopathology showed that the principal lesions that were considered unique to this stranding event were in the brain and in the heart, with the most severe necrosis being recorded in the dentate gyrus and pyramidal layers of the hippocampus in the anterior ventral region, with other areas in the brain appearing less severely or consistently affected. However, it is feasible that the extent of the lesions in other brain areas was underestimated.

In 2000, a further 184 sea lions stranded with similar clinical signs, including seizures, ataxia, and head weaving, decreased responsiveness to stimuli, and scratching behavior. Affected animals had high hematocrits, and eosinophil counts, and high activities of serum creatine kinase. They were treated supportively by using fluid therapy, diazepam, lorazepam, and phenobarbitone, but approximately half of the affected animals died despite the treatment.

In addition to these well-documented cases of acute intoxication with neurological signs, there have recently been several investigations of the long-term consequences of DOM intoxication in sea lions that, similar to the human case described by Cendes et al. (1995) (see above), develop recurrent seizures featuring many of the behavioral and histopathological indicators of epilepsy. Moreover, in addition to sea lions, domoic acid has been implicated as a causative toxic agent in several other marine mammal species; notably whales and sea otters. A 2002 report by the Working Group on Marine Mammal Unusual Mortality Events describes investigations into the deaths of 350 gray whales, seven sea otters, 90 sea lions, and two humpback whales. Domoic acid was detected in body fluids from two of 11 gray whales (*Eschrichtius robustus*) sampled and in the urine of affected sea otters. While such detection is not evidence of a causal relationship, it does demonstrate bioaccumulation of domoic acid in the marine food chain.

Experimental Toxicity

Acute Behavioral Toxicity

The acute toxicity of domoic acid has been extensively studied in both mice and rats (for reviews and original citations the reader is referred to Doucette and Tasker 2008 and Pulido 2008). One important point to note, however, is that many of the data in mice (and to a lesser extent in rats) derive from early studies that used injections of extracts from contaminated shellfish (usually mussels) that had been previously analyzed for domoic acid content, rather than using chemically purified domoic acid. Consequently, some of the variabilities observed may be due to other neuroactive compounds in the extracts or methodological differences in extract preparation and/or storage. With these provisos, however, it is clear that when DOM is administered to rodents either systemically (orally, intravenously, intraperitoneally) or directly into the brain (intracerebroventricularly), it produces robust and highly reproducible signs of behavioral toxicity.

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Acute toxicity studies to date in mice have reported that there is no appreciable sex difference in DOM toxicity in mice, but there are differences of opinion on whether different mouse strains respond differently to domoic acid. Comparisons between studies suggest that there are no major differences in toxicity between studies that used Swiss-Webster, CD-1, or CF-1 strains although the DBA strain appears to be more sensitive to DOM toxicity (see Doucette and Tasker 2008). Regardless, systemic injections of domoic acid in mice elicit a graded series of behavioral change prior to seizure onset. The sequence of events is both highly reproducible and predictably dose related. Following injection, adult mice will present initially with hypoactivity, followed in order by sedation, akinesia, rigidity, stereotypy (characteristically consisting of a repetitive scapular scratching with one hind paw), loss of postural control, convulsions (initially forepaw tremors progressing to tonic-clonic convulsions), and death. Both the maximum behavior observed and the speed with which the animal progresses through these stages are dose dependent. Consequently, it is possible to construct a domoic acid dose–response curve based on behavioral toxicity, thereby allowing for pharmacological investigation (Tasker et al. 1991).

Acute behavioral toxicity in adult rats has not been studied as extensively as it has in mice, but in general, adult rats display similar behaviors to mice. There are, however, several studies in rats investigating DOM-induced changes in electroencephalographic (EEG) patterns in freely moving non-anesthetized rats. Fujita et al. (1996) administered vehicle or one of three doses of domoic acid (1, 5, or 10 mg/kg, i.p.) to male Wistar rats implanted with bipolar electrodes in the dorsal hippocampus and recorded recurrent focal hippocampal seizures beginning within minutes (high-dose group) or within 1 h (low-dose group) postinjection and lasting for about 24 h. Rats in the two higher-dose groups displayed hippocampal seizures that progressed to full limbic seizures propagating to the sensorimotor cortex and culminating in fatal status epilepticus (Fujita et al. 1996).

The behavioral toxicity of domoic acid in nonhuman primates has also been described in a limited number of publications (Tryphonas et al. 1990; Schmued et al. 1995) but is remarkably consistent with both investigations in rats and mice and the clinical manifestations of DOM intoxication in humans (see above). Cynomolgus monkeys (*Macaca fascicularis*) dosed either intraperitoneally (4 mg/kg) or intravenously (0.025–0.5 mg/kg) with domoic acid in extracts from contaminated mussels display clinical signs of neurotoxicity preceded by a short presymptomatic period (2–3 min) and an even shorter prodromal period (0.5–1 min). The symptomatic period proper is characterized by persistent chewing with frothing, varying degrees of gagging, and vomiting. Monkeys receiving higher doses exhibit additional signs including abnormal head and body positions, rigidity of movements and loss of balance, and tremors. The duration of the symptomatic period is dose dependent. Thus, the nature and timing of dose-dependent toxicity in primates is comparable to that described previously in mice and rats. This is especially true when one considers that the gagging and vomiting seen in monkeys have no equivalent behavior in rodents (mice and rats do not vomit) but are presumably mediated by the lower brainstem regions such as the area postrema, known to be affected early in the dose–response curve in mice (see below).

Histopathology

There is widespread agreement between labs regarding the histopathology of the acute brain lesions associated with DOM toxicity in rodents that is comprehensively reviewed by Pulido (2008). Acute administration of high-dose DOM results in neurodegenerative changes, consisting of neuronal shrinkage, vacuolization of the cytoplasm, cell dropout, edema, and microvacuolation of the neuropil. These changes have preferential distribution within structures of the limbic system. The hippocampus among other brain

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regions appears to be a specific target site having high sensitivity to domoic acid toxicity, particularly the pyramidal neurons in the CA3 region, followed by the dentate gyrus and CA1 region. With some exceptions, the CA2 region is reported as the least affected. Other regions affected include the piriform and entorhinal cortices, the olfactory bulbs, the nucleus accumbens, the arcuate nucleus, the area postrema, and the retina (see Iverson et al. 1989; Tryphonas et al. 1990; Schmued et al. 1995).

In addition to neuronal degeneration, there is also evidence suggesting involvement and injury of glial cells, including astrocytes and microglia (for review see Pulido 2008). Acute injury of astrocytes has been observed by light and electron microscopy in animals exposed to domoic acid and includes vacuolation and cellular necrosis. In experimental animals surviving the acute episode of intoxication, there are permanent structural lesions of the hippocampus represented by gliosis, similar to what has been observed in patients that have died as a consequence of domoic acid exposure (see above).

Histopathologically the response of nonhuman primates is also comparable to both rodent and human patients. Excitotoxic lesions consisting of vacuolation of the neuropil, astrocytic swelling, and neuronal shrinkage, and hyperchromasia were detected in the area postrema, the hypothalamus, the hippocampus, and the inner layers of the retina in monkeys given domoic acid (Tryphonas et al. 1990; Schmued et al. 1995).

Acute Toxicity of Domoate Isomers

In addition to the parent compound, there are five isomers of domoic acid that have been identified as constituents of *Chondria armata* as well as four more identified in small amounts from both plankton cells and shellfish tissue, although it is unclear whether these are metabolic products of the plankton or are artificially produced by exposure to UV light (see Quilliam 2003). Although the binding of domoic acid isomers in vitro to kainate receptors was reported a number of years ago (Hampson et al. 1992), there have been few investigations to date of the pharmacology and toxicology of domoate isomers, due in part to a lack of availability of the purified compounds. Sawant and colleagues (reviewed in Perez-Gomez and Tasker 2014) demonstrated that DOM isomers A and C alter the electrical properties of hippocampal slices in vitro and then completed several studies of the seizurogenic potential of isomers A–F following intrahippocampal injections in rats. These authors found that DOM Iso-A was almost as potent as DOM, whereas the others were much less so and further that the seizure-inducing potency correlated with the binding affinity of the isomers to AMPA/kainate receptors. Only one study to date has reported on the systemic administration of DOM isomers. A 2008 report by Munday et al. (2008) reported that isomers A, B, and C were much less potent than DOM when injected intraperitoneally in mice and concluded that these isomers confer minimal risk of toxicity following consumption.

Toxicity After Repeated Administration

As described above, there is considerable literature on the effects of acute exposure to DOM in a variety of species. The effects of repeated exposure to DOM are, however, almost unknown. No case of repeat human exposure has ever been documented, and although it can be suspected that some affected wildlife were exposed on multiple occasions, parameters such as frequency and dose cannot be determined. The effect (or lack of effect) of repetitive doses of DOM in experimental settings has been described in only four publications (reviewed by Pulido 2008). Rats dosed by oral gavage for 64 days with one of two doses (0.1 and 5.0 mg/kg/day) of DOM (or control) showed no change in either urinary excretion rates or clinical signs, although some morphological changes were noted in the hippocampus, particularly in one of the four rats at the highest dose. A similar lack of effect was reported after administration of low or moderate doses (1.0 and 2.0 mg/kg) of DOM to mice on alternating days for 7 days which resulted in no changes in serum clearance or mean toxicity scores or performance on a test of spatial working memory with repeated exposure. And lastly, a 30-day oral dosing regimen of DOM (0.5 mg/kg/day for 15 days

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followed by 0.75 mg/kg/day for 15 days) in three cynomolgus monkeys reported no changes in any of the histopathological evaluations conducted. Consequently, the limited information available suggests that repetitive dosing with DOM in adult animals does not augment toxicity.

Domoic Acid as a Potential Developmental Neurotoxin

Although most early literature on DOM toxicity investigated adult animals, there is an ever increasing interest in the potential of domoic acid as a developmental neurotoxin (for review see Costa et al. (2010) and Perez-Gomez and Tasker (2014)). This is in part because of the presumed increased sensitivity in patients with reduced renal clearance and/or reduced blood–brain barrier integrity (see section on human toxicity above) but is primarily due to renewed interest in several early publications including a report of negative consequences to DOM exposure in utero (Dakshinamurti et al. 1993) as well as studies by Xi et al. (1997) and Doucette et al. (2000) that demonstrated convincingly that the neonatal rat brain was exquisitely sensitive to the toxic effects of DOM. Further, this difference in potency relative to adult rats changes throughout early life, with dose–response relationships for DOM toxicity shifting progressively to the right between 0 and 22 days of age (Doucette et al. 2000), an effect that is presumed to be due to maturation of the blood–brain barrier. More recent studies of maternal–fetal transfer rates and fetal kinetics as well as reports of toxicity following exposure in utero in rats and sea lions have increased scientific awareness of the issue.

Moreover, low doses of DOM administered repetitively throughout the second postnatal week in rats have been used to produce a neurodevelopmental model of temporal lobe epilepsy (Doucette et al. 2004) characterized by changes in behavior, hippocampal morphology, and seizure threshold (for review, see Perez-Gomez and Tasker 2014). The same protocol has also been shown to produce apparently permanent changes in sleep patterns and tests of attentional processing (e.g., prepulse inhibition, latent inhibition) and may thus be relevant to schizophrenia and related neuropsychiatric disorders that are known to frequently be neurodevelopmental in origin (see Perez-Gomez and Tasker 2014).

Given that current regulatory limits on DOM are based on acute toxicity in adult animals, these studies raise serious questions about the potential of low concentrations of DOM in the environment or in food being a developmental neurotoxin that may warrant additional scrutiny.

Genotoxicity/Carcinogenicity

There is only very limited information on the potential for domoic acid to produce genotoxicity. In a hepatocyte-mediated assay with V79 Chinese hamster lung cells, domoic acid added to lung cell growth medium at concentrations of 27.2 or 54.4 μg/ml medium did not cause an increase in the frequency of mutations to thioguanine resistance or to ouabain resistance, either alone or in the presence of rat hepatocytes, nor did it increase the frequency of sister chromatid exchange or micronucleus frequency. This was in contrast to the effects produced by both a direct-acting (ethyl methanesulfonate) and indirect-acting (7,12-dimethylbenz[a]anthracene) genotoxin in the same assay (Rogers and Boyes 1989). These data would argue that the risk of genotoxicity and/or carcinogenicity following domoic acid is low or nonexistent, although a study by Carvalho et al. (2006) and a very recent report by Hiolski et al. (2014) reporting that repetitive low-level exposure to DOM alters gene transcription in a zebra fish model has challenged this view. More work is clearly needed before firm conclusions can be drawn, but if domoic acid is a potential carcinogen, it would seem logical that cells in the gastrointestinal epithelium would be most at risk.

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Treatment

Reports of the 1987 poisoning event give scant mention to the treatment protocols employed and their relative success (Perl et al. 1990; Teitelbaum et al. 1990). Anecdotally, seizures resulting from DOM intoxication were largely resistant to conventional anticonvulsants, and therapy was largely supportive in nature. Thus no current treatment protocol exists for humans intoxicated with DOM although some guidance can be gained from experimental animal studies. Studies in rodents have confirmed that DOM is eliminated almost exclusively by renal excretion (Preston and Hynie 1991; Suzuki and Hierlihy 1993) and has comparatively poor penetrance of an intact blood–brain barrier (Preston and Hynie 1991). These data are consistent with the patient profiles reported in 1990 in which all of the severely affected individuals were "middle-aged" or older, and the youngest of these patients had predisposing chronic conditions that could reduce renal clearance (Perl et al. 1990; Teitelbaum et al. 1990). Thus it would seem reasonable that treatments to enhance renal excretion could reduce toxicity.

In addition, it is generally conceded that the most severe neurological consequences of DOM intoxication (both short-term and long-term) are the result of seizure activity that may or may not manifest as motor convulsions; so seizure control is almost surely critical to outcome. Reports on the treatment of intoxicated California sea lions would seem to confirm that DOM-induced seizures are very resistant to conventional anticonvulsant drugs in that combinations of diazepam, lorazepam, and phenobarbitone were only moderately effective (see Ramsdell 2010). To date, newer anticonvulsant drugs have not been used in experimental animals although such experiments are probably warranted (e.g., topiramate, is known to act on the GluK1 receptor). Further, experimental drugs that interfere with AMPA/kainate and/or NMDA receptor activation presumably hold some promise for the future. A very early study by Pinsky et al. (1989) reported that the nonselective NMDA receptor antagonist kynurenic acid was neuroprotective against crude extracts of contaminated shellfish, and the experimental drug NS-102 (a selective antagonist of GluK2 receptors) reduced DOM toxicity in vivo in mice (Tasker et al. 1996). Glutamate receptor antagonists work well in experimental settings in a number of disease models but have a very poor record of translating into clinical applications, largely due to unacceptable side effects. Nonetheless the strategy would seem to be sound, and considerable effort is under way to develop antagonists that work at either much more selective sites of action (e.g., specific subunits of glutamate receptors) or at more "downstream" sites that are involved in excitotoxicity but not in normal glutamatergic function. Studies looking at these, and other, agents as potential therapeutants for DOM toxicity are surely warranted.

Conclusions and Future Directions

Domoic acid is a potent neurotoxin that can be produced by a variety of alga and phytoplankton that exist worldwide. Multiple species can serve as vectors for DOM intoxication of wildlife and humans. The pharmaco-/toxicokinetics of the compound, the mechanisms of toxicity, and the behavioral and pathological consequences of acute DOM exposure are largely known. Yet many unanswered questions remain and warrant further study.

Domoic acid (and perhaps some DOM isomers) is unique among the major shellfish toxins in that it produces significant, serious, and even lethal CNS neurotoxicity after oral administration. The characteristic amnesia seen in humans following ingestion of DOM is presumably due to the selective vulnerability of the hippocampus and associated structures, a vulnerability that is presumed to result from the high concentrations of AMPA/kainate and NMDA glutamatergic receptors in that region. Further, intoxications in wildlife species and in experimental animals show remarkable consistency

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across species in terms of both histopathology and behavior. But the hippocampus lies deep within the mammalian brain, and all studies that have investigated the pharmaco-/toxicokinetics of DOM have convincingly demonstrated that the compound penetrates tissues, including the brain, poorly in mature mammals. Thus many questions remain about the conditions under which DOM gains access to the CNS. Equally, and importantly, intriguing are the questions surrounding absorption of DOM from the gastrointestinal tract following ingestion of contaminated species. Domoic acid has now been detected in most areas of the world, and phytoplankton blooms that produce the toxin occur regularly (and presumably have for some time), yet the only documented case of human intoxication following ingestion remains the 1987 incident in Montreal. Even in that tragic event, it is generally conceded that many more people ate contaminated mussels than appeared at the local hospitals. So while the CNS toxicity of DOM can be somewhat explained by a combination of extremely high toxic potency combined with a single route of elimination that could be impaired (resulting in higher and more sustained serum concentrations), the issue of "why only a select few people in Montreal became ill" contrasted with the uncertainty of whether there are large numbers of unreported human toxicity events occurring worldwide remains one of the most intriguing (and frightening) aspects of ASP.

Two other issues require particular mention:

The first is the lack of approved therapeutics for treating DOM intoxication. It is clear that the consequences of high-dose DOM are severe and arise largely from the seizurogenic properties of the toxin, yet effective seizure control in humans and marine mammals remains elusive. There is an urgent need for further research on the ability of existing agents (newer antiepileptic drugs, injectable or inhalational anesthetics, etc.) to control DOM-induced seizures. Moreover, accelerated development of agents that can reduce or even prevent glutamate receptor-mediated excitotoxicity in the absence of compromising side effects should be a priority not only for DOM toxicity but for the many other devastating diseases characterized by excitotoxic cell death.

The second issue is the accumulating evidence that the very young (and probably the very old) are particularly sensitive to the toxic effects of DOM whether exposed directly, or indirectly "in utero," and that such exposure can lead to long-term and/or delayed-onset neurological conditions. Most countries of the world where DOM occurs now have some form of monitoring program to detect toxic blooms. But the harvesting and sale of shellfish is still subject to a regulatory limit (20 ppm) established in the late 1980s based exclusively on toxicity studies conducted in normal adult animals. Given the uncertainty surrounding the potential for DOM to be a developmental neurotoxin and the high likelihood that individuals with compromised renal function due to age or chronic illness are at greater risk of toxicity, the issue of a reduced regulatory limit or even "zero tolerance" limit for susceptible populations is an issue that must be addressed by both further investigations and regulatory agencies.

Cross-References

- ▶ Okadaic Acid and Other Diarrhetic Toxins: Toxicological Profile
- ▶ Palytoxins: Toxicological Profile
- ▶ Saxitoxin and Shellfish, and Other Neurotoxins
- ▶ Toxins Produced by Marine Microorganisms: A Mini Review

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Brevetoxins: Toxicological Profile

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Abstract

Brevetoxins (PbTxs) are polyether ladder-shaped neurotoxins produced by the dinoflagellate *Karenia brevis*. Blooms of *K. brevis* have been recorded since the mid-1800s, principally in the Gulf of Mexico but occasionally along the mid and south Atlantic coasts. Blooms may be accompanied by public health issues as well as significant mortalities of marine mammals, such as bottlenose dolphins and manatees, fishes, sea birds, and sea turtles. PbTxs bind to the voltage-gated sodium channels (VGSCs), leading to persistent activation of neuronal, muscle, and cardiac cells. In humans, after consumption of contaminated shellfish (oysters, clams, whelks), these toxins cause a syndrome known as neurotoxic hellfish poisoning (NSP), characterized by nausea, diarrhea, vomiting, abdominal pain, paresthesia, myalgia, ataxia, bradycardia, loss of coordination, vertigo, and mydriasis. The ingestion of contaminated seafood represents the most dangerous route of exposure for humans. However, when PbTxs are aerosolized through the disruption of *K. brevis* cells by breaking waves or winds, people can suffer from respiratory effects such as conjunctivitis, rhinorrhea, and bronchoconstriction. Due to successful shellfish monitoring programs managed by the Gulf coast states, cases of human intoxications are fortunately rather rare, and no human fatalities have been attributed to NSP.

Introduction

Brevetoxins (PbTxs) are neurotoxic polyethers produced by dinoflagellates belonging to the genus *Karenia,* in particular *K. brevis* (formerly known as *Gymnodinium breve* or *Ptychodiscus brevis*). *K. brevis* primarily occurs in the Gulf of Mexico and along the southern East Coast of the United States (especially Florida), where it is associated with a harmful algal bloom (HAB), known as the "Florida red tide." In these blooms the *K. brevis* algal cells cause the water to take on a red-brown coloration (Brand et al. 2012).

PbTxs are odorless, tasteless, and heat-resistant toxins. They present two different structural backbones, commonly called A and B type, first described by Lin and Risk (1981) and Shimizu et al. (1986), respectively. The A-type backbone is characterized by ten transfused cyclic rings: one terminal five-membered lactone in the A position and nine ether rings containing various numbers of atoms (respectively, by 8, 6, 7, 9, 8, 8, 6, 6, 6 atoms from the B to J rings) (Fig. 1). Among these, the ether ring in the E position confers high flexibility on the structure. The same flexibility is bestowed on the B-type backbone by the eight-membered H ring. Unlike the A backbone, the B structure has 11 transfused rings (respectively by 6, 6, 6, 7, 7, 6, 6, 8, 6, 6, 6 atoms from the B to K rings), but the lactone ring in the A position remains (Ramsdell 2008). PbTxs can also present additional structures in which the A lactone rings are open. Depending on the structural backbone and on the terminal side chain, 15 different PbTxs can be distinguished, all deriving from PbTx-1 (type A) or PbTx-2 (type B), which are considered the parent algal toxins (Fig. 1) (Plakas and Dickey 2010).

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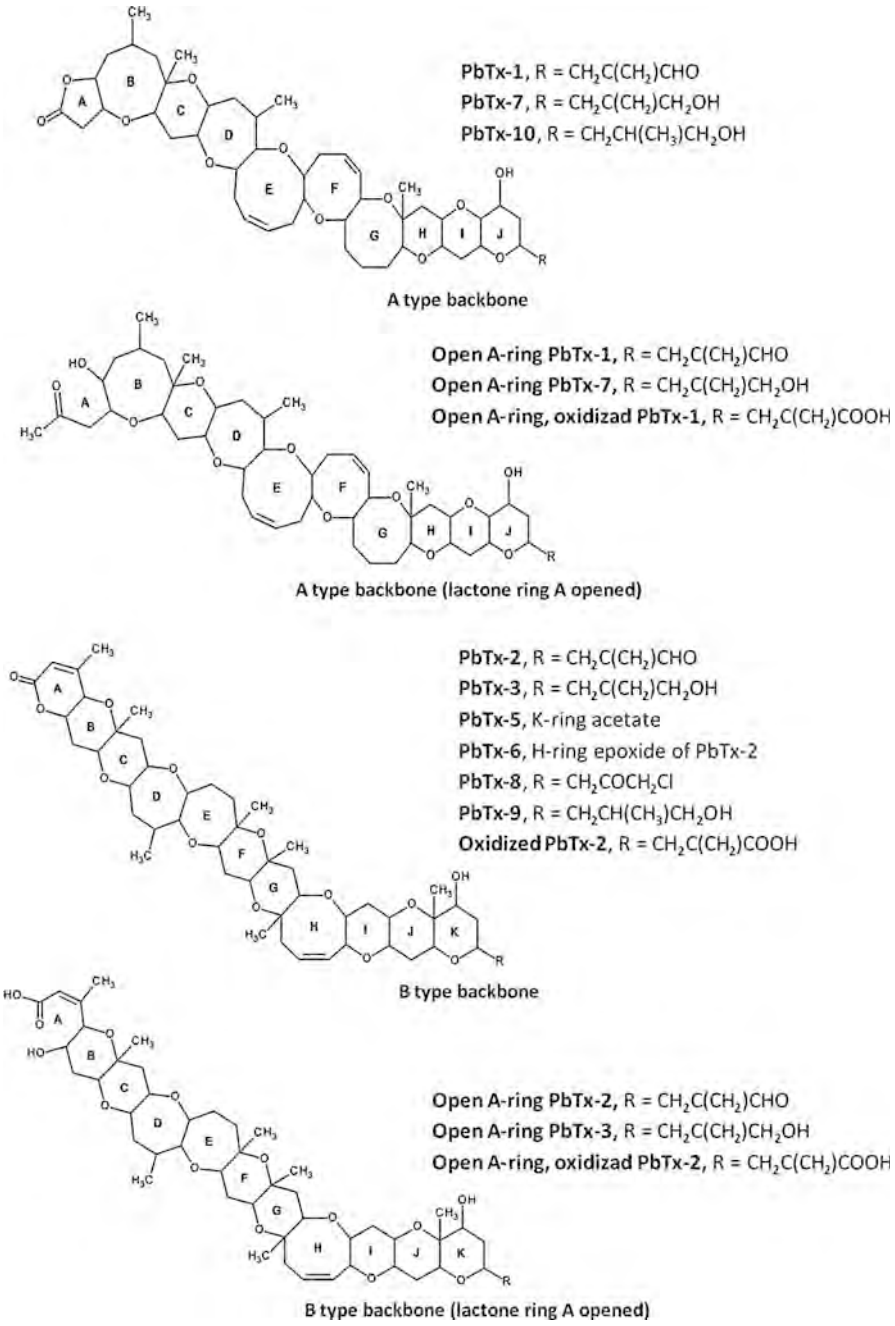

Fig. 1 Structure of PbTxs found in natural samples and cultures of *K. brevis*

K. brevis can also produce other related compounds called hemi-brevetoxins and brevenals. These toxins are characterized by a ring structure shorter than that of PbTxs and, for this reason, can be considered as incomplete products of PbTx biosynthesis. Specifically, hemi-brevetoxins present the same structure as the latter four rings of type-B backbone and brevenals have a backbone of five transfused ether rings (Fig. 2) (Ramsdell 2008).

PbTxs are metabolized by shellfish and various metabolites (resulting from oxidation, reduction, hydrolysis, and conjugation reactions) have been identified and structurally characterized. The B-type toxins are the most abundant in *K. brevis* cultures, and consequently BTX-B1 and BTX-B3 (metabolites of PbTx-2) were the first identified. Years later, BTX-B2 and *S*-desoxy-BTX-B2 were identified as the

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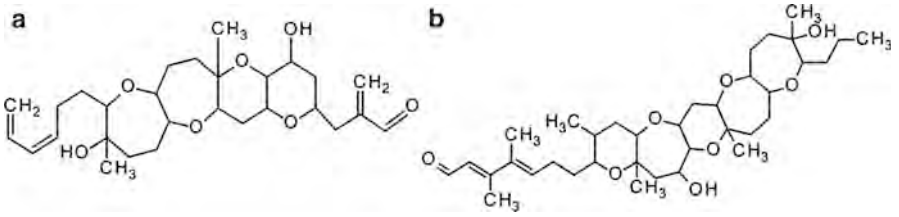

Fig. 2 Hemi-brevetoxin and brevenal structures. The backbone structure consists on four fused cyclic ether ring called hemi-brevetoxin (**a**) or five fused cyclic ether ring called brevenal (**b**)

major metabolites of PbTx-2 in Eastern oyster (*Crassostrea virginica)* in the Gulf of Mexico (Plakas and Dickey 2010).

Since 1844, *K. brevis* has been associated with high mortality in various marine species (Feinstein et al. 1955) such as fishes, aquatic birds (*Phalacrocorax auritus*, *Mergus merganser*, *Aythya affinis*), sea turtles (*Caretta caretta, Chelonia mydas,* and *Lepidochelys kempii*), invertebrates such as blue crabs (*Callinectes sapidus*) and lion's mane jellyfish (*Cyanea capillata*), and marine mammals, in particular bottlenose dolphins (*Tursiops truncates*) and manatees (*Trichecus manatus latriostris*) (Landsberg et al. 2009). Raphidophytes are also known to produce PbTxs and PbTx-like compounds. A massive mortality of fish in Japan, China, and Australia was caused by a bloom of *Chattonella marina*, one of the most toxic raphidophyte species. Using different chromatographic and analytic techniques, PbTx-like compounds have also been identified in *Chattonella antiqua*, *Heterosigma akashiwo,* and *Fibrocapsa japonica* (Shen et al. 2010).

Seagrasses (*Thalassia testudinum*) and filter-feeding shellfish, such as clams (*Macrocallista nimbosa* and *Mercenaria mercenaria*), whelks (*Busycon* sp.), and oysters (*Crassostrea gigas* and *virginica*), which do not exhibit adverse effects when exposed to PbTxs, can accumulate these toxins and act as vectors, leading to intoxication of aquatic species or humans (Flewelling et al. 2005).

Consumption of naturally contaminated organisms is the major risk factor for NSP, a syndrome that presents with gastrointestinal and neurological symptoms and an onset ranging from a few minutes to 18 h post ingestion. Nausea, diarrhea, vomiting, headache, tingling in the face, difficulty in speaking, mydriasis, paresthesia in the extremities, ataxia, loss of coordination, and bradycardia are symptoms most frequently reported. Neither chronic effects nor death have been recorded, and the majority of patients recover within 48–72 h (Watkins et al. 2008).

Although ingestion of contaminated foodstuffs represents the most common and dangerous route of exposure, respiratory exposure can also lead to negative health effects. PbTxs can become aerosolized from the water-air interface during wind- or wave-driven cell lysis and transported by onshore winds to the coast during significant red tides. Respiration of this aerosolized toxin can cause conjunctivitis, rhinorrhea, and bronchoconstriction (Kirkpatrick et al. 2004). Although rarely serious, such symptoms can result in reduced beach recreation and thus a significant loss of tourist revenues. Estimates of the economic effects of red tides in Florida have been estimated at $600,000–$700,000 annually (Hoagland et al. 2014).

Pharmacokinetics/Toxicokinetics

PbTx-3 is the most widely used of the brevetoxins in in vivo studies (Baden et al. 2005). Early toxicokinetic studies focused on characterization of distribution, metabolism, and elimination. The first toxicokinetic research in mammals was performed by Poli et al. (1990) following intravenous (i.v.) administration of the toxin in rats. One minute after administration of [^{3}H]-PbTx-3, less than 10 % of the

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administered toxin remained in circulation. After 30 min, toxin distribution was assessed as follows: about 70 % in skeletal muscle, 18 % in the liver, and 8 % in the intestinal tract. Smaller concentrations were found in the heart, kidney, testes, brain, spleen, and lung. Reverse-phase high-performance liquid chromatography (HPLC) analyses of muscle tissue extracts demonstrated only parent toxin in skeletal muscle, suggesting this tissue could be a storage compartment for the toxin. Conversely, metabolites were found in the feces and bile, suggesting that the toxin was metabolized and biliary excretion could be the most prominent route of elimination. Indeed, the elimination occurred predominantly through feces and secondarily through urine and was almost complete 3 days post exposure.

These results were then confirmed with intratracheal instillation of 2.6 μg [^{3}H]-PbTx-3/kg in mice. After 30 min, PbTx-3 was rapidly cleared from lungs and distributed to the liver, skeletal muscle, and gastrointestinal tract. The elimination of the toxin (almost complete after 4 days) occurred through feces (64 %) and urine (11 %). These results were subsequently confirmed with only minor differences in rats (Tibbetts et al. 2006).

Taken together, the results of these studies suggested that PbTx-3 accumulated mainly in the skeletal muscle tissue and underwent hepatic metabolism and biliary excretion. However, based upon the lipophilicity of these toxins, other accumulation compartments could not be excluded. For instance, in vivo and in vitro percutaneous absorption studies demonstrated that [^{3}H]-PbTx-3 accumulated in the derma after 4 h (Kemppainen et al. 1991). These studies are consistent with the hypothesis of brevetoxin association with cell membranes due to their inherent lipophilicity.

Other studies focused on the distribution of PbTxs in the blood compartment. Some results suggested that toxin could bind to plasma carrier proteins. Although PbTxs reached blood concentrations equal to 25–30 nM after 12 h following intraperitoneal (i.p.) injection in mice – values much higher than toxin concentrations active on the VGSC in vitro – no toxic effects were observed (Woofter et al. 2005). In fact, 39 % of [^{3}H]-PbTx-3 was bound to components in mouse plasma, of which about 7 % was associated with serum albumin. In the same research, the possible association between PbTxs and lipoprotein was studied in vitro by spiking mouse plasma with PbTx-3 (100 ng/ml) and in vivo by injecting mice i.p. with PbTx-3 (310 μg/kg). In both cases, a large amount of PbTx-3 was associated with plasma high-density lipoproteins (HDLs). Similarly, HDLs showed the highest binding capacity for PbTx; binding was approximately 50 % higher compared to that of low-density and very low-density lipoproteins (LDL and VLDL) in human plasma (Woofter and Ramsdell 2007). Thus, the lipophilic characteristics of the PbTx backbone make lipoproteins the most important carriers of PbTxs in plasma.

The interaction between PbTxs and serum proteins was further characterized by Wang and Ramsdell (2011) by studying possible binding of PbTx-3 to human serum albumin (HSA). Covalent and noncovalent interactions were found between toxin and protein; by liquid chromatography-mass spectrometry (LC-MS) analysis, the formation of an adduct made up of one or two toxin molecules and one HSA molecule was demonstrated.

Amino acid- and lipid-PbTx conjugates resulting from shellfish metabolism are identified in mammalian toxicity, although their involvement is not well characterized. Therefore, some studies focused on the distribution of these metabolites after i.v. administration in mice. Radiolabeled PbTx conjugates (the amino acid conjugate *S*-desoxy-BTX-B2 and the lipid conjugate *N*-palmitoyl-*S*-desoxy-BTX-B2) were administered i.v. to mice at sublethal doses. Tissue distribution and elimination of amino acid and lipid conjugates were compared to those of PbTx-3. After 48 h, elimination (via feces) was nearly complete. In contrast, amino acid and lipid conjugates presented different and peculiar tissue distribution and elimination patterns. *S*-desoxy-BTX-B2 was primarily concentrated in the kidney (8–22 % of the administered dose) and in the gastrointestinal tract 3 h after dosing, whereas *N*-palmitoyl-*S*-desoxy-BTX-B2 was concentrated in the liver and in the lungs after 3 and 12 h, respectively. In addition, the concentration of the lipid-PbTx conjugate in the spleen increased over time starting from 30 min post administration. Unlike

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PbTx-3, which was principally eliminated through feces, *S*-desoxy-BTX-B2 (amino acid conjugate) favored urinary elimination over fecal and the *N*-palmitoyl-*S*-desoxy-BTX-B2 (lipid conjugate) was eliminated in equal percentage through feces and urine. The unique tissue distribution and elimination patterns of these two metabolites are due to different structural characteristics resulting from different metabolic reactions occurring in shellfish (Leighfield et al. 2014).

Human pharmacokinetic and toxicokinetic data on PbTxs are still scarce. Poli et al. (2000) quantified these toxins by a radioimmunoassay (RIA) up to 4 days after the ingestion of contaminated seafood in the urine of two young children (ages 2 and 3 years) affected by NSP, suggesting that the toxin is mainly eliminated after 4 days post exposure. PbTx-2 and PbTx-3 were then identified in urine samples of patients by high-performance liquid chromatography-mass spectrometry (HPLC-MS).

Mechanism of Action

It has long been known that PbTxs bind to the voltage-gated sodium channels (VGSCs) in excitable cells (Poli et al. 1986). The VGSCs are transmembrane proteins formed by an α-subunit (220–260 kDa) and one or more auxiliary β-subunits (20–40 kDa), depending on the tissue of origin. The α-subunit is formed by four homologous transmembrane domains (I-IV), consisting of six transmembrane α-helices (S1–S6) connected by internal and external polypeptide loops. The S5 and S6 helices form the ion-conducting pore, while the S4 helix, which is positively charged and works as voltage sensor, is involved in function-related conformational changes. The β-subunits are not directly involved in ion conductance but serve to stabilize α-subunit conformations, allowing ion gating and ion fluxes. The VGSC can exist in three distinct states: activated, inactivated, or deactivated. Normally, when a membrane depolarization takes place, the VGSC opens its gates, allowing an intracellular flux of Na^+ ion. The transmembrane potential rises until the inactivation potential is reached, at which point the channel inactivates by closing the gate, the Na^+ flux is blocked, and membrane repolarization occurs until the normal resting potential is re-established. At that point the channel returns into its deactivated state with closed gates (Zhang et al. 2013).

PbTx binding to the VGSC leads to (a) a shift of the voltage dependence of activation to a lower membrane potential, such that, in the presence of toxin, the channel opens under conditions in which it is normally closed; (b) an inhibition of channel inactivation, leading to a sustained channel activation; (c) an increase in mean open times; and (d) multiple subconductance states. Such effects alter the membrane properties of the excitable cells, and this represents the basis of the toxic effects of this group of toxins (Zhang et al. 2013).

The binding of PbTxs to VGSCs in excitable tissues was characterized only after the development of the first radiolabeled probe, [^{3}H]-PbTx-3, synthesized in 1984 by Baden's group by the reduction of PbTx-2 to PbTx-3 using NaB^3H_4. In this manner, Poli and coworkers carried out competition experiments in rat brain synaptosomes using [^{3}H]-PbTx-3 in association with other natural toxins that specifically bind to sites 1–4 on the VGSCs. They found that PbTx-3 did not compete for any of the known sites on the channels but instead bound to a new site, denoted as neurotoxin receptor site 5 (Poli et al. 1986). Further studies in rat brain synaptosomes (Trainer et al. 1991) and purified VGSCs from rat brain (Trainer et al. 1994) have confirmed that PbTxs act on site 5 on the α-subunit of VGSC with a "head-down" orientation, with the A-ring extending toward the intracellular opening and the J or K-ring near the extracellular surface of the pore. The maximum binding capacity occurs when PbTxs are in a 1:1 stoichiometry with VGSCs.

The A and B backbones present differences in the internal ring systems, whereas they share a lactone in the A ring ("head") and a conserved structure on the "tail" ring. Thus, it was hypothesized that these

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common elements should play important roles in the interaction with the receptor. Various structure-receptor studies were conducted in order to define the relevant elements for this interaction. The favorite conformations were investigated using the Monte Carlo method. It was discovered that the "straight" shape is preferred with respect to the "bent" one, and the optimal conformation is a "cigar-shaped" molecule 30 Å in length (Rein et al. 1994a).

The opening of the A-ring of PbTx-3 to an A-ring diol, by reductive cleavage, induced loss of potency in bioassay and in the binding to VGSCs in nodose ganglia of newborn rats. The A-ring diol did not competitively inhibit radiolabel binding and it was not toxic to mosquito fish (Rein et al. 1994b).

The truncated form of PbTx-2, lacking the BCDE rings (10 Å shorter), did not inhibit [^{3}H]-PbTx-3 binding in rat brain membranes and was nontoxic to mosquito fish. On isolated VGSCs, the truncated product did not prolong the mean open times, did not inhibit channel activation, and produced only a minor shift of the activation potential (Gawley et al. 1995).

In contrast, the desoxy form of PbTx-3, which lacks the carbonyl oxygen in C1 on the A-ring, inhibited [^{3}H]-PbTx-3 binding and was toxic to mosquito fish (Gawley et al. 1995).

Other studies demonstrated that polar substitutions in the K-ring R group such as the cysteine conjugate BTX-B2 and its desoxy derivative led to a marked decrease of the affinity for the receptor in brain, skeletal muscle, and heart cells (Dechraoui et al. 2007). On the other hand, three different derivatives of PbTx-3 with hydrophobic substitutions on K-ring side chain (a benzoyl derivate and the α- and β-napthoyl derivates) showed an increased affinity for the receptor. All of these displaced [^{3}H]-PbTx-3 from the receptor site, suggesting that these K-ring derivatives do not affect the ability of the toxin to bind to its receptor. Further, the affinity of two derivatives (benzoyl and β-napthoyl derivatives) for the receptor is higher than that of [^{3}H]-PbTx-3, and the benzoyl derivate was able to activate the channel. In contrast, the α- and β-napthoyl derivates did not activate the channel but demonstrated antagonism (Purkerson-Parker et al. 2000).

Taken together, these results suggest that an intact A-ring and the full length are necessary for the biological activity of these toxins. Moreover the conformation of the backbone, the presence of the carbonyl oxygen in the A-ring, and the size and polarity of the K-ring side chain are critical for the binding and for the development of the biological effects (Poli 2002; Ramsdell 2008).

Toxicity

Human Toxicity

PbTxs and their metabolites cause NSP after ingestion of contaminated shellfish, in particular clams, oysters, and whelks. Human poisonings have occurred only on the West Coast of Florida, North Carolina, and New Zealand. Clinically similar to ciguatera, but less severe, the syndrome is a combination of gastrointestinal (nausea, vomiting, diarrhoea, and abdominal pain) and neurological (paraesthesia, headache, muscle weakness, myalgia, incoordination, tingling in the face, difficulty speaking, reversal of hot-cold temperature sensation, vertigo, double vision, and mydriasis) effects. Other effects are bradycardia, respiratory difficulties, and coma. Symptoms appear from 30 min to 3 h post ingestion and recovery is typically complete in 2–3 days. Fortunately, no lethalities or chronic outcomes related to NSP have been reported (Isbister and Kiernan 2005).

Cases of human intoxications are fortunately rather rare, undoubtedly due to extensive monitoring programs in Florida and other Gulf states. The regions mostly involved appear to be the Gulf Coast of the United States and New Zealand. A major event in the United States occurred in North Carolina in 1987 when a strong inshore eddy of the Gulf Stream carried *K. brevis*-laden water from Florida northward to North Carolina, where the organism propagated and caused an extensive red tide. Forty-eight people

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presented classic symptoms of NSP after consuming contaminated shellfish. Common symptoms included paresthesia, vertigo, malaise, and abdominal pain (Morris et al. 1991). In 1992, 180 cases of NSP were documented in New Zealand, but this wide-ranging outbreak involved other marine toxins as well as PbTxs (Watkins et al. 2008). The ingestion of lightning whelks (*Busycon contrarium*) led to another NSP outbreak in Florida in 1996. Whelks were collected from Saratosa Bay and then consumed by three family members, a father and two young children. The children showed the most severe symptoms: loss of consciousness, convulsions, vomiting, and tachycardia. PbTxs were confirmed by analysis of patient urine collected upon hospital admittance. The analysis confirmed the presence of 42 (±2) ng/ml PbTxs-like toxins (most likely metabolites) in one patient and 117 (±30) ng/ml in the other (Poli et al. 2000).

Additional cases occurred again in Florida in 2005 and in 2006 after a long-lasting red tide bloom. Paresthesia, muscle weakness, and nausea were the most common symptoms. In addition, some patients reported blurred vision, uncontrollable muscle dysfunction, and psychotic-like outburst (Watkins et al. 2008). Southwest Florida is currently considered the region with the highest risk of health hazards related to red tides.

Although oral exposure carries the greatest risk, people can be exposed to these toxins through the respiratory route as well. Indeed, *K. brevis* cells are relatively fragile, so during a red tide the breaking waves may cause cell membrane lysis and the release of PbTxs into seawater. The combined action of wind and surf can produce a contaminated sea-salt spray which can be inhaled by beachgoers during a coastal bloom. In this case, symptoms are conjunctive irritation, rhinorrhoea, nonproductive cough, and bronchoconstriction. Volunteers exposed to contaminated seawater, sprayed as an aerosol into their nose and throat, showed cough and a burning sensation similar to that experienced by affected people on the beach during blooms. In healthy individuals, the symptomatology rapidly disappears after leaving the beach area or entering an air-conditioned area. Asthmatics may be more susceptible. The susceptibility of patients with preexisting airway disease to PbTxs was suggested by recent in vivo experiments on aerosolized red tide toxins. Specifically, inhaled PbTxs induced a more severe bronchospasm in asthmatic sheep with respect to normal sheep (Kirkpatrick et al. 2004).

A no observable effect level (NOEL) for PbTxs in humans has not yet been determined. A maximum allowable level in shellfish (20 mouse units (MU)/100 g of shellfish tissue) has been established by the Food and Drug Administration (FDA) (Watkins et al. 2008).

Experimental Toxicity

In Vivo Toxicity After Single Administration

Baden and Mende reported in 1982 the first data concerning the acute toxicity in mice of PbTxs isolated from *Gymnodinium breve* (later known as *Ptychodiscus brevis,* and then *Karenia brevis*). The LD_{50} for PbTx-2 was 200 μg/kg, 200 μg/kg, and 6,600 μg/kg after i.p, i.v., and oral exposure, respectively. For PbTx-3 the LD_{50} values obtained were 170 μg/kg, 94 μg/kg, and 520 μg/kg after i.p., i.v., and oral exposure, respectively (Kirkpatrick et al. 2004).

Mice treated i.p. with PbTx-3 presented with SLUD syndrome: hypersalivation, lachrymation, excessive urination, and defecation. Orally, PbTx-3 caused tremors followed by marked muscular contractions or muscle twitch, straub tail, labored breathing, and death. After i.p. and oral administrations, symptoms appeared in 30 min and 5 h respectively, while by i.v. administration, they were immediate (van Apeldorn et al. 2001). In i.v. treated anesthetized cats, bradycardia, hypotension, and bradypnea were observed, abolished by vagotomy or administration of atropine (van Apeldorn et al. 2001).

The acute toxicity of PbTx metabolites isolated from shellfish has been investigated after i.p. administration to mice. The minimum lethal doses (MLD) of the taurine conjugate BTX-B1 and the

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cysteine sulfoxide conjugate BTX-B2 were 50 μg/kg and 306 μg/kg respectively. Moreover, LD_{50} values were 400 and 211 μg/kg for BTX-B2 and S-desoxy-BTX-B2, respectively (Plakas and Dickey 2010). BTX-B1 caused immediate irritability, followed by hind-quarter paralysis, dyspnoea, salivation, lachrymation, urination, defecation, and death from respiratory paralysis; BTX-B2 and BTX-B4 caused hind limb paralysis, diarrhoea, dyspnoea, and convulsions (van Apeldorn et al. 2001).

The side-chain fatty acid conjugate BTX-B4 was threefold more toxic than BTX-B2 with a MLD i.p. in mice of 100 μg/kg. The D-ring fatty acid conjugate BTX-B3 was not toxic after i.p. administration at doses up to 300 μg/kg. The MLD of BTX-B5 was 300–500 μg/kg (Plakas and Dickey 2010).

In Vivo Toxicity After Repeated Administration

There are few studies of toxicity after repeated exposure to PbTxs. Some studies were conducted in rats after inhalation of PbTx-3. Male F344/Crl/Br rats were exposed to 500 μg PbTx-3/m^3 by nose-only inhalation for 0.5 or 2 h/day, for 5 consecutive days. Calculated deposited PbTx-3 doses for the low- and high-dose groups were 8.3 and 33 μg/kg/day, respectively. At the end of the exposure, a reduction in body weight was noted but only at the highest dose. Neither gross or microscopic lesions nor cytotoxicity were observed in all tissues examined. Moreover, no inflammation in bronchioalveolar lavage fluid was observed. However, alveolar macrophages showed some evidence of activation and the humoral-mediated immunity was suppressed, as indicated by a >70 % reduction in splenic plaque-forming cells. These results suggested that immune system dysfunction might result from respiratory PbTx exposure. A second set of experiments expanded the exposure time to 22 consecutive days, with calculated deposited PbTx-3 doses of 0.9 and 5.8 μg/kg/day for the low- and high-dose groups, respectively. Similar results were found: no clinical signs of toxicity, no histopathological lesions in the nose, brain, liver, or bone marrow but reduced body weight in both groups, suppression of humoral-mediated immunity, as well as minimal alveolar macrophage hyperplasia and an increase in blood reticulocytes. This indicated again the immune system as a potential target after chronic PbTx inhalation (Benson et al. 2004, 2005).

Recently, effects of repeated inhalation exposure to aerosolized PbTx-3 in sheep were investigated in order to delineate putative mechanisms leading to pulmonary dysfunction. Groups of nonallergic and allergic sheep were exposed to 20 breaths of increasing concentrations of PbTx-3 (30–300 pg/ml) for 4 consecutive days. Both groups developed airway hyper-responsiveness 1 day after challenge, with a concentration-dependent severity, particularly marked in the allergic group. Both groups developed an inflammatory response after exposure to 300 pg/ml of PbTx-3, with a prominent lung neutrophilia, that persisted for 7 days (Zaias et al. 2011).

Other Toxic Effects

PbTxs represent a risk during times of blooms for a variety of species. During or immediately subsequent to *K. brevis* blooms, mortalities have been reported in fishes, sea turtles (*Caretta caretta, Chelonia mydas,* and *Lepidochelys kempii*), sea birds, particularly double-crested cormorants (*Phalacrocorax auritus*), red-breasted mergansers (*Mergus merganser*), and lesser scaup (*Aythya affinis*), and marine mammals, such as bottlenose dolphins (*Tursiops truncates*) and manatees (*Trichecus manatus latriostris*). PbTxs are potent ichthyotoxins; fish are killed by the absorption of toxin through the gills, by ingestion of *K. brevis* cells or toxins present in the water, or by consumption of other species acting as vectors in the food chain (Landsberg et al. 2009). Affected fish show violent defecation and regurgitation, twisting and corkscrew swimming, pectoral fin paralysis, caudal fin curvature, loss of equilibrium, quiescence, vasodilatation, convulsions, and death due to respiratory failure. Chronically intoxicated fish show little pathology aside from slight hemolysis, detected via anemia, cyanosis, viscous blood, splenomegaly, hepatic hemosiderosis, and dehydration (Landsberg 2002).

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In marine mammals, the most likely lethal exposure route is ingestion. However, in some cases, inhalation of aerosolized PbTxs has been reported. Manatees exposed to PbTxs displayed severe nasopharyngeal, pulmonary, hepatic, renal, and cerebral congestion; rhinitis, pulmonary hemorrhage, and edema. Multiorgan hemosiderosis and nonsuppurative leptomeningitis were also seen (Bossart et al. 1998).

More recently, the mutagenic and genotoxic potential of PbTxs have been investigated. In the human T-lymphocyte Jurkat E6-1 cell line, PbTx-2, −3, and −6 induced DNA damage in the comet assay (Murrell and Gibson 2009). PbTx-2, −3, and −9 were clastogenic in human lymphocytes, inducing both single-strand and double-strand DNA breaks. Chromosomal aberrations were also observed in CHO-K1-BH4 Chinese hamster ovary cells. In human monocytes, PbTx-2 caused a depletion of glutathione in U-937 human monocyte cells, suggesting that they may be subjected to oxidative stress. Since PbTx-2 is largely metabolized, oxidative damage may derive from cytochrome P450-mediated metabolism by formation of nucleophilic intermediates potentially able to form covalent bonds with DNA. For instance, PbTx-2 forms DNA adducts with cytidine in isolated lung cells and forms DNA adducts with adenosine and guanosine in lung tissue from rats after intratracheal exposure. However, not every DNA adduct results in a mutation; normally they are removed and repaired. PbTx-2 was clastogenic in rats dosed intratracheally but did not show mutagenic potential at the SP-98/100 Ames test (Leighfield et al. 2009).

Recently, Tian and coworkers (2011) reported that, in medaka fish exposed to PbTx-1, the abundances of 14 and 24 different proteins were significantly altered in the gills and brains, respectively. The altered proteins were categorized into diverse functional classes such as cell structure, macromolecule metabolism, signal transduction, and neurotransmitter release. Since PbTxs are known to be absorbed across gill membranes and exert their acute toxic effects in neuronal tissue, these findings can open the way to elucidate the possible pathways by which these toxins exert their ichthyotoxicity in marine organisms.

Treatment

Since NSP shares symptomatology with other diseases such as food allergy, ciguatera fish poisoning, paralytic shellfish poisoning, or pesticide poisoning, NSP diagnosis is challenging. Inquiring about recent food consumption is crucial. To verify the diagnosis, analysis of meal remnants or samples taken from the same harvesting area should be performed. The collection and analysis of urine samples within few hours of intoxication can be used to confirm the presence of PbTxs (Poli et al. 2000; Watkins et al. 2008).

There is no specific treatment for NSP other than supportive care. Analgesics, fluid and electrolyte replacement, and respiratory support should be considered. In the case of ingestion, gastrointestinal decontamination with activated charcoal within the first 4 h may be helpful.

In the case of exposure to aerosolized PbTxs, the respiratory irritation may be prevented by the use of particle filter masks or avoidance of the contaminated area. Air-conditioned environments will provide relief from the airborne irritation. Antiasthmatic medications may be useful for people with asthma and for other susceptible people exposed to aerosolized PbTxs in the event of a bronchoconstrictive response (Kirkpatrick et al. 2004).

Recently, a natural compound, produced by *K. brevis*, named brevenal, has been identified as an antagonist of PbTx. A potential employment of this molecule as therapeutic agent in the treatment of NSP is conceivable, but so far, its use against inhalation toxicity has been evaluated only in sheep (Watkins et al. 2008).

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Conclusion and Future Directions

Currently, the best weapon against NSP remains prevention. A NOEL for PbTxs in humans has not yet been established, but the FDA has defined a regulatory level of 80 mg PbTx-2/100 g of shellfish tissue (20 mouse units (MU)/100 g). Studies conducted in the 1960s related the incidence of death in mice injected with crude extracts of shellfish to the incidence of human illness. Since then, the mouse bioassay has become a critical tool in risk assessment (van Apeldorn et al. 2001). Since the 1980s, the Florida Department of Natural Resources has employed a monitoring and control program for *K. brevis* consisting of both cell concentrations in the water column and the mouse bioassay. Shellfish beds are closed when *K. brevis* concentrations reach or exceed 5,000 cells/L and remain closed until cell concentrations drop below 5,000 cells/L and the results of MBA fall below 20 MU/100 g shellfish tissue. In Florida, approved harvesting zones are monitored by sampling the predominant commercial shellfish species (oysters, clams, and mussels). Unmonitored and unregulated species are prohibited to shellfish harvesters (Watkins et al. 2008). Because the mouse bioassay is slow and requires the use of animals, immunological assays (such as ELISA) and instrumental methods of analysis (such as LC-MS) are being validated as replacement techniques (Plakas and Dickey 2010).

Cross-References

► Ciguatoxin and Ciguatera
► Saxitoxin and Other Paralytic Toxins: Toxicological Profile

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Spirolides and Cyclic Imines: Toxicological Profile

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Abstract

In this chapter, available evidence on the toxicological profile of spirolides and other lipophilic cyclic imine toxins is reviewed, highlighting their chemical structure, the phytoplankton species involved in their production, their pharmacokinetics/toxicokinetics and experimental toxicity, and their molecular targets and mechanisms of action. These phycotoxins belong to an emerging class of chemical agents associated with marine algal blooms and shellfish toxicity. Their chemical structure is represented by a macrocycle, with the ring size between 14 and 27, and two conserved features that include the cyclic imine group and spiroketal ring system. The producers of spirolides, gymnodimines, and pinnatoxins have been identified as being the dinoflagellates *Alexandrium ostenfeldii*/*peruvianum*, *Karenia selliformis*, and *Vulcanodinium rugosum*. Their acute toxicity, appraised by the mouse bioassays, classifies them as "fast-acting" toxins because they induce rapid onset of neurological symptoms followed by death within a few minutes. The spirolide congeners are the most toxic after intraperitoneal injection, while there are indications that pinnatoxins are the most toxic group after oral administration. The neurotoxic effects reported for these phycotoxins are mostly due to their specific interaction with the muscle and neuronal types of nicotinic acetylcholine receptors which are the principal molecular targets of spirolides, gymnodimines, and pinnatoxins, so far studied. Hence, these phycotoxins exhibit both high affinity and broad specificity on nicotinic receptors, indicating that their sites of interaction in the receptors include amino acid residues highly conserved among animal species.

Introduction

Spirolides were first discovered in the 1990s from the Atlantic coast of Nova Scotia in Canada when unusual toxicities were detected by mouse bioassay in mussel and scallop extracts. Since then, the spirolides have been found to have a worldwide distribution, with a remarkable diversity in their chemical profiles. Spirolides belong to the so-called cyclic imine group of neurotoxins that also include the gymnodimines, pinnatoxins, pteriatoxins, portimine, spiro-prorocentrimine, and prorocentrolides (for a recent review, see Stivala et al. 2015). The generic chemical structure of the cyclic imine toxins is represented by a macrocycle, with the ring size between 14 and 27, and two conserved features that include two subunits: the cyclic imine group (which in most cases is found as a spiroimine) and the spiroketal ring system. The cyclic imine, which has been shown to be a key pharmacophoric feature, can

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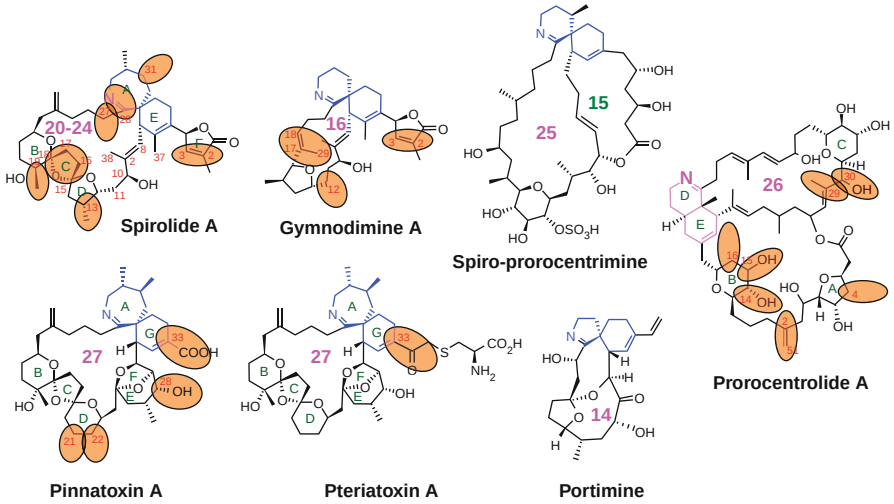

Fig. 1 Chemical structures of the spiroimine toxins. For each subfamily, the first member is shown, and the positions for which variations in the substitution pattern were reported are numbered and highlighted in orange. The characteristic spiroimine and cyclic imine features are colored in blue and magenta, respectively, and the size of the macrocyclic ring system in each subfamily is shown in magenta

be a five-membered (portimine), six-membered (gymnodimines, spiro-prorocentrimine, prorocentrolides), or seven-membered (spirolides, pinnatoxins, pteriatoxins) ring that in many cases is substituted with one or two methyl groups. The spiroketal cyclic ether ring system can be a simple tetrahydrofuran (i.e., portimine, gymnodimine) or tetrahydropyran group (i.e., spiro-prorocentrimine, prorocentrolides), but also a more complex 6,5- (spirolides H and I), 6,6,5- (spirolide G), 6,5,5- (spirolides A–F), or 6,5,6-spiroketal (pinnatoxins, pteriatoxins) (Fig. 1).

Spirolides and the cyclic imine toxins studied so far are "fast-acting" phycotoxins, because of the rapid beginning of neurological symptoms and the short time to death. The basis for these neurotoxic effects is in direct relation to their molecular antagonist action on nicotinic acetylcholine receptors. In this chapter, the authors' current understanding of the toxicological profile of spirolides and other cyclic imine toxins is summarized, with emphasis on their chemical structure, producing organisms, pharmacokinetics/toxicokinetics, experimental toxicity, molecular targets, and mechanism of action.

Spirolides

Chemical Characteristics of Spirolide Toxins

Spirolides constitute the largest family among cyclic imine toxins. As shown in Fig. 2, to date 16 spirolide analogues have been reported, which can be classified into four distinct groups according to the structure of the spiroimine and spiroketal ring systems. The first group of spirolides has ten members with a characteristic 6,5,5-spiroketal ring system. As compared to spirolides A and B, the spirolides C and D and their analogues have an extra methyl group at the position 31 on the imine ring, which is believed to render them more resistant to enzymatic and acidic hydrolysis (Christian et al. 2008). Spirolides A and C and their analogues have a double bond between the positions 2 and 3 on the lactone ring which is not present in the structure of spirolides B and D and their derivatives. Other positions where structural variability was observed for spirolides C and D are 13 and 19 on the spiroketal ring system, with the presence or absence of methyl substituents, and 27, where hydroxy and keto substituents were evidenced in some cases. The second spirolide group contains the biologically inactive spirolides E and F, which present the same

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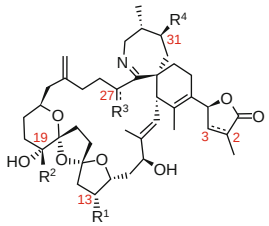
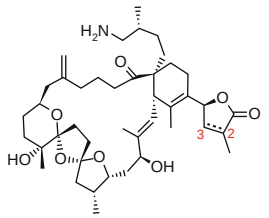
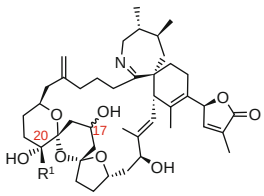


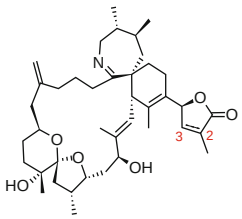
Spirolide	Δ	R^1	R^2	R^3	R^4
Spirolide-A	$\Delta^{2,3}$	CH_3	CH_3	H	H
Spirolide-B	–	CH_3	CH_3	H	H
Spirolide-C	$\Delta^{2,3}$	CH_3	CH_3	H	CH_3
Spirolide-D	–	CH_3	CH_3	H	CH_3
13-desmethyl spirolide C	$\Delta^{2,3}$	H	CH_3	H	CH_3
13-desmethyl spirolide D	–	H	CH_3	H	CH_3
13,19-didesmethyl spirolide C	$\Delta^{2,3}$	H	H	H	CH_3
27-hydroxy-13 desmethyl spirolide C	$\Delta^{2,3}$	H	CH_3	OH	CH_3
27-hydroxy-13,19-didesmethyl spirolide C	$\Delta^{2,3}$	H	H	OH	CH_3
27-oxo-13,19-didesmethyl spirolide C	$\Delta^{2,3}$	H	H	O=	CH_3



Spirolide	Δ
Spirolide-E	$\Delta^{2,3}$
Spirolide-F	–



Spirolide	R^1
Spirolide-G	H
20-methyl spirolide G	CH_3



Spirolide	Δ
Spirolide-H	$\Delta^{2,3}$
Spirolide-I	–

Fig. 2 Chemical structures of the known spirolide toxins

chemical features as spirolides A and B, respectively, except for the cyclic imine that becomes an acyclic aminoketone (reviewed by Guéret and Brimble 2010). These structural changes suggest that they are probably shellfish metabolites of spirolides A and B. The third spirolide group, represented by spirolide G and 20-methyl spirolide G, has a 6,6,5-spiroketal ring system with a hydroxyl group at C17 for which the stereochemistry could not be determined (MacKinnon et al. 2006) and a methyl substituent at C20 that is present only in the second member of this group. The rest of the structure is identical with spirolide C, with the dimethyl substituted seven-membered cyclic imine and the unsaturated lactone ring. The fourth spirolide group includes spirolides H and I whose structures are identical to spirolides C and D, respectively, apart from the presence of a 6,5- instead of the 6,5,5-spiroketal ring system (Roach et al. 2009). Spirolide H is the 2,3-unsaturated counterpart of spirolide I.

Spirolide-Producing Organisms

The producers of spirolides and other cyclic imine toxins were identified as dinoflagellates, a large and diverse group of marine eukaryotic microalgae known to be the major causative agents of harmful algal blooms. Although many dinoflagellate species can produce various natural toxins that impact humans, those involved in the production of cyclic imine toxins are limited to mainly *Alexandrium*, *Karenia* (formerly *Gymnodinium*), *Vulcanodinium*, and *Prorocentrum* species (for reviews, see Molgó et al. 2014; Stivala et al. 2015).

All known spirolides have been so far linked to *Alexandrium* species, and differences in spirolides content and profiles have been reported for dinoflagellate strains from different coastal regions. Hence, the globally distributed species *Alexandrium ostenfeldii* (Cembella et al. 1999) was formerly identified as the biological source of all known spirolides (Table 1). However, during the past six years, another dinoflagellate species, *Alexandrium peruvianum*, was also recognized as the producing organism for some spirolide congeners in the Northwestern Mediterranean Sea waters, Irish coastal waters (Touzet et al. 2008), and US coastal waters (see Table 1). It is worth noting that spirolides E and F were not detected in dinoflagellates, but were attributed to be metabolically modified products present in shellfish (Hu et al. 1996).

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Table 1 Dinoflagellate species known to produce spirolides, their location, and produced compounds

Alexandrium ostenfeldii	Canada	Spirolides A, B, C, C2, C3, D, D2, D3, H, and I
		13-Desmethyl spirolides C and D
	Norway	20-Methyl spirolide G
	Denmark	Spirolides D and G
		13-Desmethyl spirolides C and D
		13,19-Didesmethyl spirolide C
	Italy	Spirolide D
		13-Desmethyl spirolides C and D
		13,19-Didesmethyl spirolide C
		27-Hydroxy-13,19-didesmethyl spirolide C
		27-Oxo-13,19-didesmethyl spirolide C
		27-Hydroxy-13-desmethyl spirolide C
	Scotland	Spirolide C
	Ireland	Spirolides C and D
		13-Desmethyl spirolide C
		20-Methyl spirolide G
Alexandrium peruvianum	Mediterranean Sea	Spirolides B, C, and D
		13-Desmethyl spirolides C and D
	Ireland	13-desmethyl Spirolides C and D
	United States	Spirolide D
		13-Desmethyl spirolides C and D

Pharmacokinetics/Toxicokinetics of Spirolide Toxins

Among the spirolides, both 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C have been shown to cross a Caco-2 cell monolayer (Espiña et al. 2011). Caco-2 trans-epithelial permeability assays represent a good model to predict the intestinal absorption of drugs and toxins. When administered orally with a single nonlethal dose to mice deprived of food (24 h before, but given a nutritive solution with salts and glucose), both spirolides were detected in blood and urine samples after 15 min and 1 h of administration, respectively, as revealed by liquid chromatography-tandem mass spectrometry (Otero et al. 2012). The 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C were also detected 1 h after intraperitoneal (i.p.) administration in urine and trace amount of both toxins in blood; after 24 h important amounts were detected in feces. These results indicate that spirolides are absorbed when administered orally, and are excreted through the urine and feces. The histological evaluation by light microscopy of the heart, kidneys, liver, spleen, stomach, and intestines, performed 24 h and 7 days after oral administration, did not reveal any observable alteration (Otero et al. 2012). Further studies, using ultra-performance liquid chromatography-mass spectrometry and proton magnetic resonance spectroscopy, have shown that 13-desmethyl spirolide C is found in the brain a few minutes after its intraperitoneal injection to 3xTg mouse (a well-known model of Alzheimer's disease) and remains measurable even 24 h post-administration, indicating that the spirolide traverses the blood-brain barrier. Furthermore, a decrease in the intracellular amyloid beta levels in the hippocampus of treated 3xTg-mice was observed, emphasizing the positive effects of the molecule in this mouse model (Alonso et al. 2013).

Toxicity of Spirolide Toxins

Although in vitro cytotoxicity tests have been used, the most appropriate assays for appraising the toxicity of cyclic imine toxins and estimating their risk to human health are in vivo methods. Hence, to date, most if not all studies regarding the acute toxicity of cyclic imines have been performed using mainly the mouse

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Table 2 The 50 % lethal doses (LD_{50}) of spirolide congeners according to their administration route

Spirolide	Administration mode	LD_{50} (mg/kg mouse)	Ratio (compared to i.p.)
Spirolide A	Intraperitoneal injection	0.037	1
	Gavage	0.24–0.55	6.5–14.9
	Feeding	1.2–1.3	32.4–35.1
Spirolide B	Intraperitoneal injection	0.099	1
	Gavage	0.440	4.4
Spirolide C	Intraperitoneal injection	0.008	1
	Gavage	0.053–0.18	6.6–22.5
	Feeding	0.5–0.78	62.5–97.5
13-Desmethyl spirolide C	Intraperitoneal injection	0.007–0.028	1
	Gavage	0.13–0.16	4.6–22.9
	Feeding	0.5–1	17.9–142.9
13,19-Didesmethyl spirolide C	Intraperitoneal injection	0.032	1
27-Hydroxy-13-desmethyl spirolide C	Intraperitoneal injection	>0.027	1
27-Oxo-13,19-didesmethyl spirolide C	Intraperitoneal injection	>0.035	1
Spirolide E	Intraperitoneal injection	>1.000	1
Spirolide F	Intraperitoneal injection	>1.000	1
20-Methyl spirolide G	Intraperitoneal injection	0.008–>0.063	1
	Gavage	0.088–0.16	<1.4
	Feeding	0.5–0.63	<7.9

bioassays – with synthetic toxins or toxins extracted from either cultured dinoflagellates or contaminated shellfish, administrated to animals by i.p. injection, gavage administration, or voluntary consumption (feeding) – and determining their 50 % lethal dose (LD_{50}) values. It is worth noting that cyclic imine oral administration, with feeding taking priority over gavage, is a more relevant method since, first, it closely reproduces the situation in humans, and second, it avoids the use of high concentrations of solvent (i.e., ethanol) to dissolve the toxins (for reviews, see Molgó et al. 2014; Stivala et al. 2015).

Spirolides, as the other cyclic imines, are classified as "fast-acting" toxins because they induced rapid onset of neurological symptoms – including hyperactivity, jumping, piloerection, hyperextension of the back, stiffening and arching of the tail toward the head, tremors progressing to spasms, as well as skeletal muscle paralysis and extension of the hind limbs, respiratory distress with marked abdominal breathing, tremors of the whole body, and severe dyspnea – followed by death due to respiratory arrest within 3–50 min, whatever the route of administration is. It is worth noting that animals that did not die usually fully recovered with no detectable long-term effects, confirming that the cyclic imine toxins are rapidly detoxified and/or excreted (see above).

The LD_{50} of some spirolide congeners, after i.p. injection to mice, revealed that these compounds are the most toxic among cyclic imines toxins. In particular, spirolide C and, although contested (Munday et al. 2012a; Otero et al. 2012), 13-desmethyl spirolide C and 20-methyl spirolide G were the most toxic with LD_{50} values as low as 0.007–0.008 mg/kg mouse, followed by 13,19-didesmethyl spirolide C and spirolide A (with LD_{50} of 0.032 and 0.037 mg/kg mouse, respectively). The other spirolide congeners exhibit an apparently much lower toxicity (Table 2). Interestingly, the comparison of the acute toxicity of spirolides and their chemical structure points out the importance of some structural requirements, such as the imine ring with the C31 methyl group and the methyl group at C19, for the toxicological potency of the molecule. Although showing similar rank orders in the acute toxicity response, the LD_{50} of spirolide congeners after oral administration to mice *via* gavage are higher than those by i.p. injection and even higher when administered by feeding (Table 2). The significant reduction in oral toxicity *versus*

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i.p. administration of pure spirolide congeners is consistent with the absence of harmful effects in humans consuming shellfish contaminated with these cyclic imine toxins, with the exception of nonspecific symptoms, such as gastric distress and tachycardia, reported in Canada (Richard et al. 2001). However, the risk to human health of spirolide accumulation in shellfish is evidenced by the two following findings. First, the LD_{50} of lipophilic shellfish extracts, containing mainly 13-desmethyl spirolide C, was shown to be higher after i.p. than oral administration to mice (Richard et al. 2001), and second, examination of the brains of mice and rats after i.p. injection with this spirolide congener revealed neuronal damage in the hippocampus and brain stem, with increased mRNA levels of muscarinic and nicotinic acetylcholine receptors (Gill et al. 2003).

Molecular Targets and Mechanism of Action of Spirolide Toxins

From the large number of compounds found in nature known to interact with nicotinic acetylcholine receptors (nAChRs), including a number of alkaloids and peptides from various origins, only a few are known to be produced by dinoflagellates. Dinoflagellate toxins known to interact with nAChRs include the spirolides, gymnodimines, and pinnatoxins (for a recent review, see Stivala et al. 2015).

The nAChRs are ligand-gated ion channels mediating fast neurotransmission in the central and peripheral nervous systems (reviewed in Corringer et al. 2000; Albuquerque et al. 2009). At the skeletal neuromuscular junction, the nAChR is a pentamer consisting of two α1-subunits, single β1- and δ-subunits, and either a γ or a ε subunit in the embryonic or adult receptor-type, respectively (Fig. 3). The neuronal nAChRs in the peripheral and central nervous systems are composed of pentameric combinations of α and β subunits and exhibit a great heterogeneity in subtypes. To date, nine nAChR α subunits (α2–α10) and three nAChR β subunits (β2−β4) have been cloned from distinct animal species.

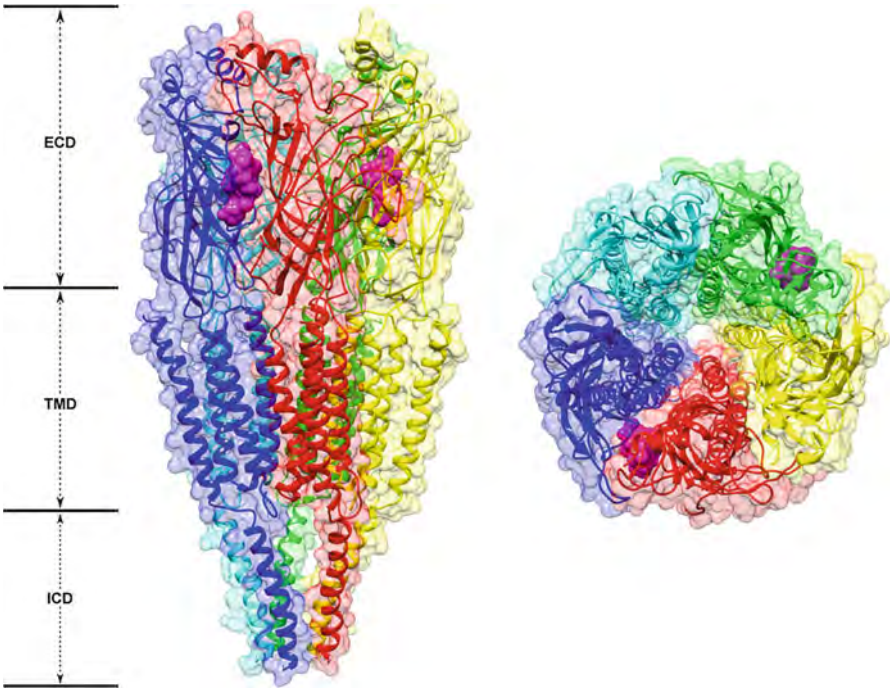

Fig. 3 Three-dimensional structure of the muscle-type nAChR (*Torpedo marmorata*) obtained by cryoelectron microscopy (Protein Data Bank code 2BG9). The five subunits are colored in *red*, *yellow*, *green*, *cyan*, and *blue*. The two antagonist sites where the spiroimine toxins can bind are shown as *magenta*-colored surfaces. *Left*: lateral view showing the extracellular (ECD), transmembrane, (TMD) and intracellular (ICD) domains. *Right*: top view showing the organization of the five subunits arranged around the central pore

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In the peripheral nervous system, the primary subtype is the heteromeric α3β4 subtype, which is present in autonomic ganglia neurons. In addition, the α3 subunit may assemble with α5 and/ or β2 subunit to form a variety of nAChRs. The other major subtype of neuronal nAChR in the peripheral and central nervous system comprised α7 subunits forming a homomeric nAChR, as well as heteromers made up from the combination of different α subunits, or α and β subunits. These arrangements include the α4β2 nAChR, which is abundant and widely distributed in the central nervous system, and more complex subunit combinations with the presence of additional subunit types may exist. Thus, a major characteristic of neuronal nAChRs is their wide heterogeneity, based on both diverse stoichiometry and subunit composition.

Using both electrophysiological techniques and competition ligand-binding assays, it has been shown that 13-desmethyl spirolide C and analogue spirolide compounds interact with muscle-type nAChRs existing at the neuromuscular junction of the skeletal muscle and with the major neuronal nAChRs present in the central and peripheral nervous systems.

The functional characterization of the action of spirolides on nAChRs has been performed using the voltage-clamp technique and *Xenopus* oocytes that have incorporated into their membrane or express a given nAChR subtype. These studies revealed that neither 13-desmethyl spirolide C nor 13,19-didesmethyl spirolide C exhibits an agonist action on the muscle-type ($\alpha 1_2\beta 1\gamma\delta$) nAChR incorporated to the oocyte membrane or in oocytes expressing the human homomeric α7 and the human heteromeric α4β2 nAChR. Acetylcholine (ACh), the endogenous agonist of nAChRs, is known to trigger conformational changes in the receptor which lead to the opening of the intrinsic cation channel, producing an inward current due to simultaneous entry of Na^+ and Ca^{2+} ions and the exit of K^+ ions that leads to membrane depolarization. Under voltage-clamp conditions, an agonist effect is seen as an inward current relative to the baseline holding current that keeps the membrane potential constant. Thus, in contrast to ACh, spirolides produced a concentration-dependent block of the ACh-evoked current in muscle-type ($\alpha 1_2\beta 1\gamma\delta$) and neuronal α7 and α4β2 nAChR subtypes (Aráoz et al. 2009; Bourne et al. 2010). The antagonist activity of 13-desmethyl spirolide C is long-lasting, since it was not annihilated even after a 30–40 min of washout of the spirolide from the nAChR subtypes studied (Bourne et al. 2010) (Fig. 4).

Competition-binding studies performed on membranes from cells expressing different nAChR subtypes using the radiolabeled probes [^{125}I]α-bungarotoxin, [^{3}H]epibatidine or [^{3}H]nicotine, and standard ligand-binding methods (reviewed in Molgó et al. 2013) allowed to get a better understanding into the interaction between spirolides and nAChRs. These competition-binding assays, performed at equilibrium, demonstrated that 13-desmethyl spirolide C totally displaced [^{125}I]α-bungarotoxin, in a concentration-dependent manner, not only from *Torpedo* membranes expressing the muscle-type nAChR but also from HEK-293 cells expressing the chicken chimeric α7-5HT$_3$ neuronal nAChR. This phycotoxin interacts also with high affinity and displaced [^{3}H]epibatidine binding from heteropentameric human α3β2 and α4β2 neuronal nAChRs (Bourne et al. 2010). Similar results have been obtained with other subtypes of nAChRs in which the 13-desmethyl spirolide C has been studied with a rank order for binding affinities (Ki) as follows: α7 > α6β3β4α5 >> rat α3β4, $\alpha 1_2\beta 1\gamma\delta$ > α4β4, human α3β4 > human α4β2 > rat α4β2 (Hauser et al. 2012). These results indicate that spirolides are competitive antagonists with a broad specificity on neuronal nAChRs.

Important advances have been made in the molecular and structural characterization of nAChRs and in the understanding of the molecular pharmacological profile of spirolides. A major step to understand the structural determinants of the interaction between 13-desmethyl spirolide C and nAChRs was the publication of the X-ray crystal structure for this phycotoxin in complex with the *Aplysia californica* acetylcholine-binding protein (AChBP) (Bourne et al. 2010). The AChBPs are water-soluble pentameric proteins, produced by glial cells in the nervous system of mollusks (freshwater snails *Lymnaea stagnalis* and *Bulinus truncatus* and the marine snail *Aplysia californica*). Their physiological role is to bind the

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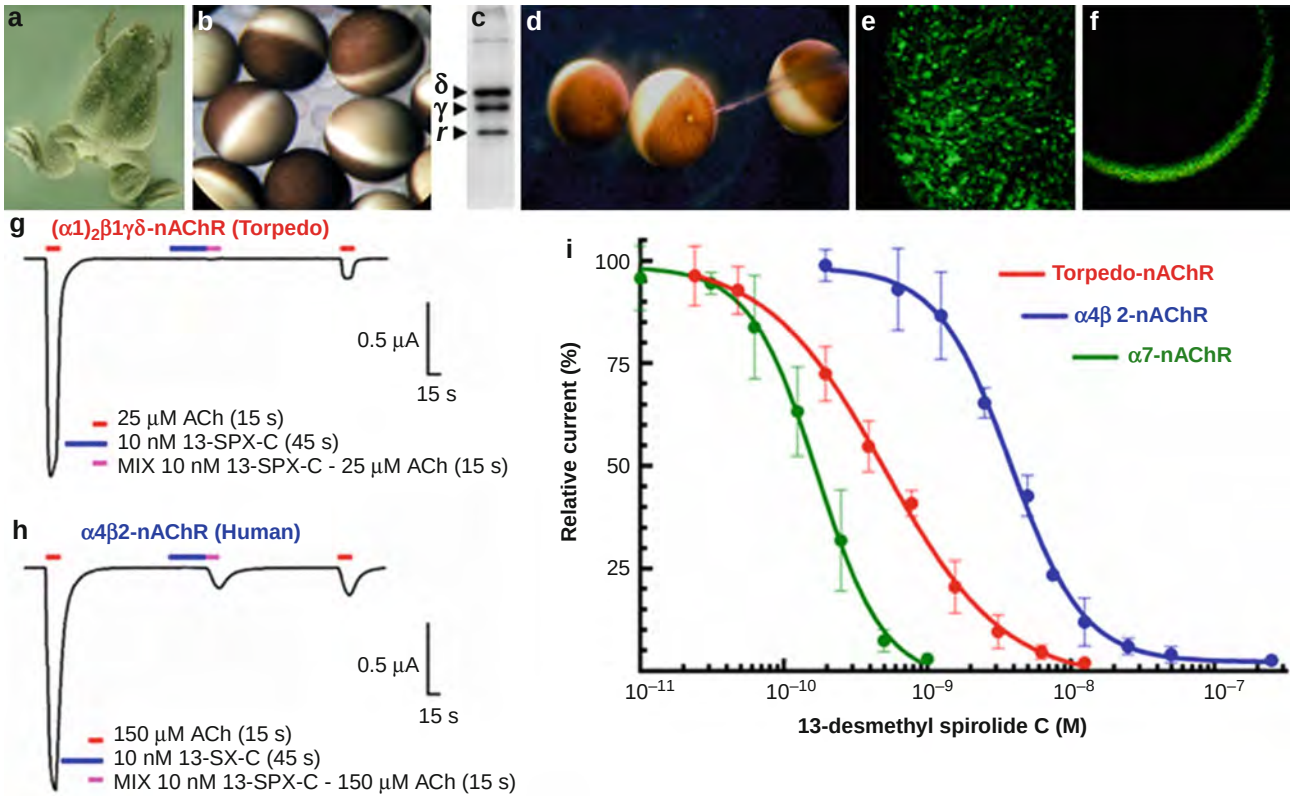

Fig. 4 Experimental approaches used to study the action of spirolides and other spiroimine phycotoxins using electrophysiological techniques. (**a**) Mature female *Xenopus laevis* frog. (**b**) Oocytes at stages V to VI of development, surgically removed from an anesthetized mature female *X. laevis* under anesthesia. (**c**) Western blot of *Torpedo marmorata* purified membranes showing the γ and δ subunits of the nAChR and the rapsyn (*r*) protein. (**d**) Manually defolliculated oocytes in which one is microinjected with 50 nL of purified *Torpedo* membranes using a Nanoliter 2010 Micro 4 Controller injector mounted on a microscope. (**e**, **f**) Staining of nAChRs incorporated to the membrane of an oocyte using fluorescent α-bungarotoxin, Alexa Fluor® 488 conjugate. (**g**) Inward nicotinic current triggered by an ACh pulse of 15 s duration, recorded in an oocyte microinjected 4 days previously with purified *Torpedo* membranes. Perfusion of 13-desmethyl spirolide C (13-SPX-C) had no agonist action (*blue tracing* above recording), but completely blocked the ACh-evoked current when applied together with ACh (*pink tracing*). A further ACh pulse (*red tracing* above recording) only evoked a small current response consistent with low dissociation rate of the spirolide from the receptor. (**h**) ACh-evoked currents recorded in another oocyte expressing the human α4β2 nAChR before and after the perfusion of the spirolide. Color tracing above recordings indicate protocols used. All recordings were performed at a holding potential of –60 mV. The ACh concentration used corresponded to the EC_{50} determined for each nAChR subtype. (**i**) Inhibition of ACh-evoked currents recorded from *Torpedo* $\alpha 1_2\beta 1\gamma\delta$ nAChR incorporated into the oocyte membrane and human α7 and α4β2 nAChRs expressed in oocytes. The amplitudes of the ACh-evoked currents recorded in the presence of 13-desmethyl spirolide C (mean ± SEM, 3–4 oocytes per concentration) were normalized to control currents and fitted to the Hill equation

neurally released ACh participating in the modulation of cholinergic synaptic transmission and represent structural and functional homologues of the amino-terminal extracellular ligand-binding domain of nAChRs (reviewed in Sixma and Smit 2003). Virtually all conserved amino acid residues existing in the nAChR family are present in the AChBPs, including those that are important for the ligand binding to the agonists ACh and nicotine, as well as for the competitive antagonists such as α-bungarotoxin and d-tubocurarine, and therefore have similar pharmacological properties. The binding of 13-desmethyl spirolide C to the *Aplysia*-AChBP displayed picomolar affinities governed by diffusion-limited association and slow dissociation, accounting for the apparent irreversibility detected in electrophysiological experiments (see above). The crystal structure of the 13-desmethyl spirolide C bound to *Aplysia*-AChBP

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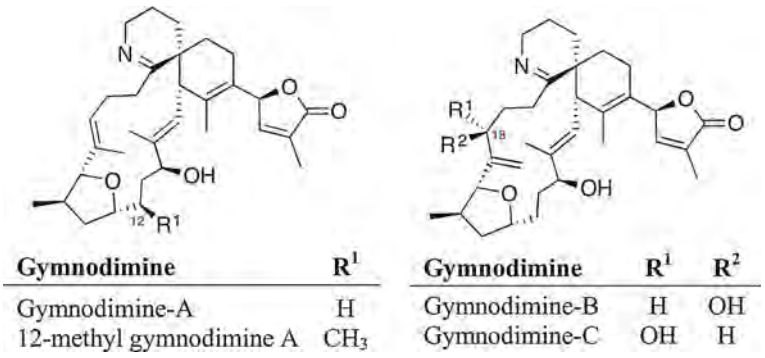

Gymnodimine	R^1
Gymnodimine-A	H
12-methyl gymnodimine A	CH_3

Gymnodimine	R^1	R^2
Gymnodimine-B	H	OH
Gymnodimine-C	OH	H

Fig. 5 Chemical structures of the known gymnodimine toxins

Table 3 Dinoflagellate species known to produce gymnodimine congeners

Karenia selliformis	New Zealand	Gymnodimines A, B, and C
	Australia	Gymnodimine A
Alexandrium peruvianum	United States	12-Methyl gymnodimine

at 2.51 Å resolution showed the phycotoxin precisely imbedded within the nest of aromatic side chains contributed by loops C and F on opposing faces of the subunit interface, which in normal conditions provide accommodation to ACh. The structure of 13-desmethyl spirolide C, and other related phycotoxins, guarantees an ideal positioning of the central cyclic imine as a pivot point. In addition, the architectural restraints of the uncommon macrocyclic ring structures stabilize conformers with favorably positioned substituents conferring high surface complementarity and minimize the loss of entropy as phycotoxins bind to the *Aplysia*-AChBP (Bourne et al. 2010).

Gymnodimines

Chemical Characteristics of Gymnodimine Toxins

The gymnodimines present a six-membered cyclic imine, bearing no methyl substituents, as part of the spiroimine ring system, an unsaturated lactone, and a tetrahydrofuran ring (Fig. 5). Two groups can be distinguished according to the substitution pattern at the positions C17 and C18. The first gymnodimine group has a methyl substituent at C17 and an *E*-configured endocyclic double bond between C17 and C18. Gymnodimine A and its 12-methyl derivative are part of this group. The second gymnodimine group contains gymnodimines B and C, which have an exocyclic double bond at C17 and a hydroxyl substituent at C18 with *S* and *R* stereochemistry, respectively.

Gymnodimine-Producing Organisms

The production of the gymnodimines identified so far (gymnodimines A, B, and C) was exclusively attributed to the dinoflagellate *Karenia selliformis* (Haywood et al. 2004) (formerly known as *Gymnodinium selliforme*) in New Zealand and Australia (Table 3). However, the gymnodimine congener 12-methyl gymnodimine was reported as originating from another dinoflagellate in the United States, *Alexandrium peruvianum* (Van Wagoner et al. 2011) (Table 3), which was proven to also produce some spirolide congeners (see Table 1).

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Table 4 The 50 % lethal doses (LD_{50}) of gymnodimine congeners according to their administration route

Gymnodimine	Administration mode	LD_{50} (mg/kg mouse)	Ratio (compared to i.p.)
Gymnodimine A	Intraperitoneal injection	0.08–0.096	1
	Intracerebroventricular injection	0.003	0.03–0.038
	Gavage	0.755	7.9–9.4
	Feeding	4.057–>7.5	42.3–>78.1
Gymnodimine B	Intraperitoneal injection	0.800	1

Toxicity of Gymnodimine Toxins

The gymnodimines are among the least toxic cyclic imines according to the mouse bioassay irrespective of the route of administration (i.p. injection, gavage, or feeding administration). Their acute oral toxicity, in particular that of gymnodimine A, was shown to be between about 8- and more than 78-fold lower than the i.p. toxicity (Munday et al. 2004) (Table 4). It is worth noting that gymnodimine A was reported to be about tenfold more toxic than gymnodimine B after i.p. injection to animals. Remarkably, the LD_{50} for gymnodimine A was decreased by about 30-fold when administered to mice by intracerebroventricular administration, as compared to i.p. injection (i.e., 0.003 and 0.08–0.096 mg/kg mouse, respectively) (Kharrat et al. 2008) (Table 4). This indicates that gymnodimine A preferentially affected the rodent central nervous system, further highlighting the risk of the cyclic imine toxins to human health may have when accumulated in shellfish at high concentrations.

Molecular Targets and Mechanism of Action of Gymnodimine Toxins

Gymnodimine A at nanomolar concentrations was reported to produce a concentration-dependent block of twitch tension responses evoked by nerve stimulation in isolated mouse phrenic-nerve-hemidiaphragm-muscle preparations, without affecting muscle excitability and directly elicited muscle contraction. Also, gymnodimine A, according to the nanomolar concentration used, either reduced the amplitude or completely blocked miniature end plate potentials generated by the release of a single ACh quantum (Kharrat et al. 2008). Similarly, gymnodimine A blocked the amplitude of end plate potentials without affecting the resting membrane potential of muscle fibers. Such an action prevented end plate potentials to reach the threshold potential for action potential generation in muscles fibers, strongly suggesting that gymnodimine A blocked end plate nAChRs. Direct evidence for such an action was obtained, using both the patch-clamp technique and iontophoretic ACh pulses of constant duration delivered by a micropipette on the surface of *Xenopus* skeletal myocytes expressing the embryonic muscle-type nAChR in their surface (Kharrat et al. 2008). Gymnodimine A was shown to have no agonist action on such receptors, but to block reversibly the inward nicotinic currents elicited by the iontophoretic ACh pulses. Furthermore, the inhibition of ACh-activated currents by gymnodimine A was clearly independent of the holding membrane potential, in other terms no voltage dependence. Interestingly, the neuromuscular block produced by gymnodimine A could be reversed by continuous washout of the phycotoxin from the medium within 30 min. Also, the block of K^+ channels in motor nerve terminals by 3,4-diaminopyridine (100 μM), which increases evoked quantal ACh release by nerve impulses (Van der Kloot and Molgó 1994), was able to antagonize the action of the phycotoxin. In addition, gymnodimine A, in a reversible manner, blocked ACh-evoked currents in *Xenopus* oocytes expressing heterologous human α7 nAChRs, and this block was independent of the holding membrane potential (Kharrat et al. 2008).

Further insight into the interaction between gymnodimine A and the muscle-type $\alpha 1_2\beta 1\gamma\delta$ nAChR was gained in competition-binding studies using [^{125}I]α-bungarotoxin as a tracer and HEK-293 cells expressing this nAChR. Gymnodimine A totally displaced, in a concentration-dependent manner, [^{125}I]-α-bungarotoxin from HEK-293 cells expressing the muscle-type $\alpha 1_2\beta 1\gamma\delta$ nAChR or the chimeric

neuronal α7-5-HT3 nAChR. Furthermore, gymnodimine A inhibited the specific [^{3}H]epibatidine binding on HEK-293 cells expressing either α4β2 or α3β2 neuronal nAChRs. These data are consistent with the fact that gymnodimine A was highly toxic when applied directly into the mouse central nervous system by intracerebroventricular injection. The rank order of potency for gymnodimine A on the various nAChRs studied was α7-5HT3 > $\alpha1_2\beta1\gamma\delta$ > α3β2 > α4β2 (Kharrat et al. 2008). In another study, the rank order for binding affinity (Ki) was α7, α6β3β4α5 > rat α3β4 > human α3β4, α4β4 > rat α4β2, human α4β2 (Hauser et al. 2012).

Some 6,6-spiroimine analogues of gymnodimine A have been chemically synthesized and were shown to block ACh-evoked nicotinic currents in *Xenopus* oocytes having incorporated the *Torpedo* muscle-type ($\alpha1_2\beta1\gamma\delta$) nAChR in their membrane or expressing the human α4β2 nAChR (Duroure et al. 2011). Although these spiroimine analogues are about 40–45 times less active than gymnodimine A, the fact that they block both types of nAChRs indicate that the 6,6-spiroimine moiety is a key structural factor required for blocking nAChRs in the gymnodimine family.

The X-ray crystal structure of gymnodimine A in complex with *Aplysia*-AChBP has been determined (Bourne et al. 2010). The structure obtained indicates that gymnodimine A was bound at the subunit interface, between loops C and F from opposing faces, in the same region where ACh binds. In addition, the structures highlighted the characteristic hydrogen bond between the iminium nitrogen in gymnodimine A and the carbonyl oxygen of Trp 147 from loop C and also the anchoring interactions of the tetrahydrofuran component in gymnodimine A with the apical and membrane extremities of the binding site, along the subunit interface.

Pinnatoxins, Pteriatoxins, and Portimine

Chemical Characteristics of Pinnatoxins and Pteriatoxins

To date, eight pinnatoxins (A–H) and three pteriatoxins (A–C) have been described. They all share the same common scaffold with a dimethyl substituted seven-membered cyclic imine as part of a spiroimine ring system, a 6,5,6-spiroketal ring system, and a bridged ketal which is unique within the cyclic imine phycotoxins family (Fig. 6). The pinnatoxins and pteriatoxins are differentiated by the substituent at C33, which might be carboxyl (pinnatoxin A), vinyl (pinnatoxins G and H), glycinyl (pinnatoxins B and C), butyrolactone (pinnatoxin F), or hydroxy/keto-butyric acid (pinnatoxins D and E), as well as *S*-cysteinyl derivatives (pteriatoxins A–C). Other positions with substituent variability are 21, 22, and 28, for which are observed two substitution patterns: H, H and OH (pinnatoxins A, B, C, G and pteriatoxins A, B, C), and CH_3, OH and H (pinnatoxins D, E, F, H), respectively (O'Connor and Brimble 2007; Stivala et al. 2015).

Pinnatoxin- and Pteriatoxin-Producing Organisms

Identical strains of dinoflagellates were shown to be responsible for the production of pinnatoxins E and F in New Zealand, pinnatoxins A, E, F, and/or G in South Australia, and pinnatoxin G in Japan (Rhodes et al. 2011). The recognition of the dinoflagellate producer of pinnatoxins was ultimately associated with the discovery of a new dinoflagellate species, named *Vulcanodinium rugosum* (Nézan and Chomérat 2011), in water samples of a Mediterranean lagoon in the French coast (Hess et al. 2013), which produced pinnatoxin G (Table 5). Recently, a new pinnatoxin analogue, pinnatoxin H, was purified from a culture of the dinoflagellate *Vulcanodinium rugosum* from the South China Sea (Selwood et al. 2014). It is worth noting that the fatty acid esters of pinnatoxins A and G isolated from Canadian mussels were reported to likely originate from the shellfish than from the dinoflagellate (McCarron et al. 2012). Similarly, there is

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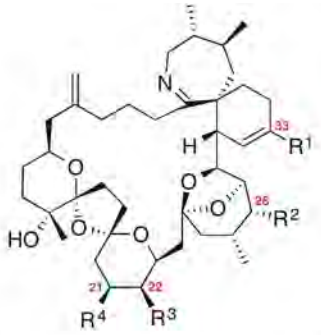


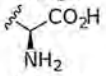
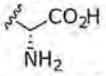
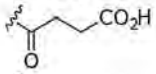
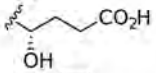
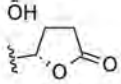
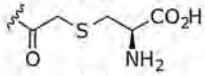
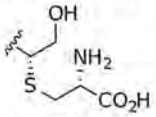
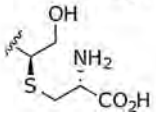
Pinnatoxin / Pteriatoxin	R^1	R^2	R^3	R^4
Pinnatoxin-A	CO_2H	OH	H	H
Pinnatoxin-B		OH	H	H
Pinnatoxin-C		OH	H	H
Pinnatoxin-D		H	OH	CH_3
Pinnatoxin-E		H	OH	CH_3
Pinnatoxin-F		H	OH	CH_3
Pinnatoxin-G		OH	H	H
Pinnatoxin-H		H	OH	CH_3
Pteriatoxin-A		OH	H	H
Pteriatoxin-B		OH	H	H
Pteriatoxin-C		OH	H	H

Fig. 6 Chemical structures of the known pinnatoxins and pteriatoxins

Table 5 Dinoflagellate species known to produce pinnatoxin congeners

Vulcanodinium rugosum	Australia	Pinnatoxins A, E, F, and G
	New Zealand	Pinnatoxins E and F
	Japan	Pinnatoxin G
	France	Pinnatoxin G
	China	Pinnatoxin H

no conclusive evidence for the dinoflagellate source of pteriatoxins, isolated from the Japanese bivalve *Pteria penguin* (Shumway 1990).

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Table 6 The 50 % lethal doses (LD_{50}) of pinnatoxin congeners according to their administration route

Pinnatoxin	Administration mode	LD_{50} (mg/kg mouse)	Ratio (compared to i.p.)
Pinnatoxin E	Intraperitoneal injection	0.045–0.057	1
	Gavage	2.800	49.1–62.2
Pinnatoxin F	Intraperitoneal injection	0.013–0.016	1
	Gavage	0.025–0.03	1.6–2.3
	Feeding	0.05–0.077	3.1–5.9
Pinnatoxin G	Intraperitoneal injection	0.043–0.05	1
	Gavage	0.150	3–3.5
	Feeding	0.400	8–9.3
Pinnatoxin H	Intraperitoneal injection	0.067	1
	Gavage	0.163	2.4

Toxicity of Pinnatoxins and Pteriatoxins

Acute toxicological studies with purified samples of pinnatoxin congeners, administered by i.p. injection, revealed that pinnatoxin F was between about 2.7 and 5.1 times more potent than pinnatoxins E, G, and H (Table 6), showing the following rank order of potency in the acute toxicity response: pinnatoxin F > pinnatoxin G > pinnatoxin E > pinnatoxin H (Munday et al. 2012b). A different rank order in the acute toxicity response (i.e., pinnatoxin F > pinnatoxin G > pinnatoxin H > pinnatoxin E) was obtained for gavage administration. The oral toxicity of pinnatoxin F was only between 5- and 6.5-fold higher than that of pinnatoxins G and H, but was about 93.3-fold higher than that of pinnatoxin E. As detailed previously for spirolide congeners and gymnodimine A, the oral toxicity of pinnatoxin E was at least 49-fold lower than the i.p. toxicity, while comparatively smaller differences were observed for pinnatoxins F, G, and H between the two routes of administration (Table 6). These findings are of great interest regarding the risk these toxins may have to human health. Only a few reports were concerned with the acute toxicity of pteriatoxins, but they revealed that these toxins induce animal lethality at doses between 0.008 and 0.1 mg/kg when administrated to mouse by i.p. injection, with toxic symptoms resembling those of pinnatoxins (see Takada et al. 2001). The atypical stability of the aliphatic cyclic imine in pinnatoxins (Jackson et al. 2012) is probably associated to their high oral toxicity, highest among the cyclic imine toxins.

Molecular Targets and Mechanism of Action of Pinnatoxins and Pteriatoxins

The use of successful strategies for the chemical synthesis of pinnatoxins A and G (Beaumont et al. 2010; Stivala et al. 2015) allowed for the production of substantial amounts of these toxins in pure form which permitted performing electrophysiological and functional studies, as well as competition-binding studies together with computational molecular modeling, leading to the revision of the mode of action previously proposed for pinnatoxins. Thus, based on such analyses, it has been unequivocally established that pinnatoxins A and G are potent antagonists of nAChRs (Aráoz et al. 2011). The rank order of potency for pinnatoxin A on nAChR subtypes determined by functional analyses was the following: human α7 > *Torpedo* $\alpha 1_2\beta 1\gamma\delta$ > human α4β2. The reversibility of the pinnatoxin A activity was faster for the α4β2 nAChR, while it was slowly reversible for the α7 nAChR subtype. Interestingly, pinnatoxin A exhibited higher affinity than pinnatoxin G for the human neuronal α7 nAChR subtype. In contrast, a synthetic aminoketone derivative of pinnatoxin A with an open imine ring showed no activity on the various nAChR subtypes studied, a feature supporting the central role of the seven-membered cyclic imine ring for the potent antagonism of this family of toxins (Aráoz et al. 2011). The molecular bases governing the unique selectivity of pinnatoxin A for the neuronal α7 nAChR have recently being reported through the analysis of the crystal structures of pinnatoxins A and G complexes with the AChBP at high

(1.9–2.2 Å) resolution (Bourne et al. 2015). Also, pinnatoxins E, F, and G were reported to produce concentration-dependent reductions in nerve-evoked twitch tension in isolated rat hemidiaphragm preparations, with a rank order of potency of F > G > E, while no effects were detected on directly muscle-evoked twitch responses. Pinnatoxins F and G moreover reduced the amplitude of spontaneous miniature end plate potentials and evoked end plate potentials at skeletal neuromuscular junctions, without affecting miniature end plate potential frequency or the resting membrane potential of the muscle fibers. These results show that pinnatoxins E, F, and G are potent neuromuscular blockers and cause toxicity by acting as antagonists at muscle-type nicotinic acetylcholine receptors (Hellyer et al. 2013).

Further ligand-binding studies were performed to examine whether pinnatoxin A had any action on other neurotransmitter receptors, including those coupled to G proteins, and neurotransmitter transporters. However, no significant action, or very little action, was detected using a high concentration of pinnatoxin A (10 μM) on the different receptors studied including: adrenergic receptors (Alpha1A, Alpha1B, Alpha1D, Alpha2A, Alpha2B, Alpha2C, Beta1, Beta2, Beta3), muscarinic receptors (M1, M2, M3, M4, and M5), serotonergic receptors (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT3, 5-HT5A, 5-HT6, 5-HT7), GABAA receptor, dopaminergic receptors (D1, D2, D3, D4, and D5), histamine receptors (H1 and H2), δ−opioid (DOR), κ−opioid (KOR), μ-opioid (MOR) and the dopamine transporter (DAT), norepinephrine transporter (NET), and serotonin transporter (SERT). Furthermore, no activity on $Ca_V1.1$ calcium channels (type-L) originally proposed to be the target of pinnatoxins was observed (Aráoz et al. 2011). These experimental results reveal an extraordinary selectivity of pinnatoxin A for the nAChRs.

The evidence that spirolides, gymnodimines, and pinnatoxins target nAChRs prompted the development of target-directed functional methods, based on the mechanism of action of competitive antagonists on nAChRs. Thus, several nonradioactive ligand-binding methods have been developed (Vilariño et al. 2009; Fonfría et al. 2010; Otero et al. 2011; Aráoz et al. 2012; Rodriguez et al. 2013) to replace the traditional mouse bioassay which has numerous drawbacks related to sensitivity and specificity, apart from the ethical concern of using large number of animals, as recently discussed (Daneshian et al. 2013).

Portimine

A new cyclic imine toxin, named portimine, has been recently discovered from the pinnatoxin-producing dinoflagellate *Vulcanodinium rugosum* from Northland (New Zealand) (Selwood et al. 2013). The structure of portimine, including the relative configurations, was elucidated by spectroscopic analyses. The cyclic imine moiety consists of an unprecedented five-membered ring with a spiro-link to a cyclohexene ring (see Fig. 1). Portimine contains only 14 carbons in its macrocycle, making it the smallest cyclic imine toxin isolated to date. Such structural factors have a large impact on the toxin toxicity, since it has a much lower toxicity in rodent bioassays after i.p. administration (LD_{50} = 1.57 mg/kg mouse) than any other cyclic imine toxin. In contrast, portimine exhibits high toxicity in vitro to mammalian cells, with a 50 % lethal concentration (LC_{50}) of 2.7 nM to mouse leukemia P388 cells, and activation of caspases indicates that it has apoptotic activity.

Prorocentrolides and Spiro-prorocentrimine

Chemical Characteristics of Prorocentrolide and Spiro-prorocentrimine Toxins

Prorocentrolides are the only members of the cyclic imine phycotoxins family that do not contain a spiroimine group. Their chemical structure includes a 26-membered carbo-macrocycle and a 28-membered macrocyclic lactone arranged around a 3,4,4a,5,8,8a-hexahydroisoquinoline that incorporates the characteristic cyclic imine group. Six prorocentrolides have been reported to date (Fig. 7), which

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Prorocentrolide	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
Prorocentrolide	H	CH_2=	H	OH	OH	H	CH_3	OH
Prorocentrolide-B	OSO_3H	CH_3	CH_2=	OH	H	OH	H	OH
9,51-dihydro prorocentrolide	H	CH_3	H	OH	OH	H	CH_3	OH
4-hydroxy prorocentrolide	OH	CH_2=	H	OH	OH	H	CH_3	OH
14-*O*-acetyl-4-hydroxy prorocentrolide	OH	CH_2=	H	$OC(=O)CH_3$	OH	H	CH_3	OH
Prorocentrolide 30-sulfate	H	CH_2=	H	OH	OH	H	CH_3	OSO_3H

Fig. 7 Chemical structures of the known prorocentrolide toxins

Table 7 Dinoflagellate species known to produce prorocentrolide congeners and spiro-prorocentrimine

Prorocentrum lima	Japan	Prorocentrolide A
	Taiwan	Prorocentrolide A
		4-Hydroxy prorocentrolide
		14-*O*-Acetyl-4-hydroxy prorocentrolide
Prorocentrum maculosum	Tropical waters	Prorocentrolide B
Prorocentrum sp.	Taiwan	Prorocentrolide A
		Spiro-prorocentrimine

differ by the substituents at the positions 4 (hydrogen, hydroxy, or sulfonate), 9 (methyl or exocyclic vinyl), 14 (hydroxy or acetate ester), 29 (methyl or hydrogen), and 30 (hydroxy or sulfonate). Prorocentrolide B shows specific structural modifications at the positions 10 (exocyclic vinyl), 15 (hydrogen instead of hydroxy), 16 (hydroxy instead of hydrogen), and 29 (unsubstituted endocyclic *E* double bond).

A single spiro-prorocentrimine compound is currently known (see Fig. 1), whose chemical structure contains a 25-membered carbo-macrocycle and a 15-membered macrocyclic lactone arranged around a 6,6-spiroimine ring system. The overall structure and the stereochemistry of substituents have been unambiguously determined by X-ray crystallography (Lu et al. 2001).

Prorocentrolide- and Spiro-prorocentrimine-Producing Organisms

To date, the production of prorocentrolide congeners and spiro-prorocentrimine is well known to be restricted to primarily benthic or epiphytic *Prorocentrum* spp. which, unlike their pelagic counterparts, does not form dense blooms. Hence, prorocentrolide A was isolated from *Prorocentrum lima* in Japan and Taiwan, and the structurally related cyclic imine prorocentrolide B from *Prorocentrum maculosum* in tropical waters. The Taiwanese *Prorocentrum lima* also produces two prorocentrolide analogues, 4-hydroxy and 14-*O*-acetyl-4-hydroxy prorocentrolides. Finally, spiro-prorocentrimine was isolated along with prorocentrolide A from another Taiwanese, still unidentified, strain of *Prorocentrum* sp. (Table 7).

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Toxicity of Prorocentrolide and Spiro-prorocentrimine Toxins

Although only a few reports were concerned with the acute toxicity of prorocentrolide and spiro-prorocentrimine toxins, they revealed that when administrated to mouse by i.p. injection, prorocentrolide A had an animal lethality of 0.4 mg/kg mouse (Torigoe et al. 1988), while spiro-prorocentrimine exhibited much less toxicity (i.e., animal lethality occurred at 2.5 mg/kg mouse) than other known marine cyclic imine toxins (Lu et al. 2001).

Molecular Targets and Mechanism of Action of Prorocentrolide and Spiro-prorocentrimine Toxins

The molecular targets for these cyclic imine toxins have not yet been determined, because of the lack of purified or synthetic toxins.

Conclusion and Future Directions

In conclusion, this short review shows that the well-chemically characterized natural products derived from some toxic dinoflagellates species constitute a novel source of potent nAChR-ligand molecules. The distinguishing feature of these phycotoxins is the presence of a cyclic imine moiety in their structure, which constitutes a chemical signature associated with their capacity to interact with muscle-types and neuronal-nAChR subtypes. Thus, spirolides, gymnodimines, and pinnatoxins, which are recognized "fast-acting toxins," are known to broadly target muscle- and neuronal-type nAChRs, generally with high affinity for the various receptor subtypes studied. The sites of interaction of these toxins on nAChRs include amino acid residues highly conserved in the sequences of muscle- and neuronal-type receptors. Another important aspect in the action of these phycotoxins is the lack of animal species selectivity, since similar actions have been reported on frog, fish, chick, rodent, and human nAChRs. The toxicological profile reported for these phycotoxins, during rodent bioassays, is mostly due to their specific interaction with the nAChRs.

The competitive binding to nAChRs has been exploited to design and develop new tests to detect spirolides, gymnodimines, and pinnatoxins in contaminated shellfish with better accuracy than the broad spectrum mouse bioassay. These tests are important in the food safety field, since shellfish constitutes a worldwide rich food resource that may be contaminated by toxins produced by harmful dinoflagellates. Additional appropriate toxicological studies must be developed to assess the risk these phycotoxins may have to human health, since at present there is no strong evidence linking the presence of spirolides, gymnodimines, and pinnatoxins in shellfish tissues with human intoxications. Also, more data is needed on the risks of chronic exposure to these phycotoxins. Shellfish frequently contains varying amounts of these toxins, at low or high concentrations, which is a risk factor for human health that cannot be neglected. Furthermore, shellfish concentrate these toxins in their edible tissues and can act as vector for transferring these phycotoxins through the marine food web, thus menacing wildlife.

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Microcystins: Toxicological Profile

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Abstract

Cyanobacteria are ubiquitous photosynthetic prokaryotic organisms, which can form blooms and scums in surface water, characterized by a wide morphological variability. Different cyanobacteria strains, i.e., *Microcystis, Planktothrix and Anabaena* spp., produce microcystins (MCs), a group of more than 100 different structural variants of toxic cyclic heptapeptides, with a common cyanobacteria-specific amino acid, Adda. MC variants differ from each other for amino acid substitutions in positions 2 and 4 (where MC-LR has leucine (L) and arginine (R), respectively) and other changes. Humans may be exposed to MCs through the oral route, by ingestion of contaminated drinking water or food (including dietary supplements) or water during recreational activities. Dermal/inhalation and parenteral exposure may also occur, the latter due to the use of contaminated water for hemodialysis, leading to fatal outcome. The acute hepatotoxicity is congener specific (range of intraperitoneal LD_{50} in mice: 50–1200 μg/kg body weight). Renal and neurotoxic effects have also been reported for some variants. Minimal structural changes among variants result in three- to fourfold differences in MC toxicokinetics: the active uptake and the biotransformation have been shown to be congener dependent. MC-LR mechanism of action is associated with inhibition of protein serine/threonine phosphatases (PP1 and PP2A): the altered phosphorylation of cellular proteins involved in signal transduction leads to cascade of events (lipid peroxidation, oxidative stress, apoptosis). MC-LR has been shown to be not mutagenic but has tumor-promoting activity; on this basis, IARC classified it as a 2B carcinogen. The WHO has defined a guidance value for drinking water of 1 μg MCs/L.

Introduction

Cyanobacterial toxins are a wide diverse group of secondary metabolites produced by Cyanobacteria, ubiquitous photosynthetic procariota organisms characterized by a wide morphological variability (Chorus and Bartram 1999). They contain chlorophyll a and accessory pigments and may be unicellular, colonial, or filamentous, with cell sizes varying from less than 2 to 40 μm in diameter. Cyanobacteria occur especially in surface waters and can form blooms or dense surface scums in the presence of conditions favoring their growth (i.e., temperature, light availability, low turbulence, or anthropogenic factors such as increased nutrient loading).

When blooms are formed, the risk of toxin contamination of surface waters increases especially for some species of cyanobacteria with the ability to produce toxins and other noxious chemicals, posing a serious health problem (Funari and Testai 2008). Although the chemical structure of many cyanotoxins and their adverse effect have been fairly elucidated and recently reviewed, the actual physiological function and ecological regulation of these toxins remain still unclear.

Microcystins (MCs) were first isolated from the cyanobacterium *Microcystis aeruginosa* from which they were named (Carmichael et al. 1990), but they are produced by several species of planktonic

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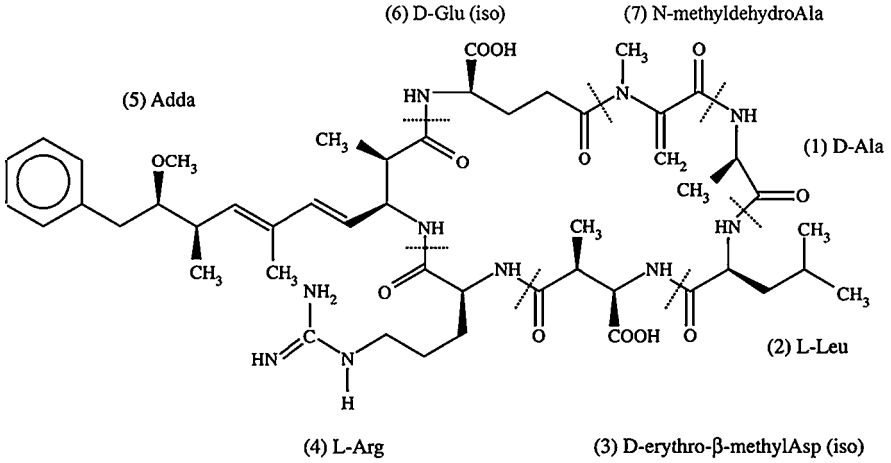

Fig. 1 Chemical structure of MC-LR

cyanobacteria. They are among the most widespread cyanobacterial toxins, frequently detected in freshwaters. They can accumulate in common aquatic vertebrates and invertebrates such as fish, mussels, and zooplankton.

They contain seven amino acids, with the two terminal amino acids of the linear peptide being condensed to form a cyclic compound. A common and peculiar characteristic of these toxins is the amino acid Adda [(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid], which is unique for cyanobacteria (Chorus and Bartram 1999). The amino acids in positions 2 and 4 can vary, giving rise to different variants, as well as other changes such as methylation/demethylation at different positions. The molecular weight of MCs varies between 900 and 1100 Da. The most common are MC-LR, MC-RR, and MC-YR, with different combinations of leucine (L), arginine (R), or tyrosine (Y) in positions 2 and 4. MC-LR is the most studied due to its ubiquity, abundance, and toxicity among the different variants (Carmichael et al. 1990). The chemical structure of MC-LR is shown in Fig. 1.

The first chemical structures of cyanobacterial cyclic peptide toxins were identified in the early 1980s, and the number of fully characterized toxin variants has greatly increased in the subsequent years. To date, approximately 100 MC variants have been described from bloom samples and isolated strains of cyanobacteria. Most of the structural variants of microcystin are highly toxic, while only a few nontoxic variants have been identified. In general, any structural modification to the Adda glutamate region of the toxin molecule renders microcystins nontoxic (Chorus and Bartram 1999).

In aquatic environments, MCs usually remain within the cyanobacterial cells and are only released in substantial amounts on cell lysis. Along with their high chemical stability and their water solubility, this containment has important implications for their environmental persistence and exposure to humans in surface water bodies (Chorus and Bartram 1999).

Although the toxin can be localized in all regions in the interior of cyanobacterial cells, it was suggested that most of cell's toxin complement is localized in the thylakoids (Young et al. 2005). The association of MCs to the thylakoids might occur through the long hydrophobic tail of the Adda residue inserted in the lipid membrane and the polar cyclic structure interacting with cytoplasmatic phase, this suggesting a role of the molecule in light harvesting and adaptation responses. Microcystins are produced by several species of the genera *Microcystis, Planktothrix (Oscillatoria)* (Fig. 2)*, Anabaena, Nostoc,* and *Aphanizomenon*. *Microcystis* sp. and particularly *Microcystis aeruginosa* are most frequently linked to hepatotoxic blooms worldwide. *Microcystis viridis* and *Microcystis botrys* strains also have been shown to produce microcystins. *Microcystis* is a non-nitrogen-fixing genus often dominant under nutrient-rich conditions (especially where there is a significant supply of ammonia), although it also forms blooms in

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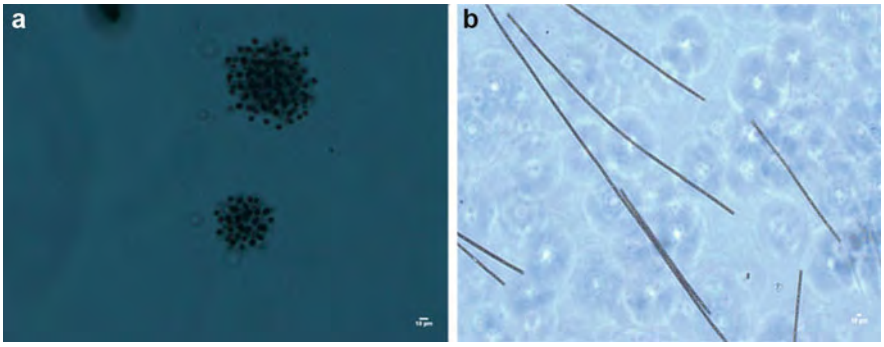

Fig. 2 Micrographs of a globular and filamentous cyanobacteria producing microcystins: (**a**) *Microcystis botrys*, (**b**) *Planktothrix rubescens* (Photos were kindly provided by M. Stefanelli)

less polluted waters. Moreover, the widespread *Planktothrix agardhii* and *Planktothrix rubescens* are frequently shown to be dominant in most microcystin-containing blooms. Indeed, in shallow lakes, populations of *Planktothrix* sp. may prevail over the other co-occurring species. In deeper, thermally stratified lakes and reservoirs with moderate nutrient pollution, they may form blooms at the interface between the warmer upper and colder deeper layers of water during summer, maintaining high, evenly distributed density throughout the entire water body during winter (Chorus and Bartram 1999).

MC production by a cyanobacterial population depends on its growth rate, with the highest amount produced during the late logarithmic phase and by other factors such as temperature, light, culture age, and nutrient availability. However, the role of these factors has been only partially clarified for *Microcystis* spp. and even less for *Planktothrix* spp. (Chorus and Bartram 1999; Manganelli et al. 2010).

Although many strains produce several MC variants simultaneously, usually only one or two of them are dominant in any single strain. However, in natural samples which usually contain many strains or more than one toxin-producing species, different combinations of MCs can be found. In general, water bodies with regular dominance of specific taxa are likely to exhibit characteristic patterns of MC variants.

Microcystins are non-ribosomal peptides synthesized by a multifunctional protein complex containing non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) domains. This multifunctional protein complex is present only in prokaryotes and lower eukaryotes and is encoded by large bacterial gene clusters which comprise at least two operons, the *mcyABC* (peptide synthetase) and *mcyDE* (hybrid polyketide–peptide synthetase) (Pearson et al. 2008) (Fig. 3).

However, within a cyanobacterial population, different genotypes can occur, and only some of them possess the genes for MC production (Kurmayer et al. 2002). Hence, the toxicity of a given bloom is strongly influenced by its strain composition, and the toxic fraction (i.e., those individuals containing genes for MC synthesis) inside a community can be extremely variable. The factors driving community succession from one genotype to the other are still unknown.

The identification of the genetic locus responsible for microcystin synthesis in several microcystin-producing cyanobacterial strains (i.e., *Microcystis, Planktothrix, Anabaena*) has improved the water resource management. Indeed, the use of genetic probes directed to selected genes within the *mcy* gene cluster allows to discriminate between nontoxic and toxic populations, and, in the latter case, the early detection of toxigenic individuals, before the bloom, is broken out.

Although PCR-based methods are a promising tool to obtain further information into the distribution of microcystin producers in cyanobacteria populations, it has to be taken into account that such a detection in field samples still runs the risk of falling into false-positive or false-negative. In fact, the presence of mcy genes (or restricted regions inside the locus conserved as pseudogenes) has been found also in non-microcystin producers, as in *M. aeruginosa* or *Planktothrix* spp.; moreover, a high degree of DNA

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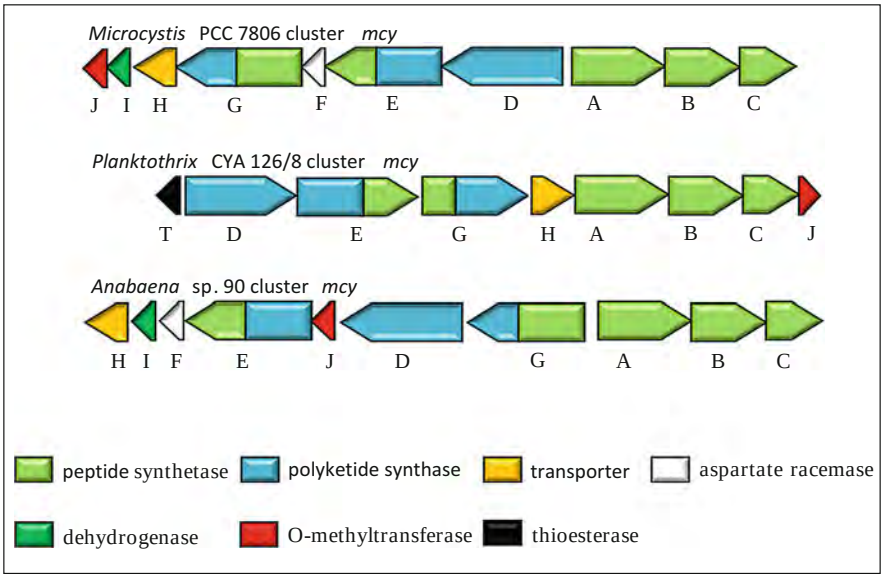

Fig. 3 Schematic representation of microcystin synthetase gene (*mcy*) cluster of *Microcystis*, *Planktothrix*, and *Anabaena*. The *light green bars* represent the peptide synthetase genes and *orange bars* the polyketide synthase genes. Genes encoding modification enzymes are indicated with other colors (Adapted from: Rantala 2007)

polymorphism has been observed to occur in mcy genes, which is responsible for a high variability of the mcy sequence. Hence, a preliminary careful analysis of the DNA is needed, to find a region in the *mcy* gene cluster that is suitable for an unambiguous detection of microcystin-producing chemotypes of a genus (Mbedi et al. 2005).

Humans may be exposed to MC through several scenarios: the main route is oral, occurring directly, by consumption of untreated infested drinking water or during recreational and bathing activities, or indirectly, through consumption of MC-containing freshwater fish and shellfish, contaminated crops, and vegetables after irrigation with infested water, as well as other food of animal origin and food supplements such as BGAS (blue-green algae supplements) (Manganelli et al. 2012). The parenteral route is relevant when water bodies containing cyanobacteria are used as the source water for hemodialysis processes. The most serious known episode associated to human exposure to microcystins occurred in Brazil, where 56 out of 130 hemodialyzed patients died after treatment with MC-contaminated water (Azevedo et al. 2002). During recreational activities, beside ingestion (e.g., during swimming, falling into water from a boat, or in case of children, playing along the shore), dermal (due to direct contact with water) and inhalation route (for bathers and professionals, e.g., lifeguards or fishermen) can all be relevant, even all at the same time. The same occurs in relation to the domestic use of contaminated water, as in the case of showering. In addition, dried scum on the shore and cyanobacteria in dried desert crusts can also be a source of inhalation exposure.

Episodes of acute/short-term human intoxications due to drinking water consumption have been reported in some countries as a consequence of failure or inefficiency of water treatment (Falconer 2005): gastroenteritis and liver damages were among the most frequently reported diseases, although other effects have been described as well. The recognition that cyanobacterial cells and MCs represent serious hazards to animal and human health led the World Health Organization (WHO) to define specific guidelines and reference values for MC-LR and the group of MCs, requiring the need to put in place risk management measures to protect human health.

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The chronic risk associated with repeated exposure to MCs in humans through drinking water is difficult to be demonstrated, as information from epidemiological studies is scant and incomplete. However, some toxicity data are available on animal models and can be used for a risk assessment.

In addition, several poisoning episodes of livestock, wild, and domestic animals have been associated with the occurrence of cyanobacterial blooms in surface waters. The cause–effect relationship identified in few studies and the knowledge of the toxin toxicological profile allow to provide hints for preventing poisonings of pets and livestock and estimate the impact on human health due to residues in edible products of animal origin.

Pharmacokinetics/Toxicokinetics

The available information on MC toxicokinetics derives from studies using either intravenous (iv) or intraperitoneal (ip) injections. Few or no data are available on the oral, inhalation, and dermal exposure routes, which are the relevant routes of exposure for the general population.

Since MCs are water soluble in most cases, they are unable to passively diffuse through the lipid membranes of animal, plant, and bacterial cells. Therefore, absorption is an active process, occurring through membrane transporters, such as the organic anion transporters, which otherwise carry essential biochemicals or nutrients. Absorption after inhalation exposure occurs mainly in the alveoli and is rapid after intratracheal instillation in mice. After oral exposure the absorption occurs primarily in the small intestine and only at a limited extent in the stomach. Once absorbed in gastrointestinal (GI) tract or injected into the blood or in the peritoneal cavity, MCs are primarily taken up into the liver. In mammals, the MC uptake into the hepatocytes (Chorus and Bartram 1999) is mediated by the organic anion-transporting polypeptide (rodent, Oatps; human, OATPs) superfamily and by the bile acid transporters (Fischer et al. 2010). Using primary human hepatocytes, the hepatic OATP1B1 and 1B3 have been shown to be congener selective transporter, with the rate of MC-LW and MC-LF entrance >>MC-LR>MC-RR, suggesting a different uptake for some MCs in the liver (Fischer et al. 2010).

The amount and the time course of administrated dose uptake by the hepatic tissue vary greatly with the different routes of exposure (oral or ip/iv injection). Intraperitoneal injection of radiolabel MC-LR resulted in rapid and continuous uptake by the liver, with approximately 72 % of the administered dose present in the liver after 1 h, 1.4 % in the small intestine, 0.5 % in the kidney, 0.4 % in the lungs, and 0.3 % in the stomach (Nishiwaki et al. 1994). Similar results were obtained after iv injection of a sublethal dose of ^{3}H-MC-LR (liver content being approximately 67 % of the dose in 60 min) (Robinson et al. 1991a). On the contrary, after oral administration only less than 1 % of the administered dose was found in the liver at either 6 h or 6 days postadministration (Nishiwaki et al. 1994). The 70 % of the hepatic radiolabel is present in the cytosol bound to high molecular weight proteins identified as the protein phosphatase enzymes (PP1 and PP2A) (Robinson et al. 1991b) resulting in retention in the liver eliciting toxic effects.

The bioavailability of the toxin seems to be largely different depending on the administration route. However, despite the low fraction entering the liver, the occurrence of hepatotoxicity and lethality after oral exposure to MCs is evident.

The distribution of MCs to organs other than the liver depends on the ability of these toxins to cross cell membranes. In fact, MCs are substantially hydrophilic compounds, although with large difference between the congeners, which have to be actively transported into cells. The OATP/Oatp transport system is present beside enterocytes and hepatocytes also in various cell types such as renal epithelial cells and in organs such as the heart, lung, spleen, pancreas, brain, and blood–brain barrier (BBB) (Feurstein et al. 2009). As a consequence, systemic distribution of MC in the organs depends on types and expression level of OATP/Oatp carriers, besides the degree of blood perfusion.

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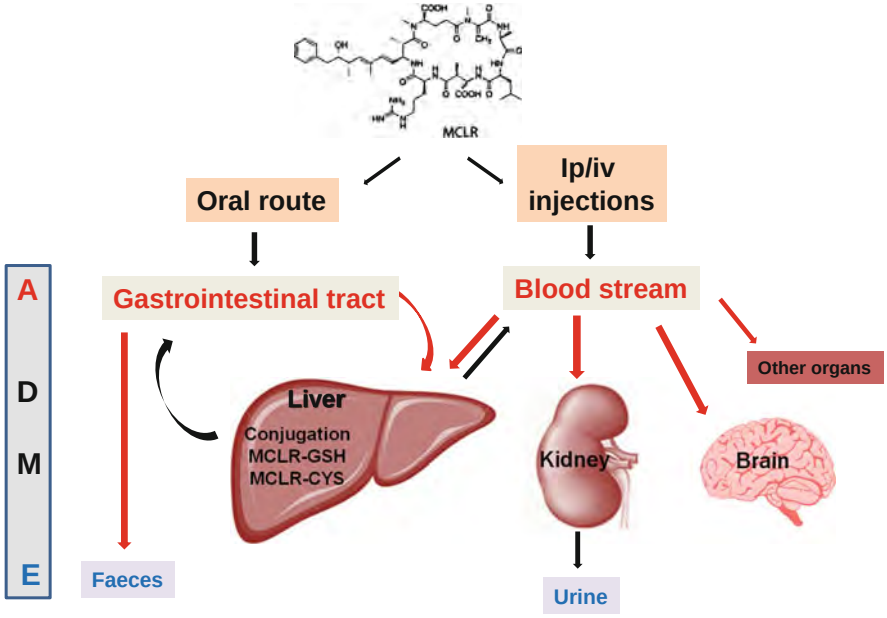

Fig. 4 Schematic MC-LR ADME in mammals (absorption, distribution, metabolism, and elimination) process. MCLR-GSH, MC-LR conjugate with GSH; MCLR-CYS, MC-LR conjugate with cysteine

In null mice, completely lacking the Oatp system, it was demonstrated that after administration of MC-LR the binding of the toxin to the hepatic proteins was much lower compared with wild-type mice, and indeed they showed complete resistance to MC-LR-induced hepatotoxicity, while when treated with the same dose, wild-type mice presented extensive hepatic hemorrhagic necrosis (Lu et al. 2008).

Beside the absorption/distribution processes, biotransformation can be the additional crucial step in defining the toxicokinetic profile of individual congeners. The accepted pathway for MC detoxication is glutathione (GSH) conjugation, which affects the degree of severity of MC-induced toxicity, being the MC conjugates much less acutely toxic in vivo than the parent compound (Ito et al. 2002). A schematic representation of MC-LR ADME (absorption, distribution, metabolism, and elimination) process in mammals is shown in Fig. 4.

The GSH adduct formation can occur spontaneously at alkaline pH and enzymatically, catalyzed by glutathione-S-transferases (GSTs). The conjugation of MC-LR (and seldom of MC-RR) has been evidenced in vitro (cell cultures from different organisms) and in vivo in invertebrates, plants, mussels, fish, and rodents. Recently, the adduct formation between MC-LR and MC-RR and GSH has been characterized also in humans, using in vitro systems as recombinant GSTs and human liver cytosols (Buratti et al. 2013, 2011). A congeners' selectivity in the conjugation efficiency has been evidenced (Buratti et al. 2013), similarly to what observed for transporters. GSTs are a polymorphic family of isoenzymes in human: this aspect may influence the interindividual susceptibility to toxic effects.

The conjugation of MCs with cysteine is also possible: the complex formed after the conjugation or after degradation of the GSH conjugate is transported to the kidneys for the elimination. From the ADME study on mice, approximately 24 % of the MC-LR-administered dose was eliminated in the urine (9 %) and feces (15 %) over 6 days with the 60 % of the excreted MC present as parent compound (Robinson et al. 1991a). In the urine, the presence of free MCs may derive also from the breakdown of conjugates due to the hydrolytic enzymes (e.g., β-lyase) present in the kidney. Consequently, also the kidney can be a potential target for MC-LR toxicity, as evidenced from several studies.

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The only data on the kinetics of MCs in humans derive from the follow-up of dialyzed patients exposed to MCs intravenously in two separate incidents in Brazil (one in Caruaru, one in Rio de Janeiro), when MCs were still present in patients' serum more than 50 days after the exposure (Hilborn et al. 2007).

In conclusion, MC congeners show minimal structural changes, but three- to fourfold differences in the uptake, tissue distribution, and excretion have been reported, evidencing the influence of individual kinetic profiles to explain the differences in toxicity among variants.

Mechanism of Action of Microcystins

Microcystin mechanism of action is associated with specific inhibition of PP1 and PP2A, by interacting with the catalytic subunits of these enzymes (Gehringer 2004). Protein phosphorylation/dephosphorylation is catalyzed by phosphatases and kinases; it is a dynamic process and plays a critical role in the regulation of cell physiology. Therefore, deregulation of specific phosphorylation events as due to the uncontrolled inhibition of PP1 and PP2A can have significant impact in cell's homeostasis. The interaction of MC-LR with PP1 and PP2A was described as a two-step mechanism, where the toxin first binds to the enzyme and subsequently forms covalent adducts (MacKintosh et al. 1990; Craig et al. 1996). The MC Adda-glu group covalently binds with a protein cysteine residue, blocking the access of any substrate in the catalytic site thus inhibiting the enzymatic activity of PP1 and PP2A. Modification on the Adda group has been shown to have very low residual toxicity. However, Adda per se is not able to inhibit PP1 and PP2A, and it is not toxic when intraperitoneally injected in mice even at very high doses (10 mg/kg body weight, wt) (Harada et al. 2004), suggesting the need for a steric hindrance, provided by the toxin molecule, to cause an efficient inhibition.

The in vitro PP1 and PP2A inhibition by different variants is quite similar, with IC_{50} values (concentration inhibiting 50 % of enzymatic activity) in the range of nanomolar concentrations (Vesterkvist et al. 2012).

The pathways involved in MC toxicity are strictly related to the functions of PP1 and PP2A (Campos and Vasconcelos 2010). The effect of MCs on liver tissue organization, hepatocyte morphology, and cytoskeleton structure/dynamics has been related with PP1/PP2A activity: at high levels of exposure (representative of acute intoxication), MC-LR produces a cascade of events (cytoskeleton alterations, lipid peroxidation, oxidative stress, apoptosis), leading to centrolobular toxicity with intrahepatic hemorrhagic areas due to damage of sinusoidal capillaries; at low repeated doses (typical of long-term exposure), phosphatase inhibition induces cellular proliferation and hepatic hypertrophy (Gehringer 2004).

Moreover, it is widely recognized that PP2A has an important tumor suppressor role, since it regulates mitogenic signaling pathways in cancer pathogenesis in mammalian cells; hence, it follows that mutations, modifications, and gain or loss of activity in distinct subunits of the enzyme may be linked to tumor promotion (Campos and Vasconcelos 2010).

In particular, the nuclear phosphoprotein p53, which plays a key role in DNA repair, apoptosis, and tumor suppression pathways, is a substrate of PP2A. Although the MC-LR-mediated p53 regulation via PP2A has not been demonstrated directly, an increase in p53 protein expression has been reported in vitro in apoptotic HepG2 cells, cultured hepatocytes, and rat liver tissues after MC exposure (Campos and Vasconcelos 2010). In addition, two pathways of DNA repair, the nucleotide excision repair (NER) and the DNA double-strand break (DSB) repair, have been shown to be inhibited by MC. Both these DNA repair systems are regulated by phosphorylation mechanisms, and in vitro experiments showed that PP1 and PP2A inhibition significantly decreases the activity of the pathways (Campos and Vasconcelos 2010). Some relevant pathways of the MC-LR mechanism of action are summarized in Fig. 5.

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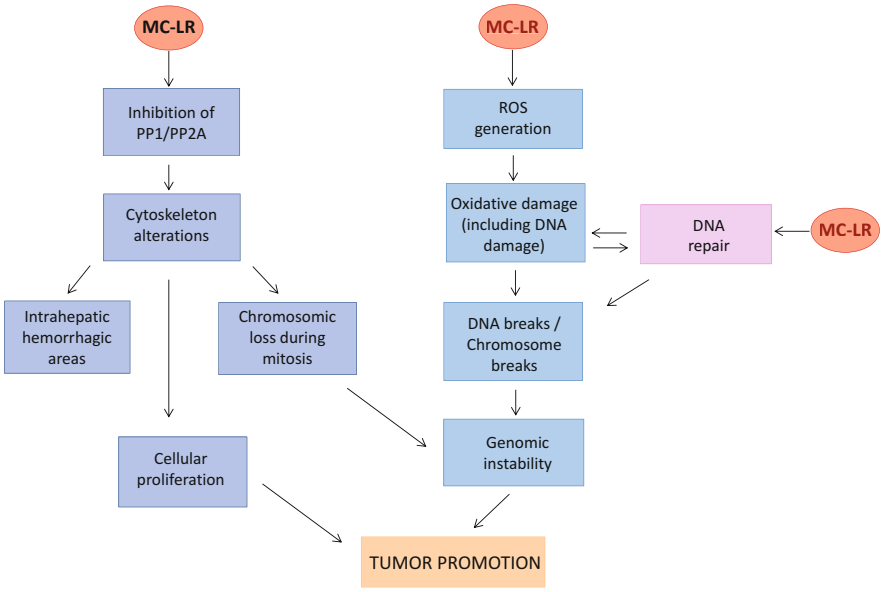

Fig. 5 Possible pathways of the MC-LR mechanism of action

Another biochemical feature of MC toxicity is the capability to produce reactive oxygen species (ROS) and therefore to induce oxidative stress. The ROS generation has been reported both from human cell lines and in several in vivo studies in mice and rat liver, heart, and reproductive system. This feature is intrinsically related with mitochondria metabolism and can conduct to indirect DNA damage and to cell death by necrosis or apoptosis (Campos and Vasconcelos 2010). The underlying mechanisms of MC-mediated ROS production and cell injury are however not clearly characterized. Some authors reported the surge of Ca^{2+} in the mitochondria of cultured rat hepatocytes, as the first event preceding apoptosis induced by MC-LR. It has been hypothesized that the surge of calcium led to the increase of mitochondrial membrane permeability, elevation of ROS formation, loss of mitochondrial membrane potential, and release of apoptotic factors with subsequent cell death; however, other proapoptotic proteins (Bax and Bid) have been also reported to be related to the cascade of events (Campos and Vasconcelos 2010). Another plausible mechanism for ROS generation is the increase of NADPH oxidase activity. In fact, it has been observed that the oxidative stress induced by MC-LR coincides with the upregulation of the CYP2E1 mRNA, an isoform of cytochrome P450 with NADPH oxidase activity, using in vitro systems such as human hepatoma cell line (HepG2). In hepatic cells (primary hepatocytes and HepG2), which likely express OATP activity, ROS induction at nanomolar toxins' concentrations is observed, which are more representative of environmental contamination and of chronic exposure at low doses (Žegura et al. 2011).

The MC toxicity is also characterized by the influx of neutrophils to affected organs, as in the majority of the lesions that occur in humans and animals. The release of proteolytic enzymes and reactive oxygen and nitrogen metabolites by the activity of neutrophils promotes tissue injury and microvascular dysfunction (Babior 2000). The role of neutrophils in the pathogenesis of MC is however poorly characterized, but it has been proposed that MC-LA, MC-YR, and MC-LR play as chemotactic agents in neuthrophil recruitments, playing therefore an important role in inflammatory responses (Kujbida et al. 2009).

Overall, it can be concluded that MC toxicity is a multi-pathway process, whose molecular mechanisms still remain partially unknown and whose mechanisms regulating the specific MC target/interacting

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Table 1 Differences in the ip acute toxicity of MC variants in mice

Toxin	Structure[a]	ip LD_{50} in mice (μg/kg bw)
MC-LR	**Cyclo-(D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)**	50
[D-Asp[3]]MC-LR	Cyclo-(D-Ala-L-Leu-D-**Asp**-L-Arg-Adda-D-Glu-Mdha-)	50
MC-LA	Cyclo-(D-Ala-L-Leu-D-MeAsp-**L-Ala-**Adda-D-Glu-Mdha-)	50
MC-YA	Cyclo-(D-Ala-**L-Tyr**-D-MeAsp-**L-Ala**-Adda-D-Glu-Mdha-)	60–70
[Dha[7]]MC-RR	Cyclo-(D-Ala-**L-Arg**-D-MeAsp-L-Arg-Adda-D-Glu-**Dha**-)	180
MC-YR	Cyclo-(D-Ala-**L-Tyr**-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	150–200
MC-AR	Cyclo-(D-Ala-**L-Ala**-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	250
MC-RR	Cyclo-(D-Ala-**L-Arg**-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	500
MC-M(O)R	Cyclo-(D-Ala-**L-Met(O)**-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	700–800
[L-MeLan[7]]MC-LR	Cyclo-(D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-**L-MeLan**-)	1000
[6(Z)-Adda[5]]MC-RR	Cyclo-(D-Ala-**L-Arg**-D-MeAsp-L-Arg-**6(Z)Adda**-D-Glu-Mdha)	>1200
[6(Z)-Adda[5]]MC-LR	Cyclo-(D-Ala-L-Leu-D-MeAsp-L-Arg-**6(Z)Adda**-D-Glu-Mdha)	>1200

Data from Zurawell et al. (2005)
ip intraperitoneal
[a]Amino acidic differences with respect to MC-LR are indicated in bold

proteins, the signaling pathways leading to the cell's response, and the downstream pathways of toxicity and cell's injury are widely elusive.

Toxicity

Experimental Toxicity

In Vivo Toxicity After Single Administration

The acute in vivo toxicity studies after single administration are generally used for the LD_{50} derivation (Table 1). LD_{50} is the dose able or expected to cause the death of the 50 % of the treated animals within 14 days after the day of treatment, so it indicates the relative intrinsic hazard of a compound when compared to other ones (Funari and Testai 2008). Gavage and intraperitoneal (ip) injection have been the most frequently adopted route of toxin exposure, but the choice of administration method influences the toxicity. In fact, the MC-LR acute toxicity after ip administration to mice results in a $LD_{50} = 50$ μg/kg bw (body weight); when given by the oral route, MC-LR is less toxic ($LD_{50} = 5000$ μg/kg bw) (Funari and Testai 2008). This lower acute toxicity (30- to 100-fold) is likely due to toxicokinetic differences. In fact, the active transport system, which permits the MC absorption through the GI mucosa, is bypassed by ip injection. Consequently, the MC-LR bioavailability is increased after ip injection, as described above in mice (Nishiwaki et al. 1994; Robinson et al. 1991a), and this administration regimen is not fully representative of the actual conditions of human exposure, which is mainly associated with consumption of possibly contaminated drinking water and food, and can be only considered as a "worst case" in the framework of risk assessment.

The MC variants are characterized by different acute hepatotoxicity: ip LD_{50} in mice is spread in a wide range of values (from 50 up to 1200 μg/kg) (Table 1) due to the presence of different substituents, although the value is not available for some congeners, such as MC-LW and MC-LF (Funari and Testai 2008). In fact, MC-RR and MC-LR differ only for the polar amino acid in position 2 (arginine vs leucine) (Fig. 1), but this minimal structural change results in a tenfold difference in the acute toxicity (ip LD_{50} in mice 500 and 50 μg/kg, respectively). On the other hand, the presence of hydrophobic amino acids, such

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as alanine or phenylalanine (MC-LA, MC-YR, YM), does not affect the acute toxicity (see Table 1) (Funari and Testai 2008). Since at least in vitro the inhibition of phosphates is similar among variants, the different acute toxicity has been attributed to differences in kinetics (Buratti et al. 2013; Vesterkvist et al. 2012).

Limited to MC-LR, it has been possible to estimate a threshold for acute damages in the liver of about 25 μg/kg bw, that is, the highest dose at which no hepatic effects are observed, derived from ip injection studies in mice (Fromme et al. 2000).

Alteration of renal functionality and physiology was also evidenced in rats, with a single ip injection of MC-LR sublethal dose (55 μg/kg) (Lowe et al. 2012).

In Vivo Toxicity After Repeated Administration

The in vivo studies after repeated exposure are used to evidence subacute, subchronic, and chronic toxicity effects and to determine the repeated toxicity threshold dose (no observed adverse effect level, NOAEL). This parameter is utilized in the risk assessment to determine a safe limit reference value (e.g., the tolerable daily intake, TDI).

The most relevant repeated toxicity study has been carried out with mice, shown to be more susceptible to MC-induced acute effects than other rodent species. When MC-LR was administered orally (by gavage) for 90 days, the NOAEL for induction of hepatic effects was 40 μg/kg bw/day (Fawell 1993). It is noted that, due to toxicokinetic differences, when mice were subchronically administered with MC-LR through the diet, a regimen more similar to human exposure, the NOAEL value was higher (333 μg/kg bw/day) (Schaeffer et al. 1999). In fact, the bolus dose, which is given by gavage, leads to higher tissue concentrations of toxin than those obtained after the more gradual introduction with a dietary treatment, giving time to the detoxification/excretion systems to be efficient.

Beside the liver, the kidney is a target for MC toxicity, as also shown in rats repeatedly treated (by ip injections) with sublethal doses (10 μg/kg) of MC-LR and MC-YR; the mechanism of renal damages is similar to the one for hepatotoxicity (Milutinović et al. 2003).

Other Toxic Effects

Potential local toxic effects at the site of contact have been tested with freeze-dried algal aqueous suspensions from both *Microcystis* and *Anabaena* blooms: very low potential for skin irritation and contrasting results for eye irritation were obtained. Although positive results were obtained in the skin sensitization test, components other than MC are likely to be present in the algal extract, with possible irritation and sensitizing potential. Indeed, no correlation was found between the MC content in the extract and the allergic reaction, and pure MC-LR showed only slight skin sensitizing potential.

When tested as a pure toxin, MC-LR did not result teratogenic in mice, in cultured mouse embryos, and in frog embryos. More recently, some studies reported the presence of in vivo toxic effects on male reproductive system with reduction of testosterone level, testicular atrophy, and a high incidence of dose- and time-dependent sperm abnormalities after subacute ip or chronic administration by oral route with MC-LR. Considering the lack of information about the OATP expression in testes, it is not clear how MC-LR might enter in the cells and cause damage. Also the studies reporting effects on the female reproductive system after ip injection have some limitations. Reproductive toxicity by MCs thus remains a potential hazard, although the weight of evidence of the available studies seems to indicate that the reproductive toxicity of MCs is, if any, limited.

Recently, some reviews have been published where, considering the available data, the direct interaction with DNA responsible for direct genotoxic activity has been reasonably excluded (Žegura et al. 2011). Indeed, contrasting results have been reported and positive results have been obtained both in vivo and in vitro at highly cytotoxic doses, suggesting the involvement of DNA endonucleases. In

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addition, as described above, induction of DNA oxidative damage and DNA repair inhibition may contribute to genomic instability (Žegura et al. 2011). Furthermore, the tumor-promoting activity of MC-LR was described early in cyanotoxin research (see in Žegura et al. 2011) and confirmed by MC-LR administration with known carcinogenic compounds, such as aflatoxin B1 and diethyl-nitrosamine (see in Funari and Testai 2008). On the contrary, MC-LR did not show any direct tumor induction when the cyanotoxin was given to mice by gavage for 28 weeks (80 μg/kg bw/day) (Ito et al. 1997).

On the basis of data on tumor-promoting mechanisms, MC-LR has been classified as a possibly carcinogenic to humans (Group 2B by the International Agency for Research on Cancer) which considered the available studies not to indicate any primary carcinogen action by MC (IARC 2006).

MC-LR is the most studied among variants, due to its high acute toxicity; recently, some more hydrophobic congeners such as MC-LW and MC-LF (with tryptophan (W) or phenylalanine (F) replacing arginine (R)) have been shown in vitro to be more cytotoxic in human hepatocytes, in embryonic cells of kidney, which express OATP, and in Caco-2 cell line (Vesterkvist et al. 2012). In addition, in a murine brain cell line, congener- and concentration-dependent pronounced neurodegenerative effects have been evidenced (MC-LF>>MC-LW>MC-LR) (Feurstein et al. 2011). These results suggested that MC neurotoxicity might be linked to neurodegeneration, and a role for cyanobacteria in the etiology of amyotrophic lateral sclerosis (ALS) and Alzheimer's disease has been also proposed (Stipa et al. 2006).

Human Toxicity

The most important episode of human health consequences has been reported in Brazil in 1996, where 56 patients out of 130 in hemodialysis treatment died after receiving water from a nearby reservoir that subsequently turned out to be contaminated by MCs (Azevedo et al. 2002). The estimated concentration of MCs in the treated water used for hemodialysis was 19.5 μg/L. Despite the increased attention and control, a further episode in Rio de Janeiro in 2001 occurred, when 44 patients were found to have been sublethally exposed to MCs. The parenteral route is a specific concern, due to the particular exposure together with the pathological conditions of patients, requiring large volumes of high-quality water for their treatment régimes.

The toxicological effects in humans exposed orally or dermally or by inhalation to water infested by cyanobacterial blooms producing MCs and to ingesting contaminated food items include symptoms ranging from gastroenteritis, abdominal pain, flu-like symptoms, ear and eye irritation, and rashes to kidney and liver damage resulting in some rare cases in fatal outcomes. However, all the studies reporting episodes of human exposure to cyanobacterial blooms in environmental settings cannot exclude co-exposure to multiple cyanobacterial toxins and/or other etiological agents which can contribute to the evidenced symptoms.

Recently, a case study of acute intoxication after recreational exposure to MCs has been reported. A young boy in Argentina accidentally remained for 2 h in a freshwater lake with an intense bloom of *Microcystis* spp. (MC-LR level in water was 48.6 μg/L). Four hours after the exposure (direct contact, ingestion, and inhalation) to contaminated water, signs as nausea, abdominal pain, and fever were evident. Subsequently, dyspnea and respiratory distress appeared, and an atypical pneumonia was diagnosed (after exclusion of the presence of bacteria, viruses, and fungi in the water). Finally, the patient developed a hepatotoxicosis with a significant increase of hepatic damage biomarkers (ALT, AST, and γ-GT) (Giannuzzi et al. 2011).

Numerous reports of skin irritations and local effects associated with recreational contact with MC-producing cyanobacteria in lakes or freshwater basins exist in addition to epidemiological studies, used by the WHO as the basis for the guidelines for recreational water (WHO 2003) to prevent the risk due to MC exposure. The study presents some limitations, including the limited number of exposed

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individuals and the lack of correlation between MC levels and the observed effects. Another study Stewart et al. (2006) considerably more accurate with 1331 recruited individuals from 19 lakes and basins in Australia and Florida evidenced a statistically significant increase of respiratory effects in groups exposed to an high cyanobacterial cell surface area (>12 mm^2/mL). Very recently, the American Center for Disease Control (CDC) has published data of health surveillance about the algal bloom – associated disease outbreaks among users of freshwater lakes for 2009 and 2010. In 11 cases (46 %) MCs were identified as the suspected (MC <20 μg/mL) or true etiological agent (MC level ≥20 μg/mL) (Hilborn et al. 2014). Whenever contact exposure was reported, affected persons developed rash or skin irritation; in each of the outbreaks for which ingestion exposure was reported, affected persons had gastrointestinal signs or symptoms, and in outbreaks for which inhalation exposure was reported, affected persons had respiratory signs or symptoms (Hilborn et al. 2014).

The consumption of raw untreated waters infested by cyanobacteria or with high cyanotoxin-dissolved concentrations in drinking water, as a consequence of either the breakdown of a natural cyanobacterial bloom or its artificial lysis followed by the failure of water treatments, can be the cause of acute/short-term effects. Several other episodes of gastroenteritis and liver damage potentially associated with drinking water contaminated by MCs have been reported. In particular, an acute episode was reported in Brazil, and a consequent gastroenteritis epidemic with 2000 cases, 88 deaths in 42 days, was attributed to the *Anabaena* and *Microcystis* bloom (see in Funari and Testai 2008).

Considering that in industrialized developed countries the water treatments are generally correct and adequate, drinking water is mainly a source of chronic exposure together with the food (as fishery products) and the food supplements.

In the past, several epidemiological studies have been carried out to link MC contamination with tumor induction, but also owing to some limitations in the study design, they failed to find any valid correlation, and it was not possible to prove that MCs were the actual etiological agents of the observed effects (IARC 2006).

On the other hand, the proved tumor-promoting mechanism of MCs in animals can help in the interpretation of data coming from an epidemiological study in Serbia. In this country more than 80 % of reservoirs used for water supply have bloomed over the past 80 years, with levels of MC-LR up to 650 μg/L in the reservoir and 2.5 μg/L in the tap water twice higher than TDI of WHO (Svircev et al. 2013). The study evidences that in the last 10 years, there was a significant increase in the incidence of primary liver cancer (PLC) in the regions where these waters were used for human consumption. Not being any correlation with other risk factors, such as cirrhosis and hepatitis viruses, it is possible that MCs, causing liver injury and acting as tumor promoter, might act synergistically with other risk factors to cause increased incidence of PLC (Svircev et al. 2013).

Regarding exposure through the diet, fish consumption is probably the main source. It is not clear how MCs can be accumulated in the fishery products considering their hydrosolubility, and it has been hypothesized to occur more a bio-dilution than a biomagnification along the food chain (Berry 2013). On the other hand, the presence of MCs in the fish used for human supply underlines the plausibility of this route of exposure also without biomagnification.

The few data available on the effects associated with the consumption of contaminated food have been obtained on Chinese population. Thirty-five fishermen were exposed chronically to MCs associated to the consumption of aquatic products (shrimps, fish, birds) and water, at a range 2.2–3.9 μg MC-LR eq (in their serum an average level of 0.39 ng/mL MC-LR eq was measured). In 11 fishermen the circulating MC level correlated positively with hepatic damage biomarkers (as ALT, AST, LDH, and ALP): although the 90 % of enrolled subjects was negative to hepatitis antibodies, other confounding factors were not considered (Chen et al. 2009).

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Another study evaluated 1322 Chinese children (7–15 years old) who consumed water, fish, and ducks from two lakes and four wells. Considering the mean concentration of MCs in the food items and their average consumption over 5 years, the estimate intake was 0.36 and 2.03 μg/day for the low- and high-exposure group, respectively. The biomarkers ALT and ALP were higher in the high-exposure group of children, excluding from the analysis HBV-positive subjects or potential hepatotoxic medicine users (Li et al. 2011).

Recently, the use of food supplements containing blue-green algae, the so-called BGAS products, begin to be assessed for their potential risk for human health associated to MC contamination. *Spirulina* spp. and *Aphanizomenon flos-aquae*, used in BGAS production, are usually collected from the natural environment, and MC-producing cyanobacteria contamination has been demonstrated, with variable levels of MC different variants (MC-LR, MC-LA, MC-RR) up to 35 μg/g dry weight (Vichi et al. 2012). They are generally proposed as health-promoting natural products for a number of purported beneficial effects and even given to children for the treatment of the attention deficit hyperactivity disorder (ADHD). The easy availability (over the counter or via Internet) and the customers' conviction that "natural is safe" can induce to assume high dosage making BGAS use a potential public health problem (Vichi et al. 2012).

Conclusion and Future Directions

The hazards of MCs toward human and animal health is nowadays recognized, and strategies to manage the health risks have been formulated, based on the quantitative data available on the toxicity of MCs to mammals via oral dosing, to enable tolerable daily intakes to be estimated. Regarding exposure via drinking water, the WHO elaborated guidelines and adopted a provisional guideline value (GV) for MC-LR, following the internationally agreed risk assessment procedure (WHO 2004). In order to derive the TDI (i.e., a dose to which an adult with a body weight of 60 kg could be orally exposed daily all lifelong, without experiencing any toxicological effect), the WHO used the subchronic NOAEL = 40 μg/kg bw/day obtained with mice by oral dosing as point of departure (PoD), since human data were not sufficiently robust to be used for the derivation of a health-based value. The PoD was divided by an uncertainty factor (UF) of 1000, obtained by using a combined factor of 10 × 10 × 10, to take into account: (1) interspecies variability, (2) intraspecies variability, and (3) lack of lifetime or chronic toxicity data, obtaining a TDI value = 0.04 μg/kg bw/day. On this basis, a GV of 1 μg/L for the total concentration of MCs in drinking water was established, by using an allocation factor (AF) of 0.8, assuming that potential daily exposure via drinking water would account for 80 % of the total ingestion of MC, via a daily consumption of 2 L of drinking water:

$$\mathrm{GV} = \mathrm{TDI} \times \text{body weight} \times \mathrm{AF} = (0.04\ \mu\mathrm{g/kg} \times 0.8 \times 60\ \mathrm{kg}) :\ 2\mathrm{L} = 1\ \mu\mathrm{g/L}.$$

Therefore, drinking water should not contain more than 1 μg MC/L; this can be achieved with adequate treatments that allow a strong reduction of both cell number (>99 %) and dissolved cyanotoxins (Dietrich and Hoeger 2005).

Since oral toxicity data for other MC variants are not known, the GV is considered valid for total MC concentrations expressed as MC-LR equivalents, although the validity of extrapolation from the acute toxicity ranking obtained via ip injection to the oral route, which is the most relevant for chronic toxicity, remains to be demonstrated.

Although MCs are the most studied among cyanotoxins, the toxicological profile has been only partially characterized only for one variant (MC-LR). Additional efforts have to be put in elucidating both the levels of human exposure to MCs under different scenarios and the identification of toxicological

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and kinetic profile for variants other than MC-LR. In addition, cyanobacterial blooms are generally characterized by the presence of multiple toxins; therefore, human exposure to a mixture of MC variants as well with other cyanotoxins via different routes and media (e.g., drinking water, contaminated food, bathing activity) is very likely. Despite this, the significance of exposure to mixtures of cyanotoxins, interactions with other cell components/bioactive compounds, or other contaminants have not been sufficiently investigated so far and represent a challenge for the future in the area of cyanobacteria.

Cross-References

- ▶ Other Marine and Freshwater Toxins
- ▶ Saxitoxin and Other Paralytic Toxins: Toxicological Profile
- ▶ Saxitoxin and Shellfish and Other Neurotoxins

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Saxitoxin and Other Paralytic Toxins: Toxicological Profile

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Abstract

Toxic microalgal blooms that produce paralytic shellfish poison (PSP) have increased worldwide in frequency, duration, and extension of affected areas, with severe impacts on human health, local economies, and exports. PSP is a variable mixture of tetrahydropurine marine biotoxins collectively named as saxitoxins (STXs) that are produced by dinoflagellates from the genera *Alexandrium, Pyrodinium,* and *Gymnodinium.* Harmful algal blooms of these species (named "red tides") produce accumulation of saxitoxins in shellfish, fishes, and other organisms. Ingestion of contaminated tissues may lead to paralytic shellfish poison intoxications in humans and deaths by muscle paralysis and cardiorespiratory failure. Tetrodotoxins (TTXs) are compounds chemically different from STXs, synthesized by cyanobacteria present in freshwater ecosystems that produce a similar paralytic syndrome in cattle, birds, and fishes. TTXs (and STXs) are also present in puffer fishes and cause also human intoxications. In addition, saxitoxins and tetrodotoxins are produced by a number of other species that may represent additional health risks. The severe public health and economic impacts of PSP intoxications brought the attention of researchers almost 80 years ago. Since then a large body of scientific studies on PSP shellfish toxins has accumulated. In recent years an international regulatory effort has been developed to assess, manage, and control health risks caused by PSP toxins and other marine toxins. However, in many countries of the developing world, monitoring and management programs for marine biotoxins in products destined for domestic consumption are limited in scope, geographical extension, frequency, and methodologies. Evidence from physiological, toxicological, and risk management studies is examined that may indicate that current approaches to manage risk and to protect consumers may be still insufficient, especially for underdeveloped countries. Strategies for future work are suggested.

Introduction

The molecular target of saxitoxins (STXs) and tetrodotoxins (TTXs) is the voltage-dependent sodium channel, a transmembrane protein that undergoes subtle conformational changes upon activation by voltage. These molecular movements open a pore within the protein that conducts sodium ions across the cell membrane generating action potentials in excitable neuronal, endocrine, and muscle cells (Catterall 2014). Saxitoxin and tetrodotoxin and their derivatives block ion conduction causing inhibition of action potentials, inducing respiratory paralysis and death (Hall et al. 1990; Haddad Jr, ► Intoxications Caused by Saxitoxin, Shellfish, and Other Neurotoxins, this volume). Saxitoxin is the most toxic parent compound of a group of over 50 known tetrahydropurines (Thottumkara et al. 2014; Wiese et al. 2010). Just 1–4 mg can cause death in humans, while other derivatives display a range of toxicities that range from 100 % to 0.05 % maximal value that are caused by structural molecular modifications (Oshima 1995). The potent toxicological effect is due to the extremely high affinity of saxitoxin for its receptor site

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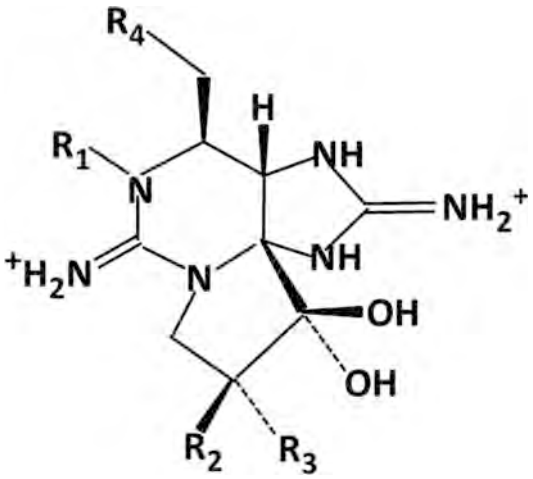

Fig. 1 The general structure of saxitoxins (Adapted from Oshima 1995)

located in the outer amino acidic structure of the sodium channel (Suarez-Isla 2008). Bioaccumulation of saxitoxins in filter-feeding bivalves and other marine species may reach concentrations that can cause human illnesses and death upon consumption. This is the cause of thousands of human cases of paralytic shellfish poisoning occurring every year, affecting mostly poor coastal communities worldwide (Callejas et al. 2015; Hernandez-Orozco and Garate 2006; Rodrigue et al. 1990; Yen et al. 2006). Monitoring programs have been developed in several countries to assess, manage, and prevent the severe health and economic risks associated with the international trade of contaminated shellfish and fish (Anderson et al. 2001; EFSA 2009a; EFSA: European Food Safety Authority). In many countries, monitoring and management programs for marine biotoxins in products destined for domestic consumption are limited in scope, geographical extension, and methodologies.

This review examines current knowledge on toxicology and toxicodynamics of saxitoxins. Recent advances on risk analysis are compared with previous comprehensive assessments on the saxitoxin group (EFSA 2009a; Paredes et al. 2011) to identify specific gaps in toxicological knowledge, analytical uncertainties, and limitations of monitoring and risk management policies that may still pose a significant public health risk.

Several comprehensive reviews are available on saxitoxins (EFSA 2009a; Lawrence et al. 2011; Otero 2014; Vale 2014) and tetrodotoxins including their chemistry (Ashihara et al. 2013; Choi et al. 2012; Ciminiello et al. 2012; Thottumkara et al. 2014; Wiese et al. 2010), pharmacology and toxicology (Daneshian et al. 2014; Llewellyn et al. 2006), detection methods (Humpage et al. 2010; Suarez-Isla 2008), and distribution and dynamics of toxic phytoplankton species (Weinstein 2013).

The Saxitoxins

Saxitoxin (STX) is an alkaloid that belongs to a large group of marine natural products that contain guanidino groups as their main structural components. The presence of two guanidino groups in STX explains the high polarity of this compound. The saxitoxin group includes over 50 analogues (Wiese et al. 2010) that differ in the functional side group R1, R2, R3, and R4 (Fig. 1). The best studied ones are hydrophilic and were isolated from contaminated shellfish and/or cultured dinoflagellates. According to the functional group on the R4, there are carbamoyl, decarbamoyl, and N-sulfocarbamoyl saxitoxins, and toxicities measured by the mouse bioassay decrease in the order carbamoyl > decarbamoyl > N-sulfocarbamoyl (Table 1) (Oshima 1995). The carbamoylated analogues

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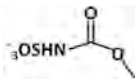
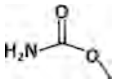
Table 1 The relative toxicities of the main saxitoxin congeners

R1	R2	R3	R4		R4		R4	
			N-sulfocarbamoyl		carbamate		decarbamoyl	
								
				MU/μmol		MU/μmol		MU/μmol
H	H	OSO_3^-	C1	15	GTX2	892	dcGTX2	382
H	OSO_3^-	H	C2	239	GTX3	1,584	dcGTX3	935
H	H	H	B1		STX	2,483	dcSTX	1,274
OH	H	OSO_3^-	C3	33	GTX1	2,468	dcGTX1	
OH	OSO_3^-	H	C4	143	GTX4	1,803	dcGTX4	
OH	H	H	B2		NeoSTX	2,295	dcNeoSTX	
H	H	H	GTX5 (B1)	160				
OH	H	H	GTX6 (B2)	180				

Adapted from Oshima 1995 and EFSA 2009a. MU: mouse unit

comprise the most toxic group members, such as STX, neoSTX, and gonyautoxins 1- and 4 (GTX1, GTX4). Loss of the carbamoyl moiety reduces toxicity significantly as is the case for the resulting analogues GTX2 and GTX3, respectively. The presence of a sulfamate group in position R4 generates the less toxic subgroup of B1 and B2 toxins (alternative designations GTX5 and GTX6, respectively). Metabolic transformations of the original mixture of saxitoxins present in the toxic dinoflagellate can take place in the shellfish (or other primary vectors) and other members of the trophic chain, including humans. These biochemical reactions can increase the total toxic burden and, rarely, decrease it. The most common modifications include N-oxidations and carbamoyl hydrolysis. Knowledge of the original composition and ensuing specific changes are essential for a correct risk assessment (Munday and Reeve 2013).

Producing Organisms and Vector of Poisoning

Saxitoxins are produced by marine dinoflagellates from the genera *Alexandrium*, *Pyrodinium*, and *Gymnodinium*, eukaryotes that are distributed worldwide (Anderson et al. 2012a, b). Dinoflagellate species such as *Alexandrium catenella*, *Alexandrium tamarense*, and *Gymnodinium catenatum* generate harmful algal blooms that can reach up to 1×10^4 cells/L or higher. These blooms that rarely produce water discolorations (red tides) display complex distribution patterns in the water column (McGillicuddy et al. 2014). Blooms of these species may lead to PSP toxin accumulation in shellfish and fishes and PSP intoxication and human deaths by cardiorespiratory failure upon ingestion of contaminated tissues. The most common toxin vectors for human intoxications are filtering bivalves, but other species such as gastropods, echinoderms, tunicates, and ascidians can accumulate toxic levels of saxitoxins (Bricelj and Shumway 1998; Deeds et al. 2008; García et al. 2015; Zamorano et al. 2013) and other marine biotoxins. Accumulation of PSP toxins in sardines (Costa et al. 2010) and salmon (Cembella et al. 2002; Sephton et al. 2007) has been established although at sub-toxic levels. In addition, saxitoxins and tetrodotoxins are produced by a number of species that represent an additional health risk. Fugu fish poisoning is caused by TTX and derivatives present in the fish tissues (Arakawa and Nagashima, ▶ Pufferfish Poisoning and Tetrodotoxin, this volume). These toxins are also produced by prokaryotes from freshwater environments, such as filamentous cyanobacteria that include *Anabaena circinalis*, *Aphanizomenon gracile*,

Cylindrospermopsis raciborskii, and *Lyngbya wollei* (Kellmann et al. 2013). Cyanobacteria blooms have caused livestock intoxications and contamination of freshwater reservoirs (Fitzgerald et al. 1999). Monitoring programs in drinking water in Australia, New Zealand, and Brazil (Costa et al. 2010; Humpage et al. 2010) have been implemented to address these hazards.

Precise knowledge of dinoflagellate bloom distribution and dynamics and possible triggering factors is limited to a few coastal systems (Crespo et al. 2011; Kleindienst et al. 2013; McGillicuddy et al. 2014; Vale et al. 2008). Causal relationships between cyst abundance and distribution with bloom initiation and toxicity levels are complex (Cox et al. 2008), and in spite of the quality of field information in specific well-studied ecosystems (Anderson et al. 2014), current results still have limited applicability for resource management decisions (Boesch et al. 1997).

Correlations between PSP accumulation and changes in global or local weather parameters are very complex, and key initiation factors for harmful algal blooms are still elusive. Moore et al. (2009) examined short term and changes in weather parameters and concluded that PSP accumulation in mussels seemed to be favored by a combination of high air and water temperatures and low stream flow, conditions that favor water column stability and stratification. McGillicuddy et al. (2014) have proposed a prediction model for HAB (harmful algal bloom) occurrence and severity in the Gulf of Maine that is based on extensive sets of environmental observations. However, predictors are rather general and do not provide sufficient spatial resolution for short-term management decisions. As a result of these difficulties, monitoring programs for PSP toxins have relied almost exclusively on toxin analyses in shellfish tissues and, in some instances, also on phytoplankton monitoring (Anderson et al. 2001).

Epidemiological Data

PSP intoxication symptoms include gastrointestinal disorders and several manifestations of neurological nature that result from sensory, cerebellar, and motor function impairment after sodium channel blockade by saxitoxins.

Depending on the severity of intoxication, first symptoms can appear within 0.5 and 2 h after ingestion, followed by a variable development phase of up 12 h (Murphy 1936; Gessner et al. 1997a; Gibbard and Naubert 1948; Montebruno 1993; Tenant et al. 1955). Patients may display oral and facial paresthesias, dysphagia, weakness of lower and upper limbs, abdominal pain, and dyspnea due to respiratory muscle paralysis. This may result in respiratory arrest, cardiovascular failure, coma, and death if untreated (Batoreu et al. 2005; Long et al. 1990; Montebruno 1993). Alert patients may report swallowing difficulties, double vision, headache, and dizziness (Hurley et al. 2014). A survival after 12 h has a good prognosis of recovery without long-term effects. The lethal average dose (LD_{50}) of saxitoxin is dependent on the animal species, age, and the administration route. In humans LD_{50} ranges from 1 to 4 mg depending upon age and physical condition of the patient (Lawrence et al. 2011). Intraperitoneal (i.p.) injection of STX in rats causes a LD_{50} of 10–12 μg STX equivalents/kg and 9–10.6 μg STX equivalents/kg in mice. However, orally administered STX has an $LD_{50}$of 260–263 μg STX equivalents/kg. These values have been critically reviewed by Munday and Reeve (2013) that advocate a complete revision to obtain a better estimation of specific toxicities in experimental animals employing oral administration.

Human Studies

Garcia et al. (2005) examined four intoxicated patients that were successfully treated during a major PSP outbreak in the Island of Chiloe in southern Chile (47°S) (March–May 2002). Patients had eaten a few ribbed mussels (*Aulacomya ater*) that accumulated 8,575 μg STX equivalents/kg. Gonyautoxins GTX2 and GTX3 were detected in shellfish tissue, serum, and urine. Pathological signs such as hypoxia, low

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arterial O_2 pressure concomitant with respiratory acidosis, and bradycardia as a consequence of respiratory failure were observed. Treatment included respiratory support, vasoactive drugs, and diuretics. In this case patients were stabilized within 8 h and recovered completely after 48 h. As reported previously (Hurley et al. 2014; Price et al. 1991), patients did not report subsequent long-lasting effects, confirming previous studies (Stafford and Hines 1995).

It is important to notice that there is no specific treatment for PSP intoxication and there are no antidotes approved for human treatment. First care symptomatic treatment during the initial phase is crucial for later recovery as PSP intoxication is a life-threatening emergency characterized by several signs and symptoms (Garcia et al. 2005; Hurley et al. 2014). There is no substitute for rapid recognition of the specific symptoms and of the circumstances of the accident by the medical team. Early diagnosis may attribute symptoms to other causes, such as intoxication with drugs of abuse, psychiatric disorders, or cerebrovascular accidents. Patients may need intubation, evacuation of stomach content, and ventilator support. The use of diuretics and close monitoring of cardiovascular parameters are important, as cases of hypertension and tachycardia have been reported (Gessner et al. 1997b; Hurley et al. 2014). Severely affected patients may become ataxic and will not respond to usual tests, but maintenance of emergency care is essential as severe cases that may resemble coma or brain death symptoms have been reported to recover. Thus, special focused training on emergency procedures and availability of respiratory aids are essential.

Toxicokinetics

Human Studies

Due to the life-threatening nature of PSP intoxications, clinical reports on human intoxications and findings about toxicokinetic parameters of saxitoxins are infrequent, as sampling and measurements have to be made in an emergency room setting. In spite of these constraints, it could be determined that saxitoxins were rapidly eliminated by urination, the main route of toxin excretion (Gessner et al. 1997a; Montebruno 1993). Initial studies suggested that elimination took place apparently without modification. However, later studies provided the first evidence of specific metabolic and detoxification routes for saxitoxins in humans that could occur in the gastrointestinal tract and liver. In fact, composition of saxitoxins found in shellfish was different from that found in blood and urine samples of patients during a toxic outbreak in Kodiak, Alaska (Gessner et al. 1997b).

Postmortem analysis of tissues of a fisherman after ingestion of highly toxic crab meat indicated the presence of saxitoxin in urine, liver, and blood (Llewellyn et al. 2002). The additional detection in urine of neoSTX that is produced by N-oxidation from STX and of decarbamoyl STX via STX hydrolysis of the carbamoyl moiety confirmed metabolic modifications of saxitoxins.

Analysis of tissues and body fluids from two fishermen that died 2–4 h after ingestion of highly toxic ribbed mussels (Aulacomya ater; 8,575 μg STX equivalents/kg as measured by mouse bioassay) detected PSP toxins such as GTX1, GTX4, GTX5, GTX2, and GTX3 in the gastric content and neoSTX and GTX4/GTX1 epimers in urine and bile (Garcia et al. 2004). As neoSTX was not detected previously in gastric fluids, it was suggested that it was an N-oxidation product from the original STX. Similarly, GTX1/GTX4 epimers should have been produced by an oxidizing metabolic step from GTX2/GTX3 during the time between toxin ingestion and death. Later in vitro studies using fresh human hepatic extracts have confirmed that saxitoxins can be N-oxidized and glucuronidated as many other xenobiotics, using a common catabolic pathway that would be expected to contribute to PSP detoxification in humans (García et al. 2009, 2010). The glucuronidated saxitoxins may have gone undetected in previous studies.

Analysis of fluid samples from a surviving intoxicated patient that ate a large portion of cockles contaminated with saxitoxins during a bloom of *Gymnodinium catenatum* in the Portuguese northwest

coast indicated also a significant modification of the original shellfish profile (Rodrigues et al. 2012). Urine samples showed an increase in the molar percentages of dcSTX, neoSTX, and GTX5. In contrast congeners with an O-sulfate at C11 that were abundant in bivalves could not be detected in urine.

Recent studies report that total PSP toxicity is eliminated with a half-life between 10.4 and 12.6 h (DeGrasse et al. 2014). These are values consistent with previous observations of patient recovery times after intoxication. These authors could also demonstrate that the sulfated derivatives of STX and neoSTX, namely, GTX1/GT4 and GTX2/GTX2 epimers, had a shorter half-life of elimination.

Animal Studies

A large fraction of saxitoxin was eliminated intact in the urine of rats after intravenous injection during the first 24 h (Stafford and Hines 1995) as determined by pre-oxidation high-performance chromatography. A slower phase that lasted another 36 h could be detected, pointing to a biphasic process. Intravenously injected STX in anesthetized cats (Andrinolo et al. 1999) was mainly eliminated via glomerular filtration without accumulation in body fluids. Similar findings were observed in cats after oral administration of sublethal doses of gonyautoxins GTX2/GTX3 epimers (Andrinolo et al. 2002a). Interestingly, STX has been detected in cat (Andrinolo et al. 1999) and rat brain tissues (Cervantes Cianca et al. 2011), a finding that has not been observed in humans. Metabolic modification of saxitoxins and involvement of xenobiotic mechanisms have been well established in rodents (Hines et al. 1993; Hong et al. 2003; Naseem 1996) and fishes (Bakke and Horsberg 2010; Gubbins et al. 2000). In vitro transport experiments of intestinal absorption in rat and human cell lines using GTX2/GTX3 epimers have provided evidence for an active transport system for saxitoxins (Andrinolo et al. 2002b).

Toxicity

In Vivo Toxicity After Repeated Administration

Several marine biotoxins are slowly eliminated, and shellfish can maintain detectable toxin levels below the regulatory limit during a detoxification phase that may last months or years (Deeds et al. 2008). Some shellfish species can retain saxitoxins for considerable periods of time (Shumway et al. 1994). In order to address this long-term exposure risk, the determination of tolerable daily intake (TDI) values is needed. TDI is defined as "the daily intake of a chemical in food that, in the light of present knowledge, can be consumed every day for a lifetime with no appreciable harmful effects" (Lawrence et al. 2011). TDI values have to be determined by repeated dosing animal studies following approved protocols (OECD 2008, 2009a). This type of normalized studies is practically absent in the marine biotoxin literature, and this has precluded EFSA (2009a) to define TDIs for this group of toxins.

Other studies using repeated doses are infrequent and contradictory. Early findings by Sommer and Meyer (1937) described that an i.p. injection of mice with a toxic shellfish extract made them more susceptible than control animals to a second dose. In contrast, many years later McFarren et al. (1961) reported that an initial sublethal dose of STX made the animals more resistant to a later administration. Recently, in a search for nontoxic levels of neoSTX to be used in experimental local anesthesia, doses of subcutaneous administration of neoSTX to rats during 12 weeks (1, 3 and 6 μg/kg) proved to be non-lethal. Under these special conditions, no signs of adaptation to previous doses were observed (Zepeda et al. 2014).

Genotoxicity

To date few studies have been reported on mutagenicity effects of marine biotoxins, and no studies on carcinogenicity employing approved standard methods using animal models or cell lines have been

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carried out (Munday and Reeve 2013; OECD 2009b, c). Tetrodotoxin gave negative genotoxicity results in the mouse bone-marrow micronucleus test (Guzmán et al. 2007). However, effects of saxitoxins have been detected in fish and shellfish species frequently exposed to these toxins in their natural habitats. Saxitoxin produced cytogenetic damage in white seabream with significant increase of nuclear abnormalities after 2 and 6 days i.c. (intracutaneous) injection as assessed by the ENA assay (erythrocytic nuclear abnormality assay) (Costa et al. 2012).

Limitations of Current Assays and Analytical Methods

Management of toxic PSP outbreaks and TTX intoxications has been based worldwide on the mouse bioassay for the last 65 years (AOAC 2000; AOAC, Association of Official Analytical Chemists; Schantz 1986; Sommer and Meyer 1937). The mouse bioassay is a semiquantitative assay that provides sufficient reproducibility and detection limit for regulatory purposes. Its major advantage is that it measures biological toxicity directly and as such should be maintained as a reference method. While reliable for regulatory purposes, this assay is costly and time-consuming and cannot be automatized, and its detection limit (320–380 μg STX equivalents/kg edible parts) is very near to the maximum permitted level of 800 μg STX equivalents/kg (AOAC 2000; Wekell et al. 2004). Some countries have decreased that level, as children and elderly people have shown signs of intoxication at lower levels (Callejas et al. 2015; Campàs et al. 2007).

Sodium channel toxins pose a significant public health threat and an enormous economic challenge to the shellfish industry worldwide. Fatal PSP intoxications represent the most serious threat of marine origin worldwide (Batoreu et al. 2005; Zhang et al. 2013) with severe public health and economic impacts in Asia, Europe, North America (Anderson et al. 2001), and South America (Sar et al. 2002). As a consequence, the large majority of coastal countries and all seafood-exporting economies have established mandatory PSP toxin screening programs.

Risk assessment analyses (EFSA 2009a, b; Paredes et al. 2011) have determined an acute reference dose (ARfD) of 0.5 μg STX equivalents/kg bw. (bw, body weight). This implies if a 70 kg person eats 400 g of mussels contaminated at the current permitted level of 800 μg STX equivalents/kg, the exposure would be 4.6 μg STX equivalents/kg bw. This is equivalent to 9–10 times the ARfD. As a consequence, EFSA (2009a) has suggested a permitted level 10 times lower at 80 μg STX equivalents/kg. Thus, future methods should have a robust limit of quantification (LoQ) lower than 80 μg STX equivalents/kg. This may be a problem as current HPLC-FD versions (HPLC, high-performance liquid chromatography; FD, fluorescent detection) of the official AOAC method (AOAC 2000) have LoQs higher (or very near to) than that value for some derivatives (STX in particular; EFSA 2009a). According to EFSA (2009b) this critical analytical limitation means that it is not possible to perform a correct exposure assessment, as many samples containing saxitoxins in the range of the newly proposed permitted level will go undetected.

Certainly, the mouse bioassay with a detection limit of 320–380 μg STX equivalents/kg cannot meet a requirement of a new maximum permitted level of 80 μg STX equivalents/kg. However, the mouse bioassay is currently used worldwide, and countries from the developing world will continue to do so due to the lack of sufficient funding to implement new methodologies in all necessary locations. These countries are facing a new difficulty. For many years standard saxitoxin dihydrochloride was used as a calibrant of the mouse bioassay, and it was provided free of charge to all accredited laboratories in the world by the US Food and Drug Administration, Office of Seafood. Since 2012 this invaluable service has ceased to exist, and laboratories that need to continue using of the mouse bioassay have to rely on a sustainable production of standard saxitoxin from the National Research Council Canada.

As it is the case with other critical supplies for regulatory work, such as assay kits and analytical standards, their continued production depends on market variables that are beyond the control of laboratories from health or fisheries services. This is a mayor risk for a sustained monitoring and management effort on these lethal marine biotoxins. Uncertainties of provision of certified analytical standards are a major risk for the sustainability of all analytical methods currently in use for marine biotoxins.

The major limitation of the mouse bioassay, however, is the controversial use of live animals. The assay measures the time to death after intraperitoneal injection of seafood extract, a procedure that has received such ethical criticism that it can no longer be carried out in some European countries. In addition, high salts and metals such as zinc, manganese, and cadmium interfere severely with bioassay determinations, suppressing the apparent PSP toxicity (Suarez-Isla 2008; Turner et al. 2012). These disadvantages stimulated the development of several alternative methods and assays (Reverté et al. 2014; Suarez-Isla 2008) that are beyond the scope of this review. Suffice it to say that in today's regulatory environment, any new method has to be validated following strict internationally agreed requirements to demonstrate that its performance is at least equivalent to the current reference method. In this context, liquid chromatographic-based methods with fluorescent detection have been most successful at validation (European Commission Decision 2002/657/EC 2002; Kruve et al. 2015).

Current analytical methods for saxitoxins have to perform with limits of detection and quantitation that are fit for their purpose. There are two AOAC-validated HPLC-FD methods for PSP toxins (DeGrasse et al. 2011), a pre-column oxidation method (AOAC 2005.06; Anon 2005) and a post-column oxidation method (AOAC 2011.02; Anon 2011). Both require oxidation of STX analogues for fluorescent detection.

The official methods display detection and quantitation limits that differ for each derivative available as a certified standard (see Table 4; EFSA 2009a) and also depend on specific implementation protocols and instruments used in each laboratory. As stated before, for some derivatives, these performance parameters may be still insufficient, especially if the proposal from EFSA (2009b) to reduce the regulatory limit 10-fold, from 800 μg STX equivalents/kg to 80 μg STX equivalents/kg, is finally accepted.

However, recent work on an improved version of the AOAC 2005.06 pre-column oxidation method (Harwood et al. 2013) will help to avoid the interference from matrix components and from fluorescent STX derivatives display similar or identical retention times that can lead to overestimation of concentrations. Recent advances in LC-MS/MS methods are also very promising and may provide the needed performance characteristic sensitivity to solve these limitations (Turner et al. 2015).

Analytical HPLC methods require analytical standards to quantitate toxins in a mixture. Availability of certified reference materials and analytical standards is critical. As mentioned before, the composition of acidic extracts from naturally contaminated PSP shellfish samples is highly variable and may contain over 18 different analogues of STX in variable proportions (García et al. 2015; Oshima 1995). To date only 12 STX analytical standards and a reference material are provided by the laudable efforts of the National Research Country Canada (www.nrc.ca).

But there is another problem. Analytical methods provide the molar composition of toxic extracts and a total toxin concentration, a quantity that has to be transformed into toxicity values. This calculation relies on toxicity factors obtained by the mouse bioassay carried out with intraperitoneal injection of pure STX analogues (EFSA 2009a; Hall et al. 1990; Oshima 1995). These STX analogues need to be certified standards in order to correlate acute toxicity in mice with a known concentration. Currently, countries in the European Union apply a set of equivalent toxicity factors (TEFs) which are based on a set of relative toxicities derived from specific toxicities determined previously by mouse bioassay (Oshima 1995) (see Table 4; EFSA 2009a). However, these agreed TEF values have poor correlation with acute toxicities measured as median lethal doses of saxitoxin, neosaxitoxin, decarbamoylsaxitoxin, and mixtures of gonyautoxins 1/4 and gonyautoxins 2/3, using several routes of administration (Munday et al. 2013).

This situation should prompt a revision of current TEF values and a renewed effort to obtain a revised set of these critical values.

In summary, results of toxicities derived from analytical measurements by HPLC-FD methods rely on a biological method that has been replaced in several countries. The lack of a complete set of analytical standards in sufficient amounts hampers the determination of equivalent toxicity factors for saxitoxin derivatives and limits the capabilities of analytical methods.

Limitations of Monitoring and Management Procedures

Monitoring and management for harmful algal blooms and marine biotoxins is a complex task (Anderson et al. 2001). Countries that export shellfish that may be potentially affected by marine biotoxins, such as New Zealand and Chile, have implemented management programs based on EU directives that are focused on monitoring of growing areas and end product testing. These procedures follow well-established international regulations (see EFSA 2009a). However, due to limitations of sampling geographical density and frequency, these programs may underestimate spatial and intrapopulational variability in live samples from growing areas. As has been reported before, high spatial variability in PSP levels in the same species has been found in samples collected from sites distant 1–10 km (Nishitani and Chew 1984; Prakash et al. 1971). In the absence of concomitant information on phytoplankton dynamics and distribution, it is very difficult to explain the mechanisms underlying intrapopulational variability.

As reported by García et al. (2015) and Zamorano et al. (2013), the distribution of PSP toxin levels may also show large interindividual variability as a function of species, specific location, and depths in natural shellfish beds or in cultures (Turner et al. 2014). Factors affecting variability have been discussed by Bricelj and Shumway (1998) and more recently by García et al. (2015). Significant factors include among other variables the degree of exposure to toxic phytoplankton, assimilation capacity of toxins by shellfish, toxin elimination rates, developmental stage of the shellfish species, and species-specific biotransformation pathways.

Large variability of PSP toxin profiles and lack of predictability of PSP events have been reported by Turner et al. (2014) after a 5-year period of study in Great Britain using official HPLC-FD methods (Turner et al. 2009). Examination of a large number of samples from 12 marine species in over 50 sampling sites indicated some regularities of PSP toxin profiles for particular species. However, the occurrence or absence of toxic events during a specific year could not be correlated with successive occurrences. It was observed that only 0.267 % of all tested samples (41/15640) showed levels above the regulatory limit (RL) of 800 μg/kg, while the number of samples above RL and another group over a reporting limit of 160 μg/kg were 249 (1.62 %). This is consistent with observations in other countries using the mouse bioassay with percentages of samples above RL below 0.5 %. Several reasons could explain the very large number of nontoxic samples found in long-term monitoring programs in several parts of the world that use the mouse bioassay. The simplest explanation is related to the high limit of detection of the mouse bioassay (ca 320–280 μg STX equivalents/kg) that would leave most samples go undetected.

Interestingly, other factors may be at play to explain the large variability of the toxicity levels found in shellfish as a function of species and geographic distribution, such as genetic forcing mechanisms affecting the primary structure of sodium channel proteins in specific amino acids that reduce the binding affinity for STX and TTXs (Soon and Venkatesh 2008). These factors have been suggested to explain the differential behavior of species that have been exposed to PSP toxins for many generations as compared to other populations that grow in areas without a previous history of toxic blooms. This is the case of the soft-shell clam *Mya arenaria* (Bricelj et al. 2005) and of razor clams (Navarro et al. 2014). Relatively

PSP-insensitive shellfish species such as mussels and clams can accumulate and sustain exceedingly high toxic levels. In contrast, PSP-sensitive shellfish species such as oysters that display inhibition of feeding mechanisms for saxitoxins during blooms, as is the case for the Pacific oyster *Crassostrea gigas* (Moore et al. 2009), may lead to accumulation of low levels of the toxins that may be undetected by the mouse bioassay. This potential genetically determined variability may add to the uncertainties of any risk analysis and forecasting effort. Therefore, it is advisable to sample PSP-sensitive and PSP-insensitive species to reduce exposure risk.

Conclusions

After almost 80 years of sustained research effort, there are still significant gaps that have to be addressed to improve public health protection with better tools for monitoring and management to protect international trade (Anderson et al. 2001). While it is essential to prioritize the implementation of analytical techniques for efficient monitoring and early warning in developing countries, it will be necessary to preserve capacities for mouse bioassays as screening tool for saxitoxins and new toxins from uncommon sources. Analytical techniques have to be validated with local matrices from potential toxin vectors that are consumed locally following long established traditions. The critical issue of availability of analytical standards should be tackled and supported by a public effort to generate low-cost reference materials. In the long term, the intriguing impact of sodium channel adaptive evolution should be addressed in those coastal areas with endemic PSP presence.

As previously stated by Boesch et al. (1997), as government support for monitoring has declined worldwide, new reliable and more sensitive methods of detection are urgently needed to insure early warning decisions. Together with a better-trained public health workforce and information technologies applied for early warning and dissemination of risks, it should be possible to mitigate impacts on public health and local economies.

Cross-References

► Intoxications Caused by Saxitoxin, Shellfish, and Other Neurotoxins
► Pufferfish Poisoning and Tetrodotoxin

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Jellyfish Venom and Toxins: A Review

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Abstract

Although countless numbers of people are being stung every year by poisonous jellyfish throughout the world, statistical data is rarely available, even for fatal cases, except for Australia. There has been a significant amount of research for the treatment of cnidarian stings, but confusion still exists as to what is the most effective first aid and clinical management. In addition, only a few toxic components of jellyfish venoms have been identified so far, suggesting it is one of the most neglected areas in toxinology research. This is probably because jellyfish are difficult to obtain; many species are randomly distributed in the wide open sea, compared with other venomous animals. Secondly, unlike the milking of snake venom, it is extremely difficult to collect jellyfish venom of high purity without contamination of other tissue debris. Thirdly, all the toxins identified from jellyfish venoms until now are proteins, and many are intrinsically susceptible to and easily denatured by harsh environmental conditions (e.g., temperature, pH), resulting in the loss of their biological activities during the extraction and purification procedures. Therefore, the isolation and characterization of jellyfish toxins has lagged behind that of many other animal toxins. A few protein toxins have been reported, mainly from box jellyfish (Cubozoa) species. In this chapter, various jellyfish venoms that have been investigated are reviewed, as well as the protein toxins which have been identified to date.

Introduction

Over the last decade, a dramatic increase in global jellyfish blooms has been observed due to climate change and other anthropogenic disturbances. Jellyfish blooms can induce direct negative effects on human enterprise, including reduction of fish populations, disturbance of fishing by tearing of nets, human stingings, and clogging of coastal power plant cooling water intakes, leading to enormous economic loss.

Jellyfish belong to the phylum Cnidaria, which can be divided into five classes, Anthozoa, Cubozoa, Hydrozoa, Scyphozoa, and Staurozoa. Except for anthozoans, most cnidarians have bells of different shapes and sizes and take the form of a free-floating medusa, the form which is commonly referred to as a jellyfish. Most jellyfish are relatively harmless to humans and some of them, typically in Asian countries, also are used for food. Jellyfish have special cells, nematocytes, which contain stinging organelles called nematocysts, that are used for prey capture, defense, and locomotion. Nematocysts have a spiralized thread immersed in the jellyfish venom. When nematocytes sense a chemical or a mechanical stimulus, the coiled thread everts, injecting venom into the prey or attacker (Fig. 1). Tentacles contain from a few 1000 to several billion nematocysts that discharge into the skin within a fraction of a second and can paralyze and kill predators (mainly zooplankton and small fish) (Malej 1982).

Furthermore, jellyfish can significantly impact human activities and ecosystems due to their envenomation. Although most jellyfish can cause envenomation, some species fail to inject sufficient amount of venom deep into skin layers or produce toxins that do not cause significant harm to humans

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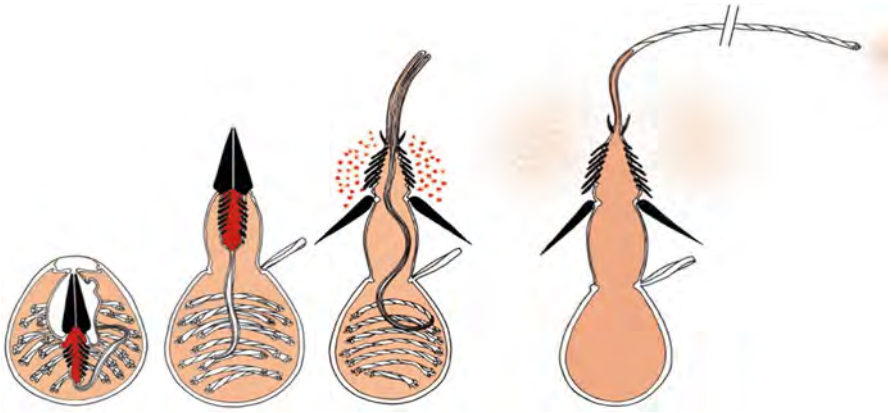

Fig. 1 Nematocyst discharge scheme. Nematocysts contain coiled threads that may bear barbs. The outer wall of the nematocyte (now shown) has hair-like projections called cnidocils that are sensitive to touch. When touched, the nematocysts are known to fire coiled threads that can either eject rapidly into the flesh of prey or predators or ensnare it. These coiled threads release toxins into the target (From Szczepanek et al. 2002)

(Barnes 1960). Human envenomation by jellyfish can induce a large variety of symptoms, ranging from a slight local effect to life-threatening symptoms (Cegolon et al. 2013). In humans, jellyfish can cause severe envenomations with extensive dermonecrosis, edema, diffused neurotoxicity, motor and respiratory problems, cardiovascular symptoms, hypotension, and even death. In particular, the cubozoan *Chironex fleckeri* (sea wasp) can cause serious cardiotoxicity within a few minutes, and several deaths have been reported along the Australian coastline during the last century (Brinkman and Burnell 2009). Additionally, some people are susceptible to allergic reactions, including immediate and delayed-type hypersensitivity responses (Tibballs et al. 2011). Stings of prominent species (*Physalia physalis*, *Chironex fleckeri*, *Carybdea arborifera*, *Carukia barnesi*, and *Pelagia noctiluca*) inject into the skin tubules which are composed of mini-collagens, glycoproteins, and polysaccharides, all of which can work as immunogens. It is well known that jellyfish stings are a particularly serious health problem along some Asian and Australian coasts and in Pacific archipelagos (Feng et al. 2010). Recently, the occurrence of venomous jellyfish species is becoming more common in the East Asia area (Kang et al. 2014).

Jellyfish venom is a mixture of toxins with a wide range of biological activities producing hemolytic, insecticidal, cardiovascular, antioxidant, and cytotoxic effects (Kim et al. 2006; Li et al. 2014; Kang et al. 2009). Various studies have revealed that cnidarian venoms contain vasoactive compounds such as 5-hydroxytryptamine (5-HT), catecholamines, histamine, neuroactive compounds such as quaternary ammonium compounds and certain amino acids and small peptides, and proteins, including proteases, phospholipases, and cytolytic and hemolytic agents. The components of jellyfish venoms and their mechanism of actions are still far from being understood.

This review will focus on the toxins or partially purified bioactive fractions that have been isolated from jellyfish venom to date. First, their effects on human health will be considered, based partly on in vitro characterization of the biological activities of their venoms. Second, identification and purification of jellyfish venom components will be presented. Third, current protocols for the management of jellyfish stings, depending on the jellyfish species, will be described. Last, biological activities of jellyfish venom with potential medical and therapeutic activity will be reported.

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Envenomation

Cubozoa

1. Cubozoans are the most venomous jellyfish and are named "box jellyfish" because of their cube-shaped bell. This class is composed of two orders, Carybdeida and Chirodropida. Carybdeida have four small tentacles, while Chirodropida are multitentacled. Carybdeida of medical importance are *Carukia barnesi*, *Tamoya* spp., and *Carybdea rastoni*. Contact with jellyfish of the order Carybdeida typically induces local cutaneous inflammation with invariably linear lesions (small blisters, erythema, and wheals) and usually, but not always, immediate pain (Cegolon et al. 2013). Erythema and wheals may appear within minutes and last several days, while moderate pain may last up to 2 h, but slight pigmentation at the sting site is observed for at least 2 weeks (Cleland and Southcott 1965). Contact with other jellyfish in this order can also induce the Irukandji syndrome. This syndrome is described as a set of delayed systemic effects. Severe symptoms appear on average 30 min after contact with the jellyfish and can last for several days (Carrette et al. 2012). Among suspected Irukandji species, *Carukia barnesi* and *Alatina mordens* are known to produce Irukandji syndrome, but it is not confined to stings by these species (Winter et al. 2008). While local effects of the sting are rarely noticed and may appear as a perceptible erythema, the systemic symptoms that include vomiting, nausea, intense muscle pain, cramps, lower back pain, headache, sweating, agitation, restlessness, and sometimes hypertension start about 30 min after contact with jellyfish. Two of 62 victims presented severe cases with systemic symptoms and tachycardia and hypertension. After 1.5–18 h, pulmonary edema and reduced cardiac output were observed (Carrette et al. 2012). The deaths in 2002 of two tourists in North Queensland, Australia, highlighted the potential risk and severity of this syndrome. Fenner et al. stated that this syndrome may be caused by an excess of circulating catecholamines. However, this mechanism has not been confirmed in human envenomation (Fenner and Hadok 2002). *Tamoya* spp. jellyfish are called "fire jellyfish." They are relatively large carybdeid cubozoans and characteristically have a taller, transparent bell. Unlike other smaller carybdeids, the symptoms that develop from this jellyfish sting are similar to Irukandji syndromes, and the lesions that may occur include skin necrosis, vesicle, and pruritus.

In the order Chirodropida, *Chironex fleckeri*, the Australian box jellyfish, is known to be the most dangerous jellyfish in the world (Tibballs 2006). At least 63 victims had sudden deaths in Australian waters up to 1994. It has been found in northern Australia and Southeast Asia. Contact with its tentacles induces a local cutaneous inflammatory reaction which is very painful and can persist for several days. The victim may scream and become irrational. The stung skin areas were large and edematous and contained erythematous wheals which became blisters and which progressed to skin necrosis during the healing process. When the skin makes contact with large *C. fleckeri with a diameter of 15 cm or more*, severe symptoms can also be observed, including an excruciating pain, dyspnea, unconsciousness, hypertension, hypotension, and cardiopulmonary arrest. *Chironex fleckeri* venom was demonstrated to induce a direct toxic effect on cardiac and vascular tissues, effects not yet confirmed for the purified lethal components (Hughes et al. 2012). Death can occur within minutes after the envenomation, as a result of cardiac and respiratory effects (Tibballs 2006). Sometimes, envenomation of *C. fleckeri* can cause immunological responses due to delayed hypersensitivity reactions; actually, 11 of 19 victims by *C. fleckeri* sting presented immunological effect which resolved spontaneously or with treatment of oral antihistamines and topical corticosteroids (O'Reilly et al. 2001).

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Chiropsalmus quadrigatus, a smaller jellyfish than *C. fleckeri*, is a very common species in the Indo-Pacific ocean from Australia to the Philippines and Japan. Stings by this jellyfish produce intense pain immediately and erythema and swelling within a few minutes, and sometimes, these symptoms last for at least 24 h. Severe envenomations may cause hypertension, bradycardia, cardiac asystole, respiratory failure with pulmonary edema, shock, and even death. Death cases have been reported in the Philippines and Japan but not in Australia (Tibballs 2006; Nagai et al. 2002). However, its effects are less than envenomation by *C. fleckeri*.

Chiropsalmus quadrumanus is found in warmer water of the Atlantic Ocean from North Carolina (USA) to Brazil. Envenomation by this species can be harmful to the cardiovascular and respiratory systems and fatal (Nagai et al. 2002; Cegolon et al. 2013).

Hydrozoa

Physalia spp. are not true jellyfish but are considered as siphonophore jellyfish and belong to the class Hydrozoa. There are two species, *Physalia physalis* (the Portuguese Man O' War) and *Physalia utriculus* (the Blue Bottle). *Physalia physalis* is distributed worldwide and is larger than *P. utriculus. Physalia utriculus* is found in the Indo-Pacific, Indian, and South Atlantic oceans. *Physalia* spp. are dangerous jellyfish because they often are stranded on the beaches but are still able to sting and envenomate, even after several days of dehydration (Tibballs 2006).

Contact with *P. physalis* usually induces sharp pain, rapid development of vesicles, systemic effects with abdominal ache, nausea, vomiting, and spasms. By contrast, *P. utriculus* sting causes mild symptoms, such as local pain and very rarely systemic effects. It was observed in most cases that the marks and skin lesions at sting sites faded within 24 h. However, severe cases of *P. physalis* sting cause neurological (confusion, lethargy, and fainting) and cardiorespiratory (dyspnea, precordialgia (pain in the precordial region), and collapse) signs. *Physalia physalis* stings are the most common type of jellyfish stings in Australia, estimated to occur 10,000 times annually on the eastern coast (Fenner and Williamson 1996), but systemic symptoms are rarely observed (Tibballs 2006). However, deaths due to respiratory arrest and cardiovascular collapse have been reported.

Scyphozoa

Although Scyphozoa are known to be less dangerous to humans than jellyfish of the class Cubozoa, contact with *Cyanea capillata*, *Nemopilema nomurai*, *and Stomolophus meleagris* can lead to systemic symptoms (Cegolon et al. 2013). *C. capillata* (named "Lion's Mane") is very commonly found in the cold seas of the Northern Hemisphere including the North Sea and in Australia. The diameter of the umbrella ranges from 30 cm to 2 m. Contact with this jellyfish produces minor or more severe pain, discomfort, swelling, and erythema. Erythematous stripes may persist for several days. Systemic symptoms are unusual, but sometimes muscle cramps, sweating, dizziness, and nausea occur (Cegolon et al. 2013). No death cases have been reported until now.

Pelagia noctiluca is distributed worldwide, being found in both tropical and cold waters (Cegolon et al. 2013), and is very common in the Mediterranean Sea (Mariottini et al. 2008). Envenomation is not fatal but produces discomfort. Local effects include painful cutaneous reactions, including blistering, edema, and erythema. Lesions can heal within 2 weeks, but scarring and hyperpigmentation can remain. This lesion can be resolved with topical hydroquinone (Kokelj and Burnett 1990). Delayed or relapsing eruptions are sometimes observed at the site of contact. Systemic effects are very rarely observed. No deaths have been reported, but allergic reactions have been observed, with possible cross-reactivity with other jellyfish (Cegolon et al. 2013).

Aurelia aurita is found in temperate and temperate-cold waters around the world. It is known to be relatively harmless to humans or even non-stinging, but cases of local cutaneous reaction associated with

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pain have been reported (Tibballs 2006). There are significant stinging cases reported for *A. aurita* in the Gulf of Mexico (Burnett et al. 1988). In China, 18 of 136 victims stung by *A. aurita* developed severe symptoms (Cao and Shao 2001).

Rhopilema esculentum is as an edible jellyfish like its relative, *Rhopilema hispidum*, and is distributed widely in the East China Sea and Yellow Sea. In general, the first species produces significant pruritus and the second causes a persistent eruption. When a subject was stung by *R. esculentum*, instant mild pain was experienced followed by a strong pruritus lasting 30 min before complete resolution (Kawahara et al. 2006).

Nemopilema nomurai is a large jellyfish with a bell size up to 2 m that is found in the East China Sea, Yellow Sea, and East Sea of Korea. Blooms have been more frequent in recent years. Common symptoms are cutaneous lesions with burning pain and erythematous eruptions with small vesicles, but not to be ignored are severe clinical symptoms (Kang et al. 2014). It also can cause cardiovascular depression in experimental animals (Kim et al. 2006) and may cause death in humans. In Chinese sea, 13 fatal cases by this jellyfish were reported (Zhang et al. 2005).

Molecular Constituents

Several jellyfish toxins have been characterized biochemically, toxicologically, and pharmacologically due to efforts of several laboratories during the last few decades. Box jellyfish (Cnidaria: Cubozoa: Cubomedusae) have been most thoroughly studied. For these reasons, several box jellyfish toxins are briefly described below.

Carybdea rastoni Toxins (CrTXs)

In 2000, two labile but potent hemolytic toxins, named as CrTX-A and CrTX-B, were purified from *Carybdea rastoni*, and their full-length cDNA sequences, which consisted of 18 amino acids as signal peptide and 432 in the complete open reading frames, were reported (Nagai et al. 2000b). These sequences were obtained for the purified toxins (which showed hemolytic activity) using peptide mapping with lysylendopeptidase and 5′/3′ RACE reaction from total RNA extracted from an intact tentacle. The two proteins were shown to be 43 and 46 kDa size by SDS-PAGE. After hydrolysis by lysylendopeptidase then separated into three fragments for CrTX-B and five fragments for CrTX-A. These fragments were identified using a PSQ-1 protein sequencer (Shimadzu, Japan) and compared with the deduced amino acid sequences. They suggested that the CrTX-A (43 kDa) toxin is modified CrTX-B (46 kDa), because CrTX-A exists only in nematocysts and CrTX-B exists only in tentacle extracts that were analyzed by the Western blot method. This supported the idea that CrTX-B is a modified form CrTX-A that is inserted into the nematocysts.

The toxicity of CrTXs varied based on the method of injection, but in case of intravenous injection into mice, the minimum lethal dose was less than 20–40 μg/kg within 40 min after injection, and it was less than 100 μg/kg within 8 h after an intraperitoneal injection. The minimum lethal dose of CrTX-A in crayfish after intra-hemocoel injection was less than 5 μg/kg. In addition, CrTX-A and CrTX-B hemolyzed 50 % of 0.8 % sheep red blood cells at concentrations of 1.9 and 2.2 ng/ml, respectively. The other toxic effects of CrTX found for partially purified *C. rastoni* toxin (pCrTX) were hemolysis, dermatonecrosis, plate aggregation, and contractions in rabbit aorta (Azuma et al. 1986). Furthermore, partially purified *C. rastoni* toxin (pCrTX) induced contractions in rat aorta by release of endothelium-derived relaxing factor.

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Carybdea alata Toxins (CaTXs)

Two hemolytic *Carybdea alata* toxins, toxin-A (CaTX-A, 43 kDa) and toxin-B (CaTX-B, 45 kDa), were purified by another method than for the purification of CrTXs, and their full-length cDNA sequences were determined (Nagai et al. 2000a). The deduced amino acid sequences have a putative signal peptide at the 18th amino acid position and a mature polypeptide of 445 amino acid residues. In a localization test of CaTX-A and CaTX-B, it was found out that only CaTX-A existed in the nematocysts and not CaTX-B. This indicated that the biochemical mechanisms of CaTX-A and CaTX-B processing were very similar with that of CrTXs of *Carybdea rastoni*. Alignment of the deduced amino acid sequence of CaTX-A had 43.7 % homology with CrTXs. By peptide mapping of CaTX-A, only the C-terminal region of CaTX-A had homology with CrTXs and not the N-terminal region. CaTX-A toxin showed an LD_{50} range of 5–25 μg/kg and hemolysis of sheep red blood cells at 70–80 ng/ml concentration range.

Chiropsalmus quadrigatus Toxin (CqTX-A)

A labile proteinaceous toxin CqTX-A (44 kDa) was isolated from another box jellyfish, *Chiropsalmus quadrigatus*, using a modified procedure developed for the CrTXs and CaTXs (Nagai et al. 2002). The deduced amino acid sequence of CqTX-A contained 462 amino acid residues and despite this showed no significant similarity to the CrTXs and CaTXs, with only 25.2 % and 21.6 % sequence homology, respectively.

The toxicity of CqTX-A showed an LD_{50} in crayfish of 80 μg/kg and an EC_{50} for hemolysis of sheep red blood cells of 160 ng/ml. These toxicities were less than for the CrTXs (5 μg/kg and 1.9 ng/ml, respectively) and CaTXs (5–25 μg/kg and 70 ng/ml, respectively). However, stinging by *C. quadrigatus* is more dangerous than stinging by *C. rastoni* or *C. alata* because the tentacles of *C. quadrigatus* are approximately three times more massive and longer than that of *C. rastoni* or *C. alata*. Since CqTX-A is the major toxin in the nematocysts of *C. quadrigatus*, it is likely the causative toxin in fatal cases of stings.

Chironex fleckeri Toxins (CfTXs)

The toxicity of *C. fleckeri* venom is known to be myotoxic, hemolytic, dermonecrotic, and lethal (Tibballs 2006). Two of the most abundant nematocyst proteins found in the box jellyfish, *Chironex fleckeri*, were identified as *C. fleckeri* toxin-1 (CfTX-1) and toxin-2 (CfTX-2) (Brinkman et al. 2007). CfTX-1 and CfTX-2 sizes were estimated as 43 and 45 kDa through SDS-PAGE of purified nematocyst proteins. The zones for these proteins were transferred onto PVDF membranes for peptide sequencing and identified by N-terminal Edman degradation amino acid sequence determination. Internal amino acid sequences were identified by MALDI-TOF/TOF mass spectrometry (Australian Proteome Analysis Facility), and these sequences were used to confirm the amino acid sequence deduced from the cDNAs. CfTX-1 and CfTX-2 had 20 and 17-residue signal peptides and 436 and 445 amino acids for the complete open reading frame, respectively. Pairwise sequence alignment analysis of the CfTXs showed significant homology with CqTX-A (89 % and 72 %, respectively), CrTXs (27 % and 26 %, respectively), and CaTXs (24 % and 25 %, respectively).

Two more venomous proteins were purified from *C. fleckeri* nematocysts by size exclusion chromatography and cation exchange chromatography and named CfTX-A and CfTX-B, respectively (Brinkman et al. 2014). The sizes of these two toxins were slightly smaller (40 and 42 kDa, respectively) than that of CfTX-1 and CfTX-2. CfTX-A and CfTX-B toxins had 18 and 24-residue signal peptides and 436 and 437 amino acid residues for a complete open reading frame, respectively. Interestingly, CfTX-A and CfTX-B showed high similarity with CaTX-A (52–55 % identity) and not with CfTX-1 and CfTX-2 (23–25 % identity). The toxicities of CfTX-A and CfTX-B were related to an expanding family of lethal, pore-forming cnidarian toxins.

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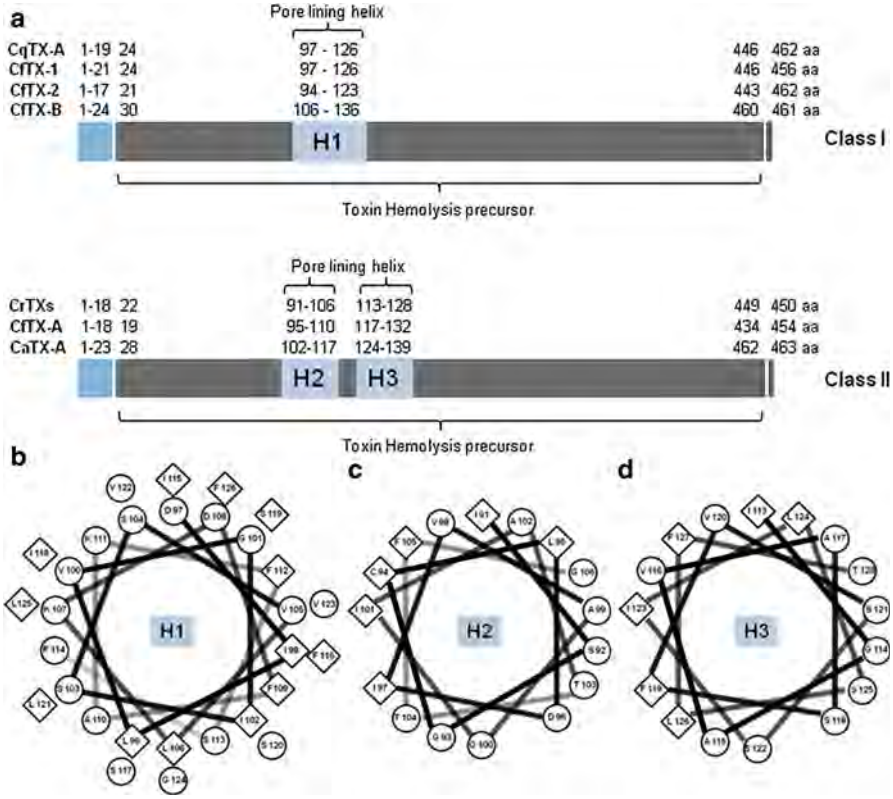

Fig. 2 Structural organization and amphiphilicity analysis of class I and class II jellyfish toxins. (**a**) Signal peptides (SignalP 4.1 server) are indicated in *blue*; putative pore-lining helices (MEMSAT3 and MEMSAT-SVM server) and toxin hemolysis precursor (InterProScan server) are indicated *up* and *below*, respectively. The sequence accession numbers are CrTXs (*Carybdea rastoni*: AB015878.1), CfTX-A (*Chironex Fleckeri*: JN695597.1), CfTX-B (*Chironex Fleckeri*: JN695598.1), CfTX-1 (*Chironex Fleckeri*: EF636902.1), CfTX-2 (*Chironex Fleckeri*: EF636903.1), CqTX-A (*Chiropsalmus quadrigatus*: AB045319.1), and CaTX-A (*C. alata*: AB036714.1). (**b**–**d**) represent putative helix sequences for CfTX-1 (from Asp-97 to Phe-126), CrTXs (from Ile-91 to Gly-106), and CrTXs (from Ile-113 to Thr-128), respectively. Hydrophobic residues are diamonds and hydrophilic residues are *circles*

Structural Analysis of Jellyfish Toxins

Analysis of jellyfish toxins by MEMSAT3 and MEMSAT-SVM (http://bioinf.cs.ucl.ac.uk/software_downloads) classified the toxins into two groups by number of helix domains (Fig. 2a), named as class I and class II, respectively. As a result, the signal peptide (SignalP 4.1 server) and toxin hemolysis precursor (InterProScan server) were included in the coding sequence. Moreover, jellyfish toxins have pore-lining helix domains which are important to make a pore in the target cell membrane. In class I toxins, the pore-lining helix exists as one part, but in class II toxins, it is divided into two helices. These results support the hypothesis that jellyfish toxins are pore-forming toxins. However, it is not yet known how many jellyfish pore-forming molecules are needed to assemble for making the pore. Generally, pore-forming toxins have some amphipathic alpha-helix structure. By helix structure analysis, jellyfish toxins have critical amphipathic sequences in both.

Classes (Fig. 2b–d). While class I toxins are predicted to make alpha-helix structure with 30 amino acids, class II toxins make helix with 20 amino acids. There are 3.6 amino acid residues per turn. In general, it is suggested that jellyfish toxins secreted from nematocysts after reaching the target cells of the victim, such as red blood cell, make pores after self-assembly. The target cell will swell following the exchange of electrolytes through these pores, which results in osmotic lysis.

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Evolutionary Relationships of Jellyfish Toxins

Brinkman et al. (2014) classified box jellyfish toxins into two groups (type I and type II). CqTX-A, CfTX-1, and CfTX-2 were classified as type I and the other (CrTX-A, CaTX-A, CfTX-A, and CfTX-B) as type II by phylogenetic analysis. Both groups are predicted to contain signal peptides and form dual-domain mature proteins. Interestingly, only the type II toxins have short propart residues followed by a classical dibasic proteolytic cleavage site (RR/KR) between the signal peptide and mature protein. In general, N-terminal proparts have important roles in protein biosynthesis, activation, correct folding, and post-translational modification. Moreover, the proparts play a role in the activation of protoxins to toxins by the cleavage proparts. Regardless, all of the box jellyfish toxins have lethal and hemolysis toxicities, and type II toxins elicit more potent hemolytic activity than type I toxins. However, type I toxins elicit more potent cardiovascular effects in vertebrates than type II toxins (McClounan et al. 2012; Saggiomo et al. 2012).

Treatments of Stings

Jellyfish stings in humans are usually painful and the symptoms of envenomation can be classified as local and systemic effects (Cegolon et al. 2013). The former may involve local skin damage (necrosis) with erythema, blister formation, pruritus, and then scar formation. When a significant amount of venom reaches the circulation, the sting can be accompanied by systemic pain, hypertension, cardiotoxicity, pulmonary edema, anaphylaxis, and even death. In general, the stings are usually not considered life threatening, except those occurring by box jellyfish species (e.g., *Chironex fleckeri*) in tropical areas. In Australia, *C. fleckeri*-associated human deaths have been relatively well documented. Although there has been a significant amount of research effort to optimize the treatment of cnidarian stings, confusion still exists as to what is the most effective first aid and subsequent clinical management. Therefore, it is urgently desirable to develop a general protocol for the management of these accidents.

The treatment of jellyfish stings has traditionally focused on two strategic goals, the relief of pain by deactivation of nematocysts and the neutralization of venom (Ostermayer and Koyfman 2015). Once jellyfish venom is injected into the skin, the use of its antivenom (in case of severe envenomation) usually by medical personnel is the only treatment that can neutralize and deactivate the venom constituents that have entered the body. Therefore, in a majority of jellyfish sting cases, the treatment mainly depends on the relief of pain by deactivation of nematocysts, namely, topical management of affected skin areas. In fact, there are numerous folk remedies proposed as a first aid of jellyfish stings. However, many of them lack scientific evidence and in some stings may accidently result in a worsened condition. It is hardly a matter for surprise to find different international organizations recommending different first-line treatments. The International Life Saving Federation suggests topical ice packs for pain relief for all stings and topical acetic acid (4–5 %) for deactivation of remaining nematocysts only in box jellyfish stings (Fenner 2000). However, there is no single protocol or golden standard for the first aid of jellyfish stings. Nematocysts from different species of venomous jellyfish may respond to the same stimulant in an opposite manner. Therefore, it appears that treatments should be customized depending on the jellyfish species of envenomation (Honeycutt et al. 2014). The Australian Resuscitation Council (2010) recommendations for treating envenomation by jellyfish stings provide geographic guidelines based on tropical Australian (box and Irukandji-causing species) and nontropical stings. For example, tropical box jellyfish (Cubozoa) stings can be treated with topical acetic acid (4–5 %) for deactivation of remaining nematocysts, whereas nontropical stings (e.g., Scyphozoa such as *Cyanea capillata*, Hydrozoa such as P*hysalia physalis*) should not be treated with acetic acid, which causes discharge of nematocysts. After immediately removing tentacles from the affected skin area, seawater is likely to be a good candidate for the first

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aid rinsing solution of all jellyfish stings regardless of their species differences, since, unlike other rinsing solutions, it does not stimulate discharges (Cegolon et al. 2013).

Potential Uses

Jellyfish venom is a research subject of great interest because many of these components may have pharmacological activities with therapeutic potential and some of them might be further developed as future drugs. However, in the last decade, despite the abundance and ubiquity of these animals, research on jellyfish venom has been limited by availability of samples and by difficulties in isolating pure venom. This may explain why the possible potential application of jellyfish venom for the therapy of human diseases has been poorly characterized in literature. With growing efforts and advances in high-throughput methods used for screening and characterizing bioactive compounds, jellyfish venom is now being studied for its promise as a therapeutic agent. Recently, research on jellyfish venom has focused on the identification of pharmacological activities and purifying active components as a new source of developing drugs. Antitumor activity has been the major area of interest in the screening of jellyfish venom.

Antitumor Activity

Reports on the cytotoxicity of jellyfish venom have been published since the early 1980s on cultured cells. Fractionated venom peptide (9 kDa) from *Chrysaora quinquecirrha* was observed to produce morphological changes in HEp2 and HeLa cells, to induce apoptosis with activation of caspase-8 and caspase-3, and to cause the downregulation of Bcl-2 and the fragmentation of DNA (Balamurugan et al. 2009). Isolated venom peptide of *Chrysaora quinquecirrha* was also shown to have an antitumor and antioxidant effects in the Ehrlich ascites carcinoma (EAC) tumor model. This venom peptide increased the survival time of the EAC-bearing mice by free radical scavenging property (Balamurugan et al. 2010).

Venom from the box jellyfish *Chiropsalmus quadrigatus* was shown to affect the growth of human U251 and rat C6 malignant glioma cells and transformed vascular endothelial ECV 304 cells. The isolated toxin (CqTX) from *C. quadrigatus* venom induced breaking of DNA bands, leading to apoptotic cell death. In particular, the expression of p53, a well-known tumor suppressor gene, was increased in U251 cells. It was suggested that treatment with CqTX could induce apoptosis in glioma cells due to regulation of p53 expression, suggesting a possible application of apoptosis-inducing venom in glioma therapy (Sun et al. 2002).

Recently, *Pelagia noctiluca* venom demonstrated antitumoral activity in human colon cancer HCT 116 cells (EC_{50} 320 μg/ml), neuroblastoma SH-SY5Y cells, and glioma U87 cells (EC_{50} 180 μg/ml). Its venom exhibited potent cytotoxicity, increasing ROS and catalase levels and inducing lipid peroxidation in HCT 116 cells. These results indicate that this venom causes cytotoxicity through oxidative stress (Ayed et al. 2011). Another group studied the effect of *P. noctiluca* crude venom in human neuroblastoma SH-SY5Y cells. Similar to the previous study, its crude venom was shown to induce oxidative stress with disruption of the mitochondrial membrane, inhibition of mitochondrial respiration, and uncoupling of oxidative phosphorylation (Morabito et al. 2012). Furthermore, crude or fractionated *P. noctiluca* venom reduced the viability and adhesion to fibrinogen in U87 cells. Among four fractions, F1, F2, and F3 showed significant antiproliferative activity in a time-dependent manner. Although the interaction of the venom with specific integrins was not investigated in the study, the authors stated that integrins αVβ3 and α5β1 are involved in the antitumor activity induced by *P. noctiluca* venom. Purification and determination of chemical structures of these active fractions are under investigation (Ayed et al. 2012a).

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Cotylorhiza tuberculata, "the fried egg jellyfish," is known to be a non-dangerous jellyfish throughout the world. However, its medusa extract was studied for cytotoxicity against human breast cancer MCF-7 cells and epidermal keratinocyte HEKa cells at concentrations raging from 0.0005 to 0.08 μg/ml. *Cotylorhiza* extracts were found to be remarkably cytotoxic and reduced the viability of MCF-7 cells but not nonmalignant HEKa cells. A remarkable time- and dose-dependent enhancement of gap junction intracellular communication (GJIC) was evident in MCF-7 cells treated with jellyfish extract (Leone et al. 2013).

Insecticidal Activity

Proteinaceous venom from tentacles of *Rhopilema esculentum* Kishinouye showed different insecticidal activity against three common pest species in China, *Stephanotis pyri* Fabricius, *Aphis medicaginis* Koch, and *Myzus persicae* Sulzer. Of the three pests, *S. pyri* was the most sensitive to *R. esculentum* venom; thus, the author stated that *S. pyri* could be a potential "target pest" for this venom. They investigated the purification of insecticidal proteins and their mechanisms of action (Yu et al. 2005). The insecticidal activity of jellyfish toxins was not studied before this study.

Insecticidal activity of crude (SFV and NSFV) and fractionated venom (Fr-1, Fr-2, NFr-1, and NFr-2) from *Stomolophus meleagris* were investigated for antiproliferative effect against the cotton bollworm *Helicoverpa armigera*, one of the most widely distributed Noctuidae worldwide. SFV, NFr-1, and NFr-2 showed inhibitory effects on the growth of *H. armigera,* while SFV, Fr-1, Fr-2, NRFV, and NFr-1 had no or low insecticidal activity against the neonate larvae of *H. armigera*. Of the six samples, NFr-2 had the strongest insecticidal activity against the neonate larvae of *H. armigera*, and the estimated mortality recorded at 7 days of NFr-2 was 74.23 % (Yu et al. 2014).

Antihypertensive Activity

Jellyfish venom has long been recognized for its cardiovascular effects. Most cubozoan jellyfish species induce hypertension, while scyphozoan jellyfish induce hypotension and bradycardia (Brinkman and Burnell 2009; Kim et al. 2006). It is conceivable that hypotensive activity induced by jellyfish venom can be used as a biological source of antihypertensive agents. To date, it was reported that two identified peptides (VKP and VKCFR) derived from *R. esculentum* hydrolysate showed angiotensin-converting-enzyme (ACE) inhibitory activity and protection effect in a H_20_2-induced endothelial cell injury model by enhancing antioxidant enzyme activity and quenching the hydroxyl radical (Li et al. 2014).

Anticoagulant Activity

Aurelia aurita tentacle extract was studied for its anticoagulant activity in vitro. The jellyfish tentacle extract (JFTE) showed very strong fibrinogenolytic activity, cleaving the α and β chains from the fibrinogen molecule. The fibrinogenolytic activity was found to be stronger than for some snake venom-derived anticoagulants. JFTE also completely liquefied fibrin clots in 24 h. The fibrinogenolysis appears to be caused by high molecular weight fractions of the extract. PMSF, a serine protease inhibitor, significantly reduced fibrinogenolytic activity, and heating totally abolished it (Rastogi et al. 2012).

Analgesic Activity

Pelagia noctiluca crude venom and its two fractions exhibited analgesic and anticholinesterase activities, inhibiting nonspecific cholinesterase (BuChE) at different doses without acute toxicity. The three venom samples showed significant analgesic effects compared to the analgesic drug, acetylsalicylate of lysine (ASL). Fraction 2 was found to exhibit higher analgesic activity than fraction 1. Furthermore, cholinesterase (ChE) hydrolyzes acetylcholine (ACh), the neurotransmitter at cholinergic synapses. Inhibition of ChE is one of the strategies for the treatment of Alzheimer's disease, senile dementia, ataxia, myasthenia

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gravis, and Parkinson's disease (Atta-ur-Rahman and Choudhary 2001; Karlsson et al. 1984). Similar to analgesic activity, fraction 2 also exhibited more anti-BuChE activity (Ayed et al. 2012b).

Conclusion and Future Directions

Jellyfish are some of the most hazardous sea creatures. Due to their toxicity, in general, the jellyfish toxins induce intense pain and swelling with surrounding redness of the skin. However, despite the research efforts of the past several decades, little is known about the toxicological effects of jellyfish toxins, which remain a mystery, including their mechanisms of action at the molecular level. Also, the biological roles of these jellyfish toxins need to be investigated to provide a more comprehensive understanding of their physiological and pathological properties.

More recently, various animals which have toxins, such as snakes, scorpions, cone snails, and spiders, have become attractive to pharmaceutical researchers in search of new drugs. Certainly, jellyfish venoms are attractive candidates for clinical applications (Turner et al. 1969). The exact mechanism of action of toxins is still unclear. However, several jellyfish species may serve as leads for new drug development in cases such as arthritis, cardiovascular medicine, and other disorders. This facet may be of interest to scientists working on these toxins.

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Cross-References

► Clinical and Therapeutic Aspects of Envenomations Caused by Sponges and Jellyfish
► Phylum Porifera and Cnidaria
► Toxins Produced by Marine Invertebrate and Vertebrate Animals: A Review

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Instrumental Methods for Paralytic Shellfish Toxins

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Abstract

Paralytic shellfish toxins (PSTs) are naturally occurring marine compounds which in some instances result in significant consumer sickness following consumption of contaminated shellfish products. The toxins are found in shellfish grown in marine waters throughout the world, and many instances of human intoxication are reported annually. This chapter describes the evolution of instrumental analytical methods for the determination of PSTs in shellfish, in the context of the global move away from reliance on live animal testing for food safety testing. Key methods are described and assessed, describing the major aspects and challenges associated with each. Fluorimetric methods, particularly those conducted following liquid chromatography separation of toxin congeners, have been formally validated and in an increasing number of countries applied to official control monitoring of shellfish harvesting zones. While these provide a reliable alternative to biological assays, these methods are complex and are unable to detect all the currently known toxin threats. While capillary electrophoresis has shown some potential, the development of rapid chromatography, particularly in tandem with mass spectrometry, has shown perhaps the greatest promise for the development of reliable, accurate, rugged, and reproducible monitoring tools. Nevertheless, further work and interlaboratory validation studies are still required to ensure such methods are fit for purpose and continue to improve the quality of the monitoring conducted to mitigate the serious risk from these highly toxic compounds.

Introduction

Saxitoxin (STX) and its analogues are a group of natural neurotoxic alkaloids, commonly known as the paralytic shellfish toxins (PSTs). The most commonly occurring toxins are hydrophilic and can be classified in three structural subgroups: the N-sulfocarbamoyl, the decarbamoyl, and the carbamoyl toxins, in increasing order of toxicity in mammalian bioassays (Oshima 1995). Figure 1 shows the structure of the main PSTs, with STX the most representative analogue, and also illustrates the structures of the more hydrophobic toxin congeners termed the *Gymnodinium catenatum* (GC) toxins. The relative potency of the analogues to STX is usually expressed using toxicity equivalency factors (TEFs). Worldwide, the most commonly used TEFs are those of Oshima (Oshima 1995) and the ones proposed by the European Food Safety Authority (EFSA) (2009). These are broadly similar, differing mainly for dcSTX (decarbamoyl saxitoxin) and dcNEO (decarbamoyl neosaxitoxin). However, more recent studies (Munday et al. 2013) question the accuracy of some of the proposed TEFs, and, at the moment, there is no global consensus on which TEFs to use.

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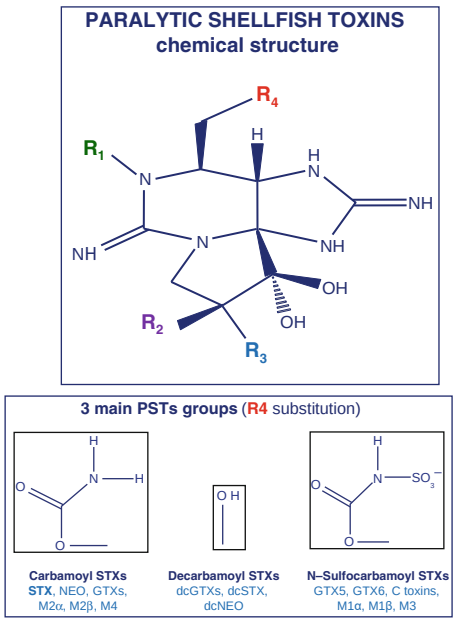

Compound	R_1	R_2	R_3	R_4
STX	H	H	H	$OCONH_2$
GTX1	OH	H	OSO_3^-	$OCONH_2$
GTX2	H	H	OSO_3^-	$OCONH_2$
GTX3	H	OSO_3^-	H	$OCONH_2$
GTX4	OH	OSO_3^-	H	$OCONH_2$
NEO	OH	H	H	$OCONH_2$
M2α	H	H	OH	$OCONH_2$
M2β	H	OH	H	$OCONH_2$
M4	H	OH	OH	$OCONH_2$
doSTX	H	H	H	H
dcSTX	H	H	H	OH
dcGTX1	OH	H	OSO_3^-	OH
dcGTX2	H	H	OSO_3^-	OH
dcGTX3	H	OSO_3^-	H	OH
dcGTX4	OH	OSO_3^-	H	OH
dcNEO	OH	H	H	OH
GTX5	H	H	H	$OCONHSO_3^-$
GTX6	OH	H	H	$OCONHSO_3^-$
C1	H	H	OSO_3^-	$OCONHSO_3^-$
C2	H	OSO_3^-	H	$OCONHSO_3^-$
C3	OH	H	OSO_3^-	$OCONHSO_3^-$
C4	OH	OSO_3^-	H	$OCONHSO_3^-$
M1α	H	H	OH	$OCONHSO_3^-$
M1β	H	OH	H	$OCONHSO_3^-$
M3	H	OH	OH	$OCONHSO_3^-$
GC1	H	H	OSO_3^-	$OCOC_6H_4OH$
GC2	H	OSO_3^-	H	$OCOC_6H_4OH$
GC3	H	H	H	$OCOC_6H_4OH$
GC4	OH	H	OSO_3^-	$OCOC_6H_4OH$
GC5	OH	OSO_3^-	H	$OCOC_6H_4OH$
GC6	OH	H	H	$OCOC_6H_4OH$

Fig. 1 Paralytic shellfish toxin structure and classification into the three main groups

PSTs are mostly associated with marine dinoflagellates (genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium*) and freshwater cyanobacteria, which form extensive blooms around the world (Wiese et al. 2010). These phycotoxins are taken up by predators feeding on plankton, either directly (i.e., mollusks) or through several trophic levels. While they do not harm the shellfish, ingestion of contaminated shellfish can cause a range of health issues including numbness and tingling of the lips. In serious cases, intoxication can result in death from respiratory arrest and cardiovascular shock due to the blockage of voltage-gated sodium channels (a syndrome known as paralytic shellfish poisoning (PSP)). Therefore, it is essential to conduct regular monitoring of the phytoplankton and the shellfish tissue toxin content in shellfish harvesting areas. Such monitoring programs rely on intensive sampling and analysis that require the availability of rapid, sensitive, accurate, and precise testing methods. For many years, the mouse bioassay (MBA) (AOAC 2005) has been used globally for determination of PSP toxicity in shellfish and has been the official reference method in several countries. However, important method drawbacks and ethical considerations have placed an increasing pressure on regulatory bodies and researchers to provide alternative methods, which do not require the use of animals for food safety testing. Instrumental methods used for the determination of PSTs include fluorimetric methods, liquid chromatography (LC) methods with fluorescence detection (FLD), LC coupled to mass spectrometry (LC-MS), and capillary electrophoresis (CE).

In 1975, Bates and Rapoport described a fluorimetric method, based on the oxidation of STX in alkaline solution to form a fluorescent pyrimidine purine, for the measurement of STX in marine bivalves. This was the basis of several LC methods with post-column oxidation (PCOX) and FLD for the determination of PSTs, among which the Sullivan et al. (1985) and Oshima (1995) methods are well known worldwide. In the last decade, there have been significant improvements in the high-performance liquid chromatography (HPLC)-FLD PCOX methods (Rourke et al. 2008; Van de Riet et al. 2009, 2011). On the other hand, a HPLC-FLD method with pre-column oxidation (PRECOX) was developed and further modified to improve its performance for routine regulatory purposes (Lawrence and Ménard 1991; Lawrence and Niedzwiadek 2001). Nowadays, the HPLC methods most widely employed for the analysis of STXs are the Association of Official Analytical Chemists (AOAC) Official Methods of Analysis (OMA) 2005.06 (AOAC 2006) and 2011.02 (AOAC 2011), both of which have been internationally validated through

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collaborative studies. In recent years, technological advances, such as the introduction of ultrahigh-performance liquid chromatography (UHPLC) and the emergence of columns packed with superficially porous particles, have allowed the development of faster LC-FLD methods for PST analysis (Hatfield and Turner 2012; Harwood et al. 2013). There have been also important advances in the use of LC-MS with electrospray ionization (ESI) for the detection and quantitation of PSTs in shellfish and for the structural elucidation of new analogues (Dell'Aversano et al. 2005; Sayfritz et al. 2008; Boundy et al. 2015, among others). Some of the latest studies (Zhuo et al. 2013; Boundy et al. 2015; Turner et al. 2015) show promising results and indicate that some initial drawbacks in the application of LC-MS for PST quantification have been overcome. CE is an alternative separation technique to LC that has been investigated in conjunction with different detectors. However, in contrast to HPLC-FLD methods and due to several drawbacks, none of the CE methods are fully quantitative for the analysis of the most known PSTs in shellfish nor have been subjected to single-laboratory validation (SLV) studies.

Fluorimetric Methods

Bates and Rapoport (1975) were pioneers in developing a method involving alkaline hydrogen peroxide oxidation of STX to 8-amino-6-hydroxymethyl-2-iminopurine-3-propionic acid. This compound reacts in acidic solution to give a fluorescent pyrimidine purine, and the fluorescence intensity can be measured. The method was applied to Alaska butter clams and to California mussels contaminated with STX. The results obtained were compared with those of the MBA and showed that the method was 100 times more sensitive than the MBA. Indrasena and Gill (1998) developed a rapid qualitative screening method with fluorimetric detection that targeted STX and also the N-1-hydroxylated PSTs (neosaxitoxin (NEO), gonyautoxin 6 (GTX6), GTX1,4, N-sulfocarbamoyl gonyautoxins 3,4 (C3,4)), and GTX2,3 (Fig. 1). They tested different oxidants and reported that hydrogen peroxide was the most convenient and efficient one. The method was useful for the screening of fractions separated by column chromatography.

On the basis of the Bates and Rapoport (1975) method, Gerdts et al. (2002) developed a fast fluorimetric method for the detection of STX in phytoplankton samples. The results obtained as total fluorescence were compared with HPLC results for a number of carbamoyl, decarbamoyl, and N-sulfocarbamoyl PSTs, and the correlation obtained was significant for most of the carbamoyl toxins. Despite the fact that other authors implemented modifications of the original fluorimetric method or developed new methods, these were soon replaced by the more sensitive and selective LC-FLD methods.

High-Performance Liquid Chromatography with Fluorescence Detection

LC is a powerful instrumental technique for the analysis of marine biotoxins, although challenges result from the fact that most toxins do not possess a chromophore for sensitive ultraviolet (UV) absorbance or fluorescence detection. PSTs are some of the most difficult compounds to analyze by LC due to their polar, multifunctional nature (Quilliam 2003). These toxins have only a weak natural chromophore that must be modified prior to fluorescence detection through an oxidation (or derivatization) reaction. Oxidation of PSTs in alkaline solution lead to purine compounds that become fluorescent in acidic solution. This derivatization can be carried out pre-column or post-column, and it is used to classify the HPLC methods for the determination of PSTs into two categories, as discussed below. The purines formed can be monitored with a fluorescence detector and individual toxin concentrations calculated. This provides one advantage for LC methods over biological and functional assays, since they enable the determination of toxin profiles within an algal bloom or toxic sample.

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In 1978, the fluorimetric method developed by Bates and Rapoport (1975) was incorporated into a detection method for PSTs after their chromatographic separation. Following the incorporation of a number of modifications, the fluorimetric method was utilized in a post-column reaction system with separation of the toxins by HPLC and subsequently improved (Sullivan and Wekell 1984; Oshima et al. 1984). These were the first reports describing the use of HPLC for the determination of PSTs and establishing the basis of current PCOX methods.

Post-column Derivatization Methods

HPLC-FLD methods with PCOX are used in several countries for the monitoring of PSTs, most notably in regions where the phytoplankton blooms are dominated by species of the genera *Alexandrium*. These methods involve the extraction of toxins from shellfish in acidic conditions, followed by the chromatographic separation of the different congeners and their on-line oxidation. The toxins are chromatographically separated and elute from the column as individual bands. This represents one important advantage over pre-column oxidation (PRECOX) methods in which the oxidation products of epimeric pairs are identical and therefore cannot be resolved chromatographically. However, for the derivatization reaction, additional instrumentation is needed: a post-column reaction system with two pumps, together with a reaction coil maintained at a constant temperature.

The first attempts with these methods were made in the 1980s. Sullivan and Wekell (1984) improved a HPLC method for PST determination initially developed by their group. The method involved the preparation of a shellfish acid extract followed by ion-interaction chromatography on a polystyrene-divinylbenzene resin column, in reverse-phase mode, using heptane sulfonic acids as ion-pairing reagents. Detection of the toxins was conducted using fluorescence following post-column alkaline periodate oxidation. In comparison with their first attempts, they achieved a better toxin separation and adequate sensitivity and were able to determine the full toxin profile from a single injection. Sullivan et al. (1985) evaluated the applicability of their method to shellfish toxicity monitoring and compared the results obtained by their method with those of the AOAC OMA 959.08 MBA (AOAC 2000). They found, in general, a good correlation between the two methods and reported that their method provided better detection sensitivity and greater sample throughput than the MBA. However, one of the disadvantages of the method was that C1 and C2 toxins eluted very close to the solvent front and could not be chromatographically resolved. Poor resolution between STX and dcSTX was also reported.

Around the same time, Oshima et al. (1984) described attempts to improve the initial oxidation reaction from Bates and Rapoport (1975) that was not adequate for N-1-hydroxylated toxins. After testing various oxidizing reagents, they found that tert-butyl hydroperoxide yielded highly fluorescent derivatives, even for the PSTs non-fluorescing following hydrogen peroxide oxidation. Since NEO and STX showed stronger basicity than GTX1–GTX6, the two groups of toxins were analyzed under different chromatographic conditions, each group using different mobile phases. Separation between the epimers GTX2 and GTX3 was achieved, but this was not the case for GTX1 and GTX4 and for GTX3 and GTX5. Later, Oshima et al. (1987) described a method that was able to separate the most important PSTs by applying three chromatographic runs. Toxin groups of different net charges were separately determined by isocratic elution using different mobile phases. Group I comprised C1–C4 toxins; group II comprised GTX1–GTX6 and decarbamoylgonyautoxins 1–4 (dcGTX1–dcGTX4); and group III consisted of NEO, dcSTX, and STX. The optimized method was applied to *Gymnodinium catenatum* cells. Later, it was improved further by Oshima (1995) who also tried the application of a solid-phase extraction (SPE) step using C18 cartridges for the cleanup of acidic scallop extracts. This allowed the removal of interfering fluorescent compounds from the sample extracts and consequently extended the analytical column lifetime. The main advantage of this method over Sullivan et al. (1985) was the successful separation

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and quantification of C1–C4 toxins. These toxins were inseparable in the Sullivan et al. (1985) method that had been initially designed for determining the PSTs related to *Alexandrium* spp. blooms.

Most of the later HPLC-FLD PCOX methods were modifications of one of these two methods. More information on these methods, together with a summary of their different chromatographic and/or oxidation conditions, are available in Ben-Gigirey and Villar-González (2008).

Another HPLC method coupled to a post-column electrochemical oxidation system (ECOS) was described by Boyer and Goddard (1999), who applied it to the analysis of cyanobacteria, dinoflagellate, and shellfish samples. A coulometric electrochemical cell, placed in-line between the column outlet and the fluorescence detector, oxidizes PSTs into fluorescent derivatives. The advantages of this system over the previous ones are the simpler instrumental setup and the avoidance of reagent instability problems. However, disadvantages are related to the requirement for care and maintenance of the oxidizing electrode. By employing, also, electrochemical PCOX, Jaime et al. (2001) developed a method able to separate the relevant PSTs in a single chromatographic run and demonstrated applicability to the analysis of biological materials. They reported the use of anion and cation exchange columns connected in series and aqueous ammonium acetate mobile phases. C1, C2, GTX1, GTX4, dcGTX3, dcGTX2, GTX2, GTX3, NEO, dcSTX, and STX could all be detected by FLD and also through MS detection. In 2005, Papageorgiou et al. developed a single-run method using high-performance ion exchange chromatography with sodium acetate mobile phases, coupled to chemical PCOX and FLD. It was successfully used to determine PSTs, at low ppb levels, in freshwater samples and cyanobacterial extracts. The toxins C1, C2, GTX1, GTX4, GTX2, GTX3, NEO, and STX were separated, although the resolution of dcSTX/STX was poorer and an unidentified peak (possibly dcNEO) coeluted with NEO in a *Cylindrospermopsis raciborskii* extract.

The HPLC-FLD PCOX method most commonly used currently is AOAC OMA 2011.02 (AOAC 2011). The first paper on the method was published by Rourke et al. (2008) and focused on the method development. PSTs are extracted from shellfish samples with 0.1 M hydrochloric acid (HCl) and heat. Afterwards, an aliquot of the supernatant is deproteinated with trichloroacetic acid (TCA), centrifuged, and pH-adjusted for LC analysis. Two different LC columns are used, with different mobile phases and LC conditions. STXs and GTXs are separated on a C18 silica column with a step gradient using a heptane sulfonic acid/phosphoric acid buffer system. C toxins are chromatographed on a C8 silica column using an isocratic tetrabutylammonium phosphate buffer. Separated toxins are subsequently derivatized post-column with a phosphoric acid, periodic acid buffer solution. The oxidized effluent is acidified using nitric acid, and the derivatives detected by FLD. The main advantage of this method over previous PCOX methodologies is the higher throughput and faster turnaround of positive samples, essential in a regulatory environment where decisions are required on a timely basis. Figure 2 shows a schematic diagram of the method.

In order to achieve international and regulatory acceptance, the method was single laboratory validated in mussels, clams, scallops, and oysters (van de Riet et al. 2009). Later, the method was internationally validated through a collaborative trial in the same matrices (AOAC 2011). The method determines individual PST concentrations (STX, NEO, GTX1 to GTX5, dcGTX2 and dcGTX3, dcSTX, C1, and C2) in edible tissues, as well as the total toxicity of the test samples above 100 μg STX dihydrochloride (di-HCl) equivalents per kg. However, method validation did not include the toxins C3, C4, GTX6, dcGTX1, dcGTX4, and dcNEO, mainly due to the lack of those reference standards. Notably, dcNEO coelutes with NEO, and a practical solution to separate both toxins was not included in the method protocol. This means that, to date, the method is not suitable for the analysis of samples which contain toxins of the *Gymnodinium catenatum* toxic profile, and this could be an important handicap for the use of the method in PST monitoring in certain countries. Figure 3 shows chromatograms of standards and a mussel sample contaminated during an *Alexandrium* spp. bloom. Another method drawback is the short

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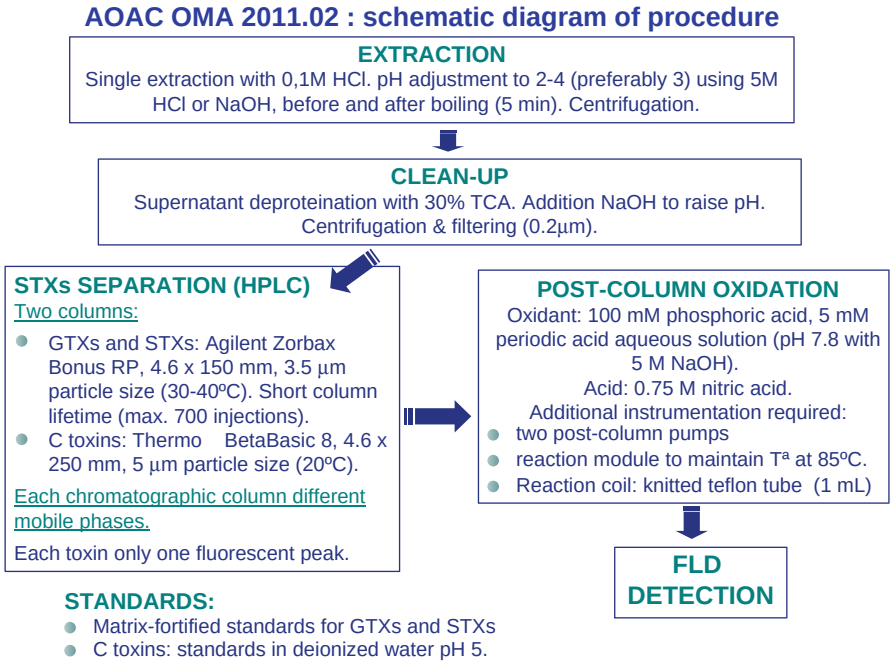

Fig. 2 Association of Official Analytical Chemists Official Methods of Analysis 2011.02: schematic diagram of procedure

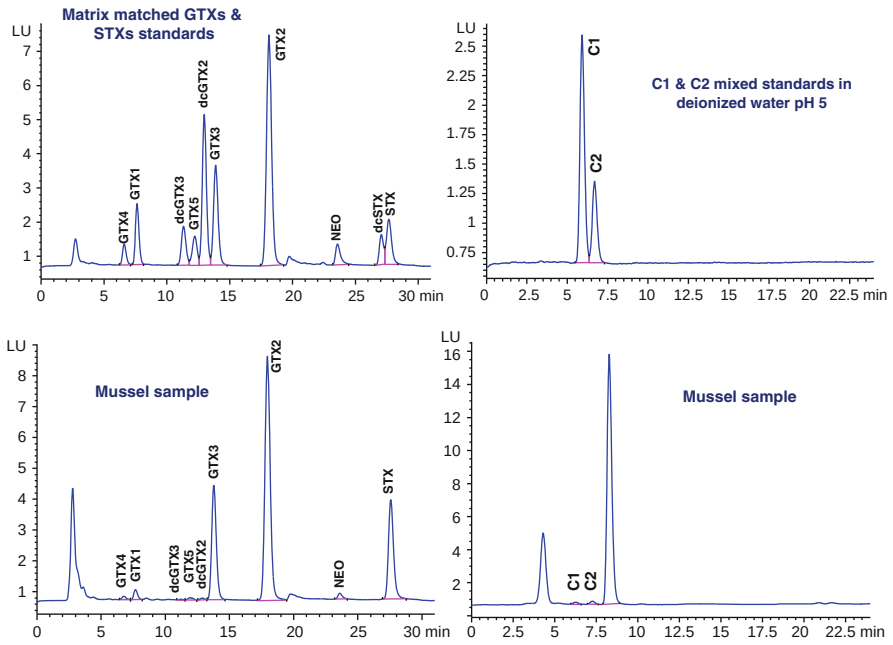

Fig. 3 Standard chromatograms obtained with two different columns and chromatographic conditions using the post-column oxidation method. Association of Official Analytical Chemists Official Methods of Analysis 2011.02. Chromatograms from a mussel sample show the typical toxins found in samples contaminated during *Alexandrium* spp. blooms

life span (300–700 injections) of the column used to separate GTXs and STXs. At the European Union Reference Laboratory for Marine Biotoxins (EURLMB), several chromatographic columns have been tested in order to find a more robust column for GTX and STX analysis. The potential for separation between GTX4 and GTX6 as well as NEO and dcNEO is also currently under evaluation (Escudeiro-Rossignoli et al. 2015).

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Pre-column Derivatization Methods

The development of HPLC PRECOX methods for PST analysis dates back to 1991. Lawrence and Menard developed a technique involving an extraction with 0.1 M HCL, followed by a pre-analysis oxidation reaction using hydrogen peroxide and sodium periodate. Derivatized extracts were subsequently subjected to chromatographic separation and FLD. Several method modifications were subsequently reported achieving an improved separation and quantification of most PSTs, as well as improving the method performance characteristics (repeatability, ruggedness, recovery) required for routine regulatory purposes (Lawrence and Niedzwiadek 2001). As well as modifying chromatographic conditions, the oxidation reaction conditions were changed to achieve the best compromise for all the PSTs, since the optimum pH for the oxidation of individual toxins varies considerably (Gago-Martínez et al. 2001). Moreover, a matrix modifier, consisting of a toxin-free oyster extract, was added to the derivatization to improve the yield and repeatability at the periodate oxidation of both standards and sample extracts. Finally, the performance of the SPE-COOH cleanup, which enables the separation of the sulfocarbamoyl toxins from their gonyautoxin and carbamoyl counterparts, was also evaluated.

After the method SLV, it was validated through a collaborative trial (Lawrence et al. 2005) and adopted as First Action AOAC Official Method 2005.06 (AOAC 2006). AOAC OMA 2005.06, known also as Lawrence method, is applicable to the determination of STX, NEO, GTX2,3 (combined), GTX1,4 (combined), dcSTX, GTX5, C1,2 (combined), and C3,4 (combined) in shellfish (mussels, clams, oysters, and scallops).

Figure 4 shows a schematic diagram of the method. First, the toxins are extracted from the shellfish through a duplicate extraction with 1 % acetic acid (HOAc) solution, with the first extraction conducted in boiling water. This is followed by a cleanup of the combined extract supernatants in SPE C18 cartridges. Toxin oxidation is conducted using hydrogen peroxide and/or periodate reagents, followed by LC-FLD. Although standards for C3,4 and GTX6 are still not commercially available, extracts containing GTX1,4, C3,4, NEO, and GTX6 must be further purified with weak cation exchange SPE cartridges, in order to obtain three fractions each containing different toxin groups (Fig. 5). Each fraction is then subjected to

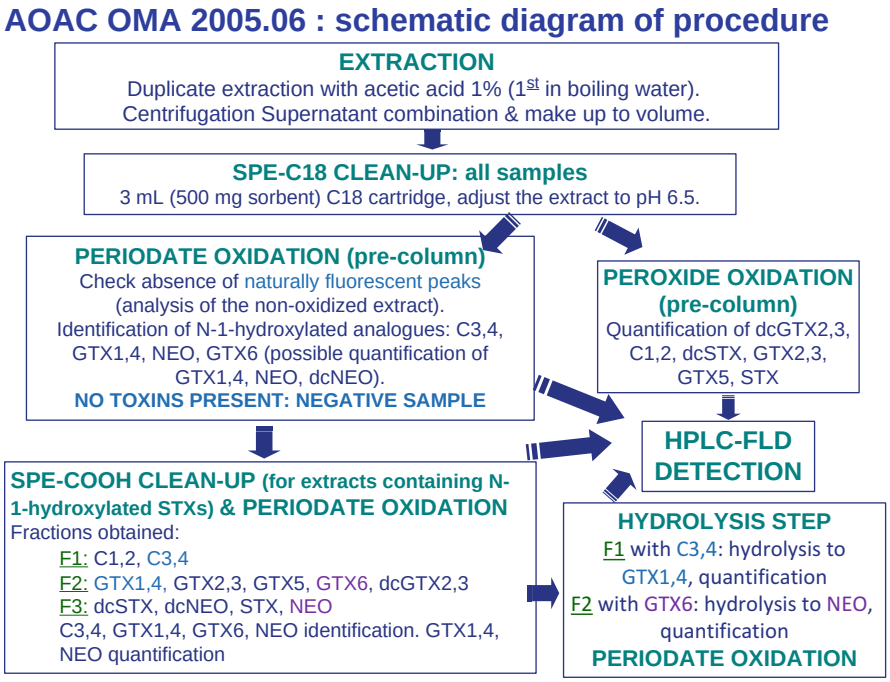

Fig. 4 Association of Official Analytical Chemists Official Methods of Analysis 2005.06: schematic diagram of procedure

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periodate oxidation and LC-FLD analysis. This SPE-COOH cleanup step is required to be able to separate and adequately quantify toxins that have common oxidation products. The whole procedure would be needed for the full quantification of abovementioned PSTs; however, a simpler procedure could be applied for screening.

In 2006, an interlaboratory study was organized to evaluate the fitness for the purpose of AOAC OMA 2005.06 for the official control of PSTs in EU laboratories (Ben-Gigirey et al. 2007). The results obtained by 18 participant laboratories were encouraging for most shellfish samples, contaminated during *Alexandrium* spp. blooms. However, some drawbacks were found for samples with *G. catenatum* toxic profile. This, together with the fact that the method was not internationally validated for all the main PSTs, prompted the creation of an expert EU working group and the organization of several studies to further refine the method and extend the international validation. These studies were designed to study the quantification performance of dcGTX2,3, dcNEO, and GTX6 (EURLMB 2007; Ben-Gigirey et al. 2012). With the absence of a suitable certified reference standard, GTX6 may be quantified through an additional

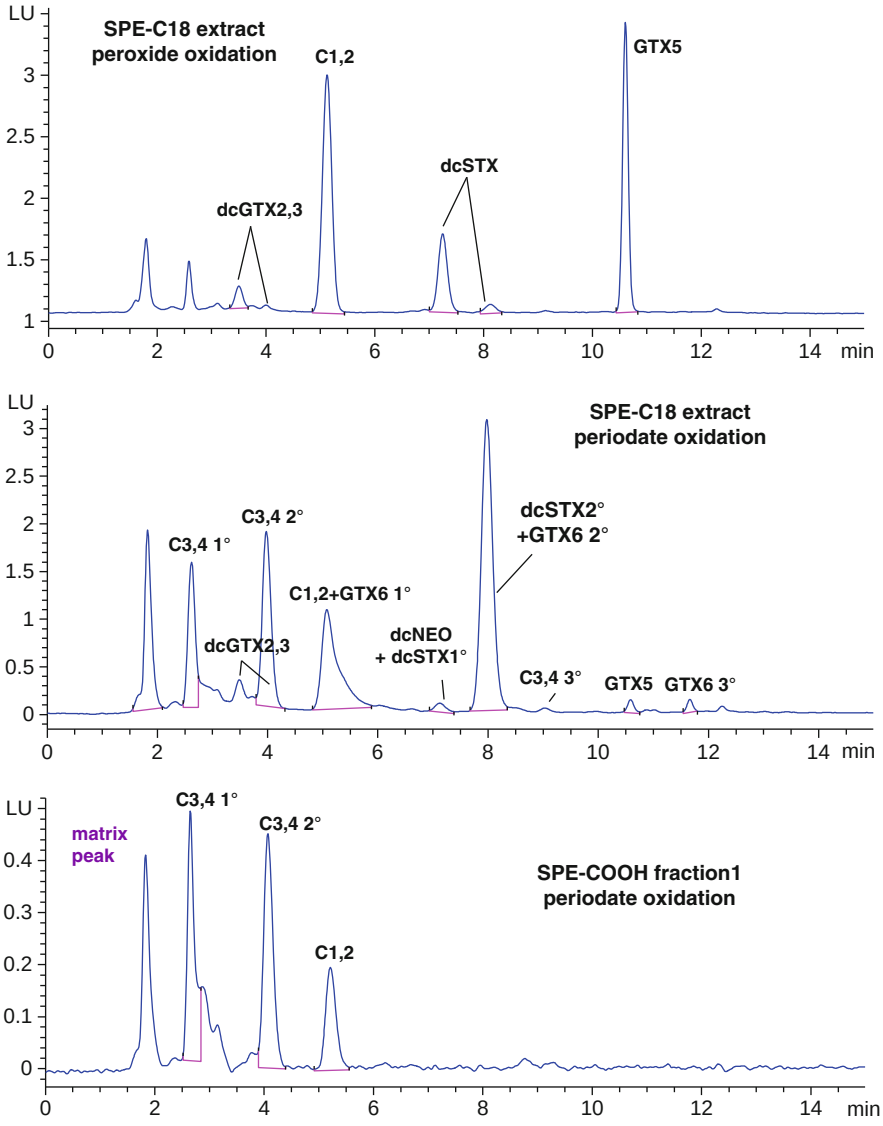

Fig. 5 (continued)

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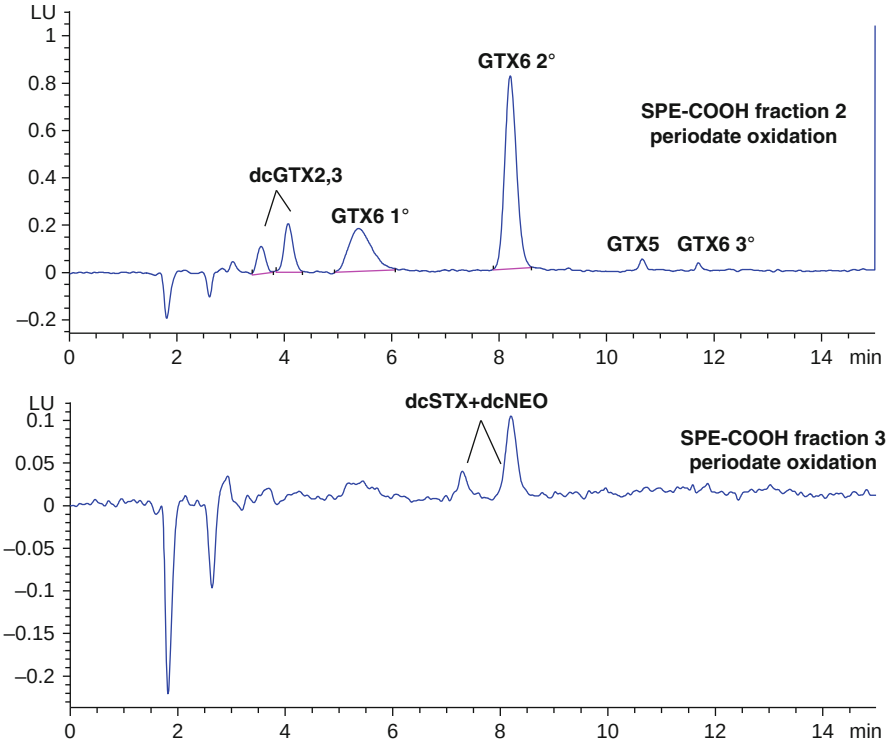

Fig. 5 Chromatograms from a Galician oyster sample contaminated during a *Gymnodinium catenatum* toxic event. Pre-column oxidation of solid-phase extraction C18 cleaned up extracts, with both peroxide and periodate oxidants, is shown, together with periodate chromatograms from the three fractions obtained after solid-phase extraction-COOH fractionation

hydrolysis step to form NEO. This step can also be applied to C3,4 allowing quantification once it is hydrolyzed to GTX1,4. One of the major challenges of the method is the proper identification of the different PSTs, which is a key issue for accurate quantification. In certain samples the interpretation of the chromatograms can be challenging due to the coelution of different oxidation products (Fig. 5) and the production of more than one fluorescent oxidation product for N-hydroxylated toxins. Proper training and highly skilled staff are key issues to successfully apply the method.

Turner et al. (2009) reported other method improvements. These authors standardized certain procedures and refined the SPE-COOH fractionation by using cartridges with a different sorbent and slightly changing the concentrations of the salt solutions employed for fraction elution. They also performed both SPE steps semiautomatically producing faster, reliable, and repeatable cleanup results and reducing manual labor at the same time. This approach is highly convenient in a high-throughput monitoring environment. The method was also refined to improve the recovery and sensitivity following the periodate oxidation step in scallops (Turner and Hatfield 2012). In this matrix, performance was improved through altering the preparation of the periodate oxidant, the use of higher volumes for SPE-C18 cleanup, the injection of higher volumes, and the use of a king scallop matrix modifier for oxidation of N-1-hydroxylated toxin calibration standards. Other studies aimed at reducing analysis time by altering the method chromatography conditions will be discussed in section "Ultrahigh-Performance and Rapid Resolution Liquid Chromatography."

AOAC OMA 2005.06 has replaced the use of the MBA in several countries, since it is applicable to shellfish samples contaminated during both *Alexandrium* spp. and *Gymnodinium catenatum* blooms. This method has been recently selected by the CODEX Alimentarius Committee on Methods of Analysis and Sampling as a reference method for PST analysis in shellfish.

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Ultrahigh-Performance and Rapid Resolution Liquid Chromatography

With multiple chromatographic analysis runs required to accurately quantify PSTs profiles in shellfish using AOAC 2005.06, the advantages of shortening chromatographic run times are significant. AOAC 2005.06 method utilizes 5 μm reverse-phase C18 HPLC columns, usually 150 mm in length with 4.6 mm internal diameter and packed with porous silica or polymer micron-sized particles. In 2004, ultrahigh-performance liquid chromatography (UHPLC) hardware was introduced, facilitating the use of lower particle size columns and high column back pressures. UHPLC has been applied to PSTs analysis, most notably in tandem with mass spectrometric detection, although there is a report of UHPLC with FLD detection based on AOAC 2005.06 enabling faster analysis times (Harwood et al. 2013).

The availability of "superficially porous" particles, also known as "core-shell," "fused-core," or "solid-core" particles, has alternatively provided opportunities for fast LC analysis using standard HPLC hardware (Hayes et al. 2014). These columns contain solid, nonporous cores, which are surrounded by a shell of porous material with similar properties to fully porous HPLC materials. Use of these columns provides chromatographic performance similar to that obtained with UHPLC columns on UHPLC hardware. Hatfield and Turner (2012) first described the application of solid-core particle HPLC columns to AOAC 2005.06. DeGrasse et al. assessed the performance of an Ascentis Express 4.6 mm × 100 mm C18 fused-core column in comparison with a fully porous SUPELCOSIL LC-18 column. A reduction in run time was achieved from 15 to 5 min. Hatfield and Turner 2012 described the validation of a fused-core method using both a Phenomenex Kinetex 4.6 mm × 100 mm 2.6 μm C18 and an Agilent Poroshell 4.6 × 100 mm 2.7 μm C18 column. Both methods provided a total analytical run time of 50 % of the time required for 5 μm HPLC. In addition, analytical sensitivity was significantly improved, although issues with column longevity were highlighted (Hatfield and Turner 2012).

Overall, UHPLC and fused-core column technologies both offer high beneficial options for improving the efficiency and throughput of PSTs LC-FLD methods, although to date there have been no published reports of such approaches being used for AOAC 2011.02.

Liquid Chromatography-Mass Spectrometry Methods

In recent years, there have been significant advances in the use of liquid chromatography with electrospray ionization mass spectrometry (ESI-MS) for the detection and quantitation of PSTs in both phytoplankton and shellfish tissues (Quilliam et al. 1989; Quilliam et al. 2001; Dell'Aversano et al. 2005; Turrell et al. 2008; Sayfritz et al. 2008; Zhuo et al. 2013; Watanabe et al. 2013; Boundy et al. 2015; Turner et al. 2015). ESI-MS is a highly suitable detection method for these toxins, due to the structural characteristics of the saxitoxins giving rise to the generation of intense ions in both the positive $[M + H]^+$ and negative $[M\text{-}H]^-$ ion modes.

Shellfish Extraction

Methods for extraction of the PSTs from shellfish tissue have been well described and validated, with the two most common methods involving weak acidic extraction using either HCl or HOAc. For injection of samples into highly organic mobile phases, extracts are often diluted post-extraction with organic solvent or very low injection volumes used to minimize sample solvent-related chromatography issues. As an alternative approach, alternative extraction methods have been reported using organic solvent into the extraction steps. The Dell'Aversano (2005) hydrophilic interaction liquid chromatography (HILIC)-MS/MS method utilized a three-step extraction with acetonitrile/water (80:20, v/v) and 0.1 % formic acid, subsequently modified by Sayfritz et al. (2008) and Zhuo et al. (2013) to incorporate additional freezing and cleanup stages. Gerssen et al. (2015) also recently reported the use of a mixture of acetonitrile, water,

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ammonium formate, and formic acid. However, recently published work has reverted to the use of acetic acid extraction to maximize recovery of toxins, with high salt concentrations subsequently removed by SPE cleanup (Boundy et al. 2015; Turner et al. 2015).

Chromatography

The use of ESI-MS in combination with current conventional LC methodologies for direct replacement of fluorescence detection provides analytical challenges due to the use of ion-pair reagents such as heptafluorobutyric acid as mobile phases (Quilliam et al. 1993). These are known to be generally unsuitable for interfacing with MS for detection of PSTs (Quilliam 2003; Dell'Aversano et al. 2005), although some reports of reverse-phase ESI-MS methods do exist (Dahlmann et al. 2003).

A different approach using HILIC in tandem with ESI-MS was first reported by Quilliam et al. (2001), Quilliam (2003), and Dell'Aversano et al. (2005). This technique has become the method of choice given its high suitability to the separation of small polar molecules. The double-charged toxins such as STX, dcSTX, dcNEO, and NEO exhibit the longest retention times, due to the greater interaction with stationary phase. Progressively shorter retentions are observed, respectively, for the singly charged gonyautoxins and the neutral C toxins (Dell'Aversano et al. 2005). Separation of epimeric pairs, with the earlier elution of α-epimers, arises due to the reduction in positively charged molecular substituents in the α-forms as a result of the interaction between the 11-hydroxysulphate group and the C8 guanidinium group (Dell'Aversano et al. 2005).

Mass Spectrometry

The choice of mass spectrometric interface is also important. Quilliam et al. (1993) reported their attempts to use a number of approaches in tandem with LC, including a moving belt interface, thermospray, and atmospheric pressure chemical ionization (APCI). However, none of these methods were successful in producing mass spectra for either parent compounds or oxidation products. Fast atom bombardment (FAB) and secondary ion mass spectrometry (SIMS) (Maruyama et al. 1984) and ion-spray MS (IS-MS) or ESI-MS were used for early reports of PSTs analysis (Quilliam et al. 1993, 1999) with ESI-MS becoming the detection method of choice for future developments. For MS detection, quadrupole mass spectrometry with single ion monitoring (SIM) or selected reaction monitoring (SRM) is the most commonly used approach for PSTs detection and quantitation.

Many authors have described issues with matrix effects resulting from the presence of salts and other sample co-extractives. These are particularly significant for PSTs analysis in shellfish extracts where both the HILIC chromatography and MS detection are compromised. Matrix components are known to significantly affect the sensitivity of PSTs analysis in shellfish extracts due to ionization suppression and cause other method performance issues such as variable chromatographic retention times and poor peak shape (Fang et al. 2004; Dell'Aversano et al. 2005; Sayfritz et al. 2008; Boundy et al. 2015). Given the high sensitivity of detection for pure PSTs standards in solvent, the use of a suitable sample preparation method can result in the development of highly sensitive detection methods. Until recently, the lack of a suitable sample cleanup method has slowed the development of a highly sensitive ESI-MS PSTs detection method (Turrell et al. 2008).

Other issues with MS detection result from the need to fully separate congeners which form interference peaks due to in-source fragmentation and the structural similarity between different toxins, particularly if detection is conducted using positive mode ESI exclusively (Dell'Aversano et al. 2005). Mass spectra were extensively characterized by Dell'Aversano et al. (2005) for 19 different PSTs and form the basis for the majority of PSTs LC-MS methods which have followed. There are relatively few examples of published methods employing negative mode ESI-MS, although recent papers evidence a considerable advance in utilizing both positive and negative SRM transitions (Boundy et al. 2015).

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HILIC-MS Development

HILIC-ESI-MS for PSTs detection was first reported by Quilliam et al. (2001) and Quilliam (2003), with subsequently other researchers utilizing the approach for PSTs detection in a range of samples including phytoplankton (Dahlmann et al. 2003; Collins et al. 2009), zooplankton (Durbin et al. 2002), octopi (Robertson et al. 2004), cyanobacteria (Foss et al. 2012; Lajeunesse et al. 2012), purified toxin solutions (Munday et al. 2013), and gastropods (Jen et al. 2014).

Dell'Aversano et al. (2005) made the major breakthrough in the area with the description of an efficient HILIC separation using a 30 min run time with a 5 μm TSKgel Amide-80 Tosoh Bioscience LLC HPLC column. The method was applied to a wide range of PSTs congeners and later used to characterize a new group of toxins, termed M toxins, which were undetectable with the use of FLD methods (Dell'Aversano et al. 2008). Tandem mass spectrometry (MS/MS) with selected reaction monitoring (SRM) detection was conducted exclusively in positive mode, with the authors noting issues resulting from in-source fragmentation and matrix-related problems including unstable chromatographic retention times and ionization suppression. Stobo et al. (2005) described the use of the same HPLC column for separation of a smaller number of PSTs using a 30 min isocratic HILIC method, but again matrix effects were significant.

Other versions of the method incorporated additional cleanup steps (Sayfritz et al. 2008). More recently, Watanabe et al. (2013) published a quantitative method for the determination of 12 different PSTs standards using a 3 μm Waters XBridge amide column on standard HPLC hardware, for the sensitive and repeatable analysis of algae samples.

Retention time variability between different shellfish matrices prompted Diener et al. (2007) to develop a new HILIC method allowing complete separation in one chromatographic run of 12 PSTs congeners using a 5 μm zwitterionic (ZIC) SeQuant HILIC column. A 40 min run time was developed, and all SRM MS/MS data was acquired in positive mode. The method was illustrated with the analysis of toxin standards, a single extract of *Gymnodinium* catenatum and a spiked mussel extract.

Both the TSKgel Amide-80 and ZIC-HILIC columns were used by Turrell et al. (2008) for the separation of toxins prior to SPE cleanup and MS detection. Developed methods were applied to the analysis of acidic mussel extracts and tested using both MS and MS/MS detection. SPE options were investigated to reduce shellfish matrix effects, with HILIC SPE using a ZIC-HILIC cartridge found to be most successful for sample extract cleanup (Turrell et al. 2008). While this optimized ZIC-HILIC SPE with HILIC-MS/MS method showed promise, it was applied only to three mussel samples and remains untested on either a larger sample set or in a high-throughput monitoring environment. Most recently, a C18 SPE cleanup method with ZIC-HILIC-MS/MS detection incorporating positive and negative ionization ESI has been published for PSTs detection and quantitation (Rossignoli et al. 2015). The chromatography was optimized for a 25 min separation and detection of 13 PSTs, without the need for fast polarity switching.

UHPLC-HILIC Methods

UHPLC with low particle size HILIC columns has enabled the development of PSTs analysis at high pressures and shorter run times. Halme et al. (2012) published a 3 μm HILIC-ESI-MS/MS method for the quantitation of STX and identification of other PSTs in algal samples, with a total run time of less than 10 min. Sub-2 μm HILIC has also been applied with full scan MS and MS/MS to the detection of PSTs in Tasmanian abalone (Harwood et al. 2014) as well as in clinical urine samples (Johnson et al. 2009; Bragg et al. 2015). Many of the reported methods for the analysis of PSTs in shellfish are, however, impractical for high-throughput routine analysts, given the long sample preparation procedures for sample cleanup and high analysis times for effective HILIC separation of PSTs analogues. More recently, a few authors have published methods with a specific focus on the development of methods which involve UPLC chromatography in tandem with faster sample processing.

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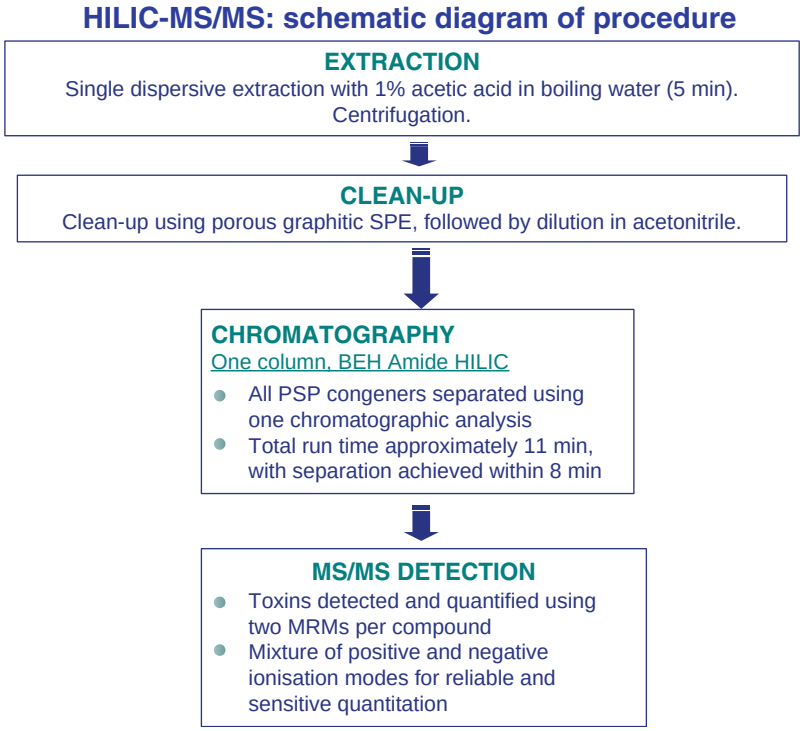

Fig. 6 Hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS/MS): schematic diagram of procedure

Zhuo et al. (2013) reported the use of QuEChERS (quick, easy, cheap, effective, rugged, and safe) cleanup methods prior to a 25 min HILIC analysis with positive mode ESI and SRM for up to ten PSTs analogues. Matrix-matched standards were required to compensate for significant levels of matrix suppression. Good toxin recoveries and method precision were reported for an oyster, clam, and mackerel species, although results were not compared with those from other PSTs detection methods. The method incorporated long sample processing and analysis times, resulting in low sample throughput.

Boundy et al. (2015) reported significant developments with a new UHPLC-HILIC-MS/MS method. A quick and simple sample preparation protocol incorporated a one-step dispersive extraction in 1 % HOAc, followed by an effective cleanup step using amorphous graphitized polymer carbon solid-phase extraction cartridges (Fig. 6). HILIC was conducted using 1.7 μm ACQUITY UPLC BEH Amide columns, with authors reporting the full separation of all interfering congeners within 11 min. ESI-MS/MS also incorporated both positive and negative mode SRMs. Analytes studied included all PSTs available as certified reference material (CRM) standards together with C3 and C4, GTX6, dcGTX1 and dcGTX4, doSTX, and the M toxins M1–M5 (Fig. 1). As a consequence, the method was found to successfully overcome the major issues affecting the speed, efficiency, accuracy, reproducibility, and sensitivity of HILIC-MS detection methods for PSTs in shellfish. Turner et al. (2015) subjected the same method to a full single-laboratory validation for the application to 12 different commercial bivalve shellfish matrices including mussels, oysters, clams, and scallops from the UK and New Zealand. The full validation covered the detection and quantitation of STX, NEO, dcNEO, dcSTX, doSTX, GTX1–GTX6, dcGTX2, dcGTX3, C1, C2, while the method also included C3, C4, dcGTX1, dcGTX4 and M toxins. Acceptable method performance characteristics were reported for ruggedness, linearity, specificity, recovery, precision, and reproducibility. The sensitivity of the method was shown to be excellent in comparison with LC-FLD methods, with a summed limit of quantification for the HILIC-MS/MS method of 54 μg STX di-HCl eq/kg in comparison with 784 and 358 μg STX di-HCl eq/kg for AOAC 2005.06 and AOAC 2011.02, respectively (Turner et al. 2015). The method also compared well

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with LC-FLD AOAC 2005.06 following the comparative analysis of over 1,100 contaminated shellfish samples. Consequently, the method has been shown to be fit for purpose for the rapid, accurate, and reproducible determination of a large number of PSTs appropriate to a variety of toxin profiles in a wide range of shellfish species. Given the success of this method, work should continue through interlaboratory validation studies.

Other MS Approaches

While the majority of reported methods to date have focused on quantitative MS/MS detection, another MS instrumentation and methodology is available and has been applied to the analysis of PSTs. Negri et al. (2003) first published the use of high-resolution ESI-MS for the measurement of accurate masses of hydroxybenzoate PSTs congeners, with Sleno et al. (2004) describing the use of quadrupole time-of-flight (qTOF) MS in comparison with a high-resolution Fourier transform ion cyclotron resonance MS, when applied to the unambiguous detection of STX and NEO. Blay et al. (2011) later reported the use of LC with high-resolution MS method (LC-HRMS) for marine biotoxins including STXs and GTXs, incorporating a 6 min HILIC separation on a 1.7 μm Waters ACQUITY BEH Amide column. While complete separation of the GTXs was not possible in such a short run time, the separation was enough to resolve isobaric epimeric pairs. A rapid qualitative screening method for PSTs using HILIC-ESI-high-resolution (HR)-MS in algal samples has also been published (Li et al. 2013). The method involves the HOAc extraction of algae samples followed by HILIC-HR-MS in positive and negative full scan modes for both molecular ions and fragment ions using TOF-MS. Chromatography was performed on a 3 μm Waters Atlantis HILIC column over a gradient run time of 45 min for the separation of ten common PSTs in both toxin standard mixes and three different algal extracts. While there is some promise for LC-HRMS methods to provide quantitative methods for PSTs, to date, none of these have been validated or even applied to the analysis of naturally contaminated shellfish samples.

One other recent development in mass spectrometer technology, which has attracted some attention in relation to the analysis of marine biotoxins, is the technique of ion mobility-mass spectrometry (IMS or IM-MS). The ion separation potential of the technique is potentially very powerful, resulting in a highly selective analysis. HILIC with 5 μm Amide columns and a 40 min run time has been used in combination of IMS for the separation and identification of PSTs in toxin reference standards using a Waters SYNAPT G2 hybrid quadrupole-IM (TOF)-MS (Poyer et al. 2015). The resulting three-dimensional separation using the coupled technique allows the full separation of analogues which were not resolved using HILIC alone, noting improved IM separation for positive GTX isomer ions as opposed to negative ions. Beach et al. (2015) described the use of a similar approach using high-field asymmetric waveform ion mobility-mass spectrometry (FAIMS), in order to overcome the matrix effects present in samples of shellfish extracts. The work involved the assessment of both a direct ESI-FAIMS-MS infusion method and a full LC-ESI-FAIMS-MS approach. Application of the method to the quantitation of a mussel reference material provided some evidence for method accuracy for some, but not all, PSTs congeners. Further work involving more sensitive MS/MS SRM detection could result in improved method performance.

Capillary Electrophoresis

Capillary electrophoresis (CE) is a family of related techniques that employ narrow-bore (20–200 μm id) capillaries to perform high-efficiency separations of both large and small molecules. These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary. The properties of the separation and

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the ensuing electropherogram have characteristics resembling a cross between traditional polyacrylamide gel electrophoresis (PAGE) and modern high-performance liquid chromatography.

Owing to its high-resolution separation capability in very short times and its ease of operation, CE has aroused considerable interest for the analysis of complex polar biomolecules including marine toxins. Thibault et al. (1991) reported the application of CE with UV detection to a range of PSTs, with the exception of C toxins, due to their overall positive charge under acidic conditions which make them ideal candidates for separation based on ionic state. Although the separation of PSTs in three groups was already achieved using old electrophoresis formats such as high-voltage paper electrophoresis (HVPE) and a more efficient separation was expected by using CE, one of the inherent difficulties in analyzing these compounds relates to the lack of a chromophore absorbing in the UV range and the unavailability of standards to confirm the electrophoretic peak identity. NEO and STX were separated by CZE (capillary zone electrophoresis) and detected at 200 nm. The study also had limitations based on the lack of sensitivity associated with low injection volumes in CE which typically are in the nanoliter range. The confirmation of the identities of the electrophoretic peaks was carried out using ion-spray MS. The success of the coupling of CE and MS was critical, given the incompatibility of the two techniques. The lack of sensitivity of this initial approach was overcame in the later studies of Locke and Thibault (1994) who applied on-column sample preconcentration through capillary isotachophoresis (CITP) using discontinuous buffer systems prior to the CZE separation of PSTs. In CITP, samples are inserted between leading and terminating electrolytes with higher and lower electrophoretic mobility, respectively, than the sample compounds. Once the electric field is applied, analytes migrate at different speeds and are separated into contiguous bands. A steady-state configuration is ultimately reached, and all sample zones migrate at the same velocity. The CITP technique was compatible with ESI-MS and provided an improvement in limits of detection (LODs) of at least two orders of magnitude in comparison to the conventional CZE format. This permitted the analysis of scallop extracts containing submicromolar levels of certain PSTs with minimal cleanup requirements. An important drawback relating to the application of this technique to a large number of crude sample extracts could be the irreversible modification of the capillary surface coating and thus a decrease in separation performance. For this reason, Buzy et al. (1994) tried alternative electrophoretic modes capable of handling samples with high salt concentrations or shellfish extracts with relatively little sample cleanup. Improvements in sensitivity were also achieved through large volume sample stacking prior to CE. This approach was reported for PSTs dissolved in high-ionic-strength buffers using on-column sample stacking, combined with either UV or MS detection, and applied to the analysis of products released during the enzymatic digestion of PSTs isolated from the viscera of little neck clams. The complexity of the matrix also plays an important role, compromising the efficiency of CE. Consequently, extraction and cleanup steps need to be optimized to minimize interferences and contribute to an increased electrophoretic efficiency. Further studies of CE optimization using CITP in preconcentration mode and UV detection to be applied to the analysis of PSTs in mussels were conducted by Piñeiro et al. (1999). The optimization was focused on parameters affecting the electrophoretic separation, such as voltage, buffer composition, and organic modifiers. A significant improvement of CE performance resulted which allowed the efficient separation of STX, NEO, and dcSTX, although separation of GTXs was not fully resolved. CITP with UV detection (CITP/UV) was also applied for the quantitative analysis of PSTs in algal extracts by Wu et al. (2006). Parameters affecting the electrophoretic separation such as pH, buffer concentration, and duration time and voltage applied during the isotachophoretic process were optimized allowing an efficient resolution of most PSTs analogues (dcSTX, STX, NEO, GTX2, GTX3, GTX1, and GTX4). Good linearity was obtained in the1.3–200 μM range for these PSTs with the LOD also improved (0.1–0.3 μM). Toxin concentrations in the algal extracts were quantified by using NEO as internal standard, and some recovery data were reported in diluted extracts of *A. tamarense.*

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Recent studies by Keyon et al. (2014a, c) show the development of CE as a screening technique that can be implemented in portable instrumentation for on-site monitoring. Keyon et al. (2014c) also compared UV, MS, and capacitively coupled contactless conductivity (C^4D) detection methods used after CZE electrophoresis.

An important part of the work carried out by Keyon et al. (2014c) has been focused on the development of an alternative CE mode such as the micellar electrokinetic chromatography (MEKC) using fluorescence detection (FLD) to the analysis of PSTs.

Micellar electrokinetic chromatography has extended the scope of the application of capillary electrophoresis to the separation of uncharged molecules, thus enabling the separation of C toxins which were unresolved through conventional CZE. MEKC offers a combination of unique features of CZE and reverse-phase liquid chromatography. The selectivity in the separation process is achieved through careful adjustment of the pseudo-stationary phase (micelles) composition. This electrophoretic mode involves the separation by partitioning between micelles and a surrounding aqueous buffer solution (mobile phase).

Advantages and disadvantages of using novel CZE approaches such as CZE-C^4D and UV detection with counterflow transient isotachophoresis and MEKC versus conventional CZE are discussed in Keyon et al. (2014c). An improved selectivity was obtained by CZE-UV and CZE-C^4D, while the sensitivity of CZE coupled with UV and MS was clearly improved when using CZE-C^4D or even MEKC-FLD. MEKC-FLD was used for PSTs screening (including C1 and C2), but the sensitivity was lower than the one obtained by HPLC-FLD. The high selectivity and efficiency typically achieved by MEKC in this particular case are compromised due to the pre-column oxidation required to obtain the PSTs fluorescent compounds. Finally, Keyon et al. (2014b) developed a post-column reaction system based on droplet microfluidics for CE. High sensitivity was achieved with this approach; nevertheless, further improvements are still required to be applied to shellfish matrices.

Conclusion and Future Directions

In recent decades, there have been many challenges facing researchers and monitoring programs that have been searching for suitable replacement methods for the PSP MBA. While the MBA has provided a useful PSP toxicity assessment tool for many years, a number of method-related performance issues exist including the prevalence of both false positives and false negatives. There is also a clear global drive toward more ethical analytical tools which do not rely on the use of live animals for food safety testing. Instrumental methods available for PSTs testing are numerous and varied in both their methodology and application. All the methods reported to date have their own advantages and disadvantages, but a few, most notably two LC-FLD methods, have been formally validated and applied to the routine official control programs for marine toxin testing in a number of countries. However, both approaches still exhibit method performance characteristics and application practicalities which make the implementation complex and not straightforward. An ideal monitoring method for routine PSTs testing of shellfish should provide rapid high-throughput analysis and sample turnaround, while providing reliable, accurate, and reproducible quantification. They should also be applicable to all elucidated toxins, although in particular capable of accurate quantitation of the more toxic analogues. In particular, such a method should be applicable to toxin profiles found globally, including shellfish contaminated with toxins from all three major toxic phytoplankton genera. Chromatographic separation based on UHPLC and/or fused-core column technologies can in particular provide ultrafast sample analysis most suited to the needs of the industry. While conventional LC methods currently provide the majority of alternative PSTs testing methods in countries worldwide, there is also a move toward the highly specific analysis achievable through the use of mass spectrometry. For such methods, extraction protocols must be rapid, suitable, and

appropriate for integration with modern MS ion sources, with a particular importance being the effective removal of matrix co-extractives which significantly affect the ionization efficiency of target analytes. Automation of key steps, including SPE cleanup, is also a highly useful tool for improving throughput. Currently, the most recent developments in UHPLC-HILIC-MS/MS detection show great promise in delivering the above desirables, although further testing is important to ensure such methods are reliable and reproducible. The applicability of high-resolution MS for PSP testing in shellfish tissues also requires further investigation. Finally, the need for accurate toxicological information of the relative toxicity of existing and newly discovered congeners is extremely important. Without such data, the accuracy and relevance of any alternative instrumental PSP testing methods may be significantly compromised.

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