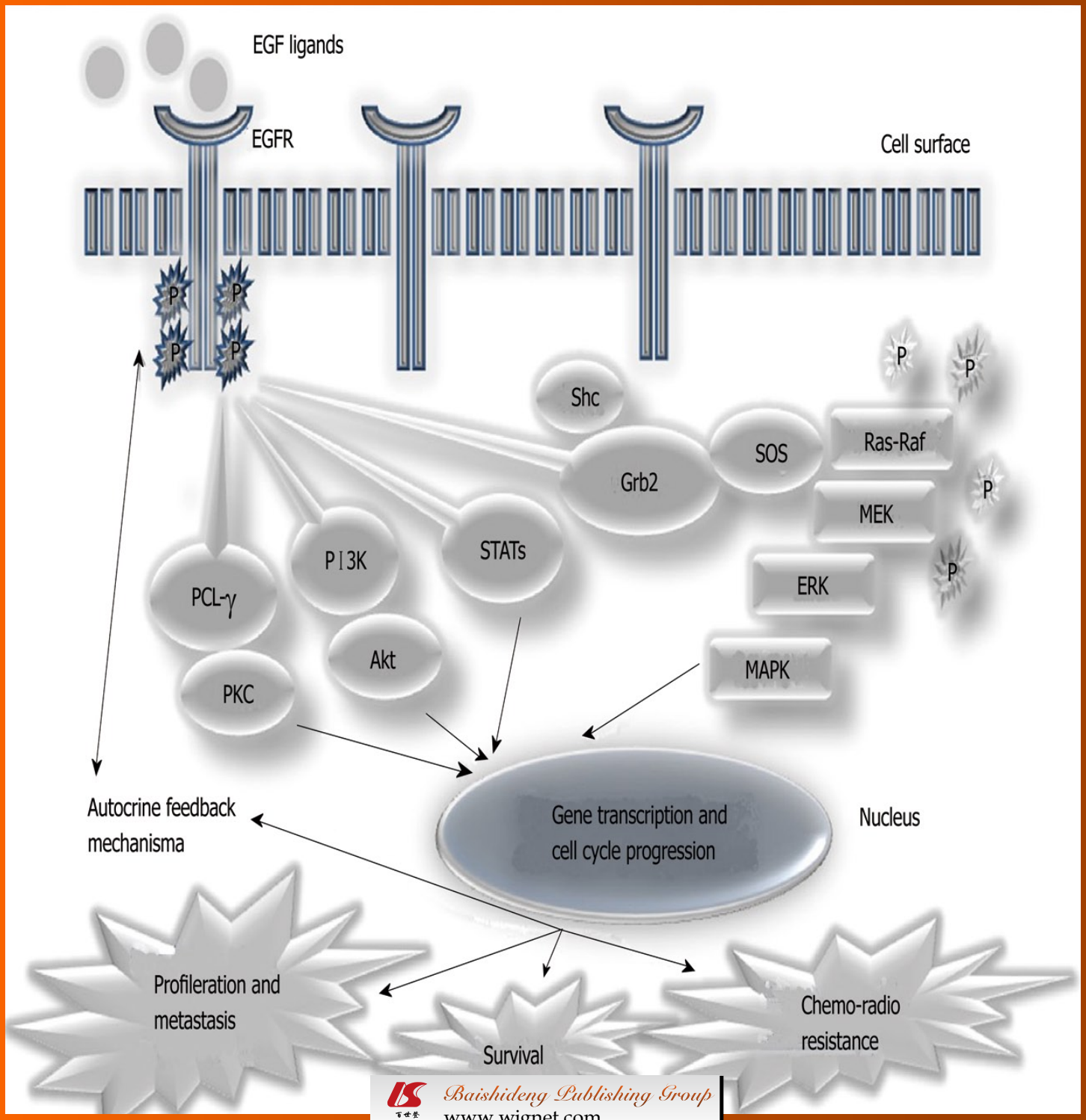
ISSN 2218-4333 (online)

World Journal of *Clinical Oncology*

World J Clin Oncol 2011 November 10; 2(11): 355-376



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WJCO World Journal of Clinical Oncology

A peer-reviewed, online, open-access journal of oncology

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World Journal of Clinical Oncology

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Monthly Volume 2 Number 11 November 10, 2011

World Journal of Clinical Oncology
Volume 2 Number 11 November 10, 2011

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NAME OF JOURNAL
World Journal of Clinical Oncology

LAUNCH DATE
November 10, 2010

SPONSOR
Beijing Baishideng BioMed Scientific Co., Ltd.,
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http://www.wjgnet.com

PUBLISHING
Baishideng Publishing Group Co., Limited,
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
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http://www.wjgnet.com

PUBLICATION DATE
November 10, 2011

ISSN
ISSN 2218-4333 (online)

EDITORIAL OFFICE
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Full instructions are available online at http://www.wjgnet.com/2218-4333/g_info_20100722172206.htm

ONLINE SUBMISSION
http://www.wjgnet.com/2218-4333office

World Journal of Clinical Oncology

Online Submissions: http://www.wjgnet.com/2218-4333office
wjco@wjgnet.com
doi:10.5306/wjco.v2.i11.355

World J Clin Oncol 2011 November 10; 2(11): 355-361
ISSN 2218-4333 (online)

EDITORIAL

Bioactive substances with anti-neoplastic efficacy from marine invertebrates: *Porifera* and *Coelenterata*

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Received: July 6, 2011 Revised: October 10, 2011
Accepted: October 17, 2011
Published online: November 10, 2011

Abstract

An ever increasing demand for new lead compounds in the pharmaceutical industry has led scientists to search for natural bioactive products. Based on this extensive research, marine invertebrates now represent a rich source of novel substances with significant anti-neoplastic activities. As the current approach of synthesizing new and chemically modifying old drugs seems to have slowed down, and the identification of new anticancer drugs is not too promising, a new approach is clearly needed. The objective of this review is to present up-to-date data on these newer compounds. Based on the data summarized in this short review, it is clear that marine invertebrates represent an extremely important source of compounds with potential anti-cancer effects. Considering that we tested only a tiny number of *Porifera* and *Coelenterata*, the best is yet to come.

Key words: Cancer; Coelenterata; Invertebrates; Porifera

Peer reviewers: Bruna Scaggiante, PhD, Assistant Professor of Molecular Biology and Aggregate Professor of Molecular Biology, Department of Life Sciences, University of Trieste, Via Licio Giorgeri 1, 34127 Trieste, Italy; T H Marczylo, PhD, Endocannabinoid Research Group, Cancer Studies and Molecular Medicine, University of Leicester, Leicester, LE2 7LX, United Kingdom

Sima P, Vetvicka V. Bioactive substances with anti-neoplastic efficacy from marine invertebrates: *Porifera* and *Coelenterata*. *World J Clin Oncol* 2011; 2(11): 355-361 Available from: URL: http://www.wjgnet.com/2218-4333/full/v2/i11/355.htm DOI: http://dx.doi.org/10.5306/wjco.v2.i11.355

INTRODUCTION

Adaptive (inducible) immunity emerged together with the appearance of jaws, and is based on the rearrangement of very variable molecules of the immunoglobulin superfamily (antibodies, T cell receptors, MHC molecules, *etc.*) which are produced in myriads of variants. While, adaptive immunity is regarded as unique among the defensive strategies of metazoans, all members of the invertebrate phyla utilize a plethora of substances in their natural immunity ranging from peptides to steroids and alkaloids for defense and preservation of their internal integrity[1,2].

Oceans comprise 70% of the Earth's area and the marine ecosystem represents 95% of the biosphere. Thirty-three of thirty-four animal phyla live in the marine environment[3]. The diversity of species is extraordinary and in tropical reef zones reaches 1000 different species per square meter[4,5]. Environmental pressures, competition for space, nutrition, and self-defense have led to the production of a diverse array of compounds-the secondary metabolites, which these organisms use for intercommunication within their environment. These communication molecules evolved within the scope of symbiotic interrelations *sensu*, and de Barry[6] studied protocooperation and neutralism followed by competition and parasitism. Many of these molecules also represent ancient defense factors[7,8].

Bioactive substances formed by marine organisms such as bacteria, algae, protozoans, and invertebrates/

vertebrates have attracted attention due to their antiviral, antimicrobial, antiprotozoal, antifungal, antihelminthic, and anticancer properties[9,10].

A vast number of these substances are produced by phylogenetically diverse organisms that have different and often unprecedented chemical structures and are able to kill eukaryotic cells. These so-called "natural substances", or synonymously "secondary metabolites," are small molecules (generally up to 3000 Da) and have been the focus of anticancer research for more than 40 years[11]. The main sources of natural metabolites which exert antitumor activity are from invertebrates, particularly marine invertebrates.

Up to the end of the first decennium of this century, more than 15 000 natural compounds have been isolated mainly from sessile animals, sponges, corals, mollusks, and chordates. These substances belong to very different families of chemical compounds. Some of them have very exotic structures and their bioactive and antineoplastic activities take effect on different levels including blocking metabolic/enzymatic reactions, interrupting cell cycle, and direct cell killing. During the past few years, a large numbers of novel compounds have been reported[12-14]. Some of them have undergone preclinical and clinical trials and it is expected that they will be used therapeutically in the very near future.

Difficulties still persist regarding the development and commercial production of pharmacologically bioactive substances. First, the samples of pharmacological interest are obtainable only in minute quantities from the source. Thus, it is necessary to gather significant amounts of such organisms which could threaten the stability of their ecosystems. In certain countries, this is often unlawful[15]. Second, bioactive substances often have very complicated structures and their isolation or synthesis is very expensive[16]. Third, the source of these bioactive compounds are from microorganisms living in symbiosis[17,18]. The determination and isolation of the responsible bacterial producers of such bioactive substances are rather complicated because microbial communities within the ecological environment containing marine sessile animals are very complex. To date, only a few studies have determined the bacterial symbionts of these substances[19-22]. Currently, only about 5% of the symbiotic bacteria present in marine specimens can be cultivated under standardized conditions. Conversely, interest in the development of novel therapeutics from marine symbiotic microbiota continues to grow[23].

It would be pointless to introduce a list of known substances with antineoplastic activities that have been derived from marine invertebrate animals. The aim of this review is only to draw attention to this relatively new branch of pharmacological science which is a rapidly developing new discipline.

PORIFERA

Marine sponges are easy to collect and therefore are among the richest sources of bioactive compounds. There are approximately 6000 species of the phylum *Porifera* living in the sea[24]. Sponges are evolutionary and one of the most successful animal groups[25]. Sponges or their associated microorganisms produce a number of secondary metabolites for self-defense[26-28]. More than 5300 chemically different bioactive substances have been discovered in sponges or their symbiotic microorganisms and every year about 200 new compounds are added. Microorganisms comprise up to 40% of the total mass of some sponge species[10].

Many of these substances have pharmaceutical activities against human diseases such as malaria, AIDS, and particularly cancer[29]. They are chemically classified as alkaloids, lipids, steroids, terpenoids or further compounds[30,31]. Cytotoxic substances described, e.g., in *Strongylophora durissima* are mainly polyacetylenic lipid derivatives, glycerol ethers, linear alcohols or fatty acids and their esters. Of these compounds, the durissimolsexert strong activity against human gastric tumor cells (NUGC)[32]. A lipid metabolite, plakorsin B from *Plakortis simplex*, is cytotoxic against COLO-250 and KB-16 carcinoma cells, and chondrillinis cytotoxicagainst the human KB-16 cell line. A macrolide, neopeltolide**,** which was isolated from *Incertae sedis* species from the family *Neopeltidae* (*Lithistida*) is cytotoxic to several cancer cell lines[33]. IC_{50} values of 1.2 nmol/L against the A549 human lung adenocarcinoma cell line, 5.1 nmol/L against the NCI/ADR-RES ovarian sarcoma cell line, and 0.56 nmol/L against the P388 murine leukemia cell line were found. Strong activity was also found in the PANC-1 pancreatic cancer cell line and the DLD-1 colorectal adenocarcinoma cell line, both of which have *p53* mutations. This substance has already been synthesized[34].

Our research is also aimed at the clinical use of secondary metabolites for the therapy of cancer. The secondary metabolites of sponges such as crambescidin-816 (*Crambe crambe*), have been shown to beactive against HCT-16 human colon *carcinoma* cells (IC_{50} = 0.24 g/mL). The additional metabolites, discodermolide (*Discodermia dissoluta*), halichondrins and isohomohalichondrins (*Halichondria okadai, Lissodendoryx sp.*), laulimalide and isolaulimalide (*Cavospongia mycofijinsis*), mycaperoxideB (*Mycala sp.*) and spongistatin (*Hyrtios erecta*) should also be mentioned. Numerous drugs from marine sponges have been identified as a source of targeting microtubules[35,36]. The anti-cancer activity of these agents may lie mainly in their inhibitory effects on spindle microtubule dynamics, rather than in their effects on microtubule polymer mass[35]. Substances such asjaspolide, dolastatin, halichondrin, spongistatin, hemiasterlin, dictyostatin, discodermolide, laulimalide, pelorusideA, and zampanolide influence function and are disruptors of the cytoskeleton similar to taxanes (major classes of tubulin polymerization promoters)[37,38] and some tunicate and molluscan molecules[13].

Pharmacologically interesting substances from sponges are lamellarins[39], which were first described in mollusks (see below). Recently, anticancer drug research has focused on a group of low molecular inhibitors of hy-

poxic signaling in tumor tissues. These substances target a key regulator of oxygen homeostasis, hypoxia-inducible transcription factor 1 (HIF-1[40]), which represents a negative factor in cancer prognosis. In addition, they induce apoptotic cell death through multi-target mechanisms, including inhibition of topoisomerase I, interaction with DNA and direct effects on mitochondria. Inhibition of its function not only suppresses tumor growth, but simultaneously enhances chemo- and radiotherapy. As of 1998, only two substances were known to inhibit HIF-1. Since that time, about 15 000 extracts of plants and marine animals have been investigated for the inhibition of HIF-1. Several groups of chemically related substances were discovered in plants and microorganisms and in marine algae, sponges, echinoderms and tunicates. These were mainly sodwanone(activity against T47D breast tumor cells (IC_{50} = 15 μmol/L), PC-3 prostate tumor cells (IC_{50} = 15 μmol/L) and MDA-MB-231 breast tumor cells (IC_{50} = 23 μmol/L), and yardenone-type triterpenoids, furanolipid, homoscalaranesesterterpenes, norsesterterpenesdiacarnoxides, and lamellarin-like substances. They were discovered in lipophilic extracts of the sponges *Axinella sp*., *Lendenfeldia sp*., *Diacarnus levii*, and *Dendrilla nigra*. Further HIV-1 inhibiting substances, meroditerpenoids strongylophorines were isolated from the sponge *Petrosia* (*Strongylophora*) *strongylata*[10].

Many of the bioactive substances from sponges are terpeneswhich havecytotoxicity against L-1210 cells (IC_{50} between 2.8 and 8.1 g/mL) and KB cells (IC_{50} between 1.2 and 7.6 g/mL)[41]. These compounds are biosynthesized from the five carbon isoprene building units and their structural modification leads to the formation of numerous derivatives with various biological properties. Steroidal terpenoids were the first marine isoprenes discovered during the 1930s-1940s[42]. Today, marine terpenoids encompass an enormous number of derivatives. Monomeric congeners of jaspolides can be classified into triterpenes, sesterterpenes, terpenes and nortriterpenes. JaspolideB showed efficacy comparable to that of paclitaxel and seems to be a promising anticancer agent for the treatment of leukemia due to its ability to block the cell cycle during transition from the G2 phase to mitosis and trigger apoptosis. Examples of bicyclic sesterterpenes are thorectandrols isolated from sponge (*Thorectandra sp*.) together with the parent compounds palauolide and palauolol. All these substances inhibited the growth of MALME-3M (melanoma) and MCF-7 (breast) cancer cell lines in the range 30-40 mg/mL. Similarly, petrosaspongiolides, the first cheilantane sesterterpene lactones isolated from a new sponge species *Petrosaspongia nigra,* exerted cytotoxicity against the human bronchopulmonary carcinoma cell line. The group of isomalabaricane-type triterpenoids representsstelletins. Stelletin A was recognized in 1981 as a yellow triterpenoidal pigment from the sponge *Jaspis stellifera*[43], and showed significant cytotoxicity against murine leukemia cells. Geoditins, stelliferin-related isomalabaricane triterpenoids, were obtained from the sponges *Geodia aponica* and *Rhabdastrella aff. Distinca*[44,45]. These substances showed significant cytotoxicity against several human promyelocytic leukemia, prostate, gastric, breast, cervical and hepatocellular carcinoma cell lines such as NSCLC-N6 bronchopulmonary cells (IC_{50} between 1.0-32.2 mol/L) leukemia P388 cell line (IC_{50} = 2.1 nmol/L), leukemia cells HL-60 (IC_{50} = 0.9 mol/L)prostate cancer cells LNCaP (IC_{50} = 260 mol/L), and stomach cancer cells AGS (IC_{50} = 2.9 mol/L). Readers seeking more details regarding the 60 cancer cell lines tested should read this comprehensive review[43].

Other chemically different groups of diterpenoid substances constitute terpenyl purines. Some of these (agelasines, asmarines) were isolated mainly from the sponge genera *Agela sp*. and *Raspailia sp*. They display a high general toxicity against protozoa and bacteria including cytotoxicity against several cancer cell lines[46]. This study found cytotoxic effects against lymphoma L1210 cells (IC_{50} = 3.1 g/mL), and MEL-28 melanoma cells (IC_{50} = 1.18 mol/L). Another group of spongian substances with antitumor activity are the alkaloids which include pyridoacridine, indole, pyrrole, pyridine, isoquinoline, guanidine and steroidal alkaloids[47].

Pyridoacridines are probably the largest class of the marine alkaloids. Pyridoacridines can be divided into tetracyclic, pentacyclic, hexacyclic, heptacyclic and octacyclic alkaloids. Almost all were isolated from sponges, coelenterates, and ascidians. They show significant cytotoxicity against different types of tumors and contain additional specific biological properties. The most pronounced effects are inhibition of topoisomerase II catalytic activity in human colon cancer cells HCT-116. Numerous additional compounds in this group were isolated; most of which are polycyclic (shermilamine, kuanoniamine, neoamphimedine, arnoamines and styelsamines).

Bisindole alkaloids, dragmacidin and dragmacidons, were extracted from the sponges *Dragmacidin sp., Spongosorites sp*., and *Hexadella sp*. Several substances in this family were found to be cytotoxic against several human cancer cell lines such as P388 (IC_{50} =15 g/mL), lung cells A-549, colon cells HCT-8 and the breast cancer cell line MDA-MB (IC_{50} =10 g/mL)[48]. New bisindole alkaloids, nortopsentins, were isolated from the sponge *Spongosorites ruetzleri*. Some exhibited cytotoxic activity against cancer cell lines. Similarly, topsentins and their derivatives from the sponge *Topsentia genitrix* inhibited proliferation of cultured human and murine tumor cells at micromolar concentrations (IC_{50} values ranged from 4 to 40 mmol/L) and were active against *in vivo* P388 leukemia (%T/C = 137, 150 mg/kg, QD1-5) and B16 melanoma (%T/C = 144, 37.5 mg/kg, QD1-9) tumors[49].Other bisindole alkaloids, hyrtinadineand hyrtosins from the sponge *Hyrtios sp*., exhibited *in vitro* cytotoxicity against murine leukemia and human epidermoid carcinoma cells. In addition, β-carboline alkaloids, hyrtioerectinesand hytriocarboline-from *Hyrtios reticulates* showed cytotoxicity against murine leukemia L1210 cells (IC_{50} = 1 μg/mL) and human epidermoid carcinoma KB cells (IC_{50} = 3 μg/mL) *in vitro*[50]. Manzamines and their congeners from the sponge *Am-*

Table 1 The most important bioactive substances with anti-neoplastic effects isolated from *Porifera*

Component	Species	Ref.
Durissimols	*Strongylophora durissima*	[32]
Plakorsin B	*Plakortis simplex*	[32]
Chondrillin	*Xestospongia sp.*	[32]
Neopeltolide	*Neopeltidae*	[33]
Jaspolide		[37,38]
Dolastatin		[37,38]
Halichondrin		[37,38]
Spongistatin		[37,38]
Hemiasterlin		[37,38]
Dictyostatin		[37,38]
Discodermolide		[37,38]
Laulimalide		[37,38]
Peloruside A		[37,38]
Zampanolide		[37,38]
Lamellarins		[39]
Sodwanone	*Axinella sp.,*	[10]
Triterpenoids	*Axinella sp.,*	[10]
Furanolipid	*Axinella sp.,*	[10]
Homoscalarane sesterterpenes	*Axinella sp.,*	[10]
Norsesterterpenes	*Axinella sp.,*	[10]
Diacarnoxides	*Axinella sp.,*	[10]
Strongylophorines	*Petrosia strongylata*	[10]
Stelletin A	*Jaspis stellifera*	[44]
Agelasines	*Agela sp.*	[46]
Asmarines	*Agela sp.*	[46]
Dragmacidin	*Dragmacidin sp.,*	[48]
Nortopsentins	*Spongosorites ruetzleri*	[48]
Topsentins	*Topsentia genitrix*	[49]
Hyrtinadine	*Hyrtios sp.*	[50]
Hyrtosins	*Hyrtios sp.*	[50]
Hyrtioerectines	*Hyrtios reticulates*	[50]
Hytriocarboline	*Hyrtios reticulates*	[50]
Cylindradines	*Axinella cylindratus*	[52]
Agelastatins	*Agelas dendromorpha*	[53]
Dibromophakellstatin	*Phakellia mauritiana*	[54]

phimedon sp. also appear to have some potential cancerogencity towards human hepatocellular carcinoma cells HEPG-2[51].

Other bioactive substances found in marine sponges are the (brom) pyrrole alkaloids. Cylindradines from *Axinella cylindratus* displayed moderate cytotoxicity against murine leukemia cells P388[52], and agelastatins (*Agelas dendromorpha*) and clathrodin (*A. clathrodes*) showed significant potent *in vitro* activity against several tumor cell lines. Agelastatin A inhibited OPN protein expression and enhanced expression of the cellular OPN inhibitor, Tcf-4. Agelastatin A treatment also reduced β-catenin protein expression and reduced anchorage-independent growth, adhesion, and invasion in R37 OPN pBK-CMV and C9 cell lines. Similar effects were observed in MDA-MB-231 and MDA-MB-435s human breast cancer cell lines exposed to (-)-agelastatin A[53]. The tetracyclic pyrrole-imidazole alkaloid, dibromophakellstatin, from *Phakellia mauritiana,* showed cytostatic activity against a panel of 36 human cancer cell lines in a cell survival and proliferation assay. The ovarian cancer cell line OVXF 899L proved to be most sensitive (IC_{50} = 0.60 mol/L), followed by the glioblastoma cell line CNXF 498NL (0.93), the non-small lung cancer cell line LXF 529L (0.96 mol/L), and the uterine cancer cell line UXF 1138L (1.21 mol/L). The selectivity profile of rac-dibromophakellstatin may be indicative of a novel mechanism of action[54].

There are many other alkaloids which exert cytotoxic and potentially anticancerogenic activities, which have been identified in various species of marine sponges. Chemically, they belong to the alkaloid groups: the pyrroloquinoline alkaloids (e.g., zyzzyanones, discorhabdins ,batzellines, prianosins, makaluvamines,tsitsikammamme s), pyrroloacridine alkaloids (plakinidines), pyrrole alkaloids (perinadines, variolins, halitulins), pyridine alkaloids (pyrinodemins, pyrinadines, amphimedosides, echinoclathrines), isoquinoline alkaloids (cribrostatins), guanidine alkaloids (ptilomycalins, netamines), aminoimidazole alkaloids (leucosolenamines, naamidines), steroidal alkaloids (plakinamines, ritterazines, cortistatins) and many other alkaloid molecules. Our detailed knowledge of these potentially interesting molecules is still limited.

In conclusion, numerous and very different antitumor substances (Table 1) have been isolated from sponges[31]. Many of them are already in clinical use or undergoing the final stages of clinical trials (e.g., antiviral vidarabin or manzaminA with antimalaric, antituberculitic and anti HIV activities)[55].

COELENTERATA

There are two phyla of *coelentrates*: *Cnidaria,* comprising more than 9000 species, and *Ctenophora* with 50 species[56]. Practically all substances with bioactive activities-mainly steroids, terpenes, and other compounds (e.g., ceramides)-were obtained from sea anemones and corals (*Anthozoa*). The glycosides, cervicosides and prostanoid-sclaviridenones, from the soft corals *Sinularia cervicornis* and *Clavularia viridis* were shown to have antitumor activity against human cancer cell lines[31]. A great number of diterpenoids classified into the dollabelane, xenicane, phenylgermacrane, and cembranegroups showing cytotoxicity against cancer cell lines were isolated from *Nephe-ta sp*., as did the clavulactones, clavirolides and clavudiols isolated from *Clavularia sp*. For example, these substances were examined for growth-inhibition activities *invitro* toward human cancer cells using the Japanese Foundation for Cancer Research 39 cell line assay. The results showedinhibition of the proliferation of NCI-H522 cells (lung cancer) with an IC_{50} of 0.66 μg/mL, and of LOX-IMVI cells (melanoma) and MKN74 cells (stomach cancer) with an IC_{50} of 0.72 and 0.81 μg/mL, respectively. The pattern of differential growth inhibition was evaluated by the Compare Program and was revealed not to

Table 2 The most important bioactive substances with anti-neoplastic effects isolated from *coelenterata*

Component	Species	Ref.
Cervicosides	*Sinularia cervicornis*	[31]
Claviridenones	*Clavularia viridis*	[31]
Dollabelane	*Nepheta sp.*	[57]
Xenicane	*Nepheta sp.*	[57]
Phenylgermacrane	*Nepheta sp.*	[57]
Clavulactones	*Clavularia sp.*	[57]
Clavirolides	*Clavularia sp.*	[57]
Clavudiols	*Clavularia sp.*	[57]
Cembrane	*Sinularia sp.*	[59]
Eleutherobin	*Eleutherobia sp.*	[60]
Sarcodictyin	*Sarcodictyon roseum*	[62]
Menverins	*Menella verrucosa*	[65]

be correlated with that shown by any other compounds including the currently used anticancer drugs. The correlation coefficient value was less than 0.5 indicating that these substances may have a new mode of action[57]. Another experiments showed moderate cytotoxic activity against human colorectal adenocarcinoma cells (DLD-1) with an IC_{50} of 5.0 μg/mL. The lobane diterpenes and lobane lacatnes, the pacifins from *Sinularia sp.* and *Lobophytum sp.* appear to have similar cytotoxicity. A number of diterpenoids of the xenicane groups from *Xenia sp.* exhibited mild-to-potent cytotoxic activities against human lung carcinoma (H460) and liver carcinoma (HepG2) cell lines[58]. The cembrane substances mainly from *Sinularia* sp. and polyoxygenated steroids from *Alcyonum patagonicum* and another coral species (*Nephtea*) represent the most numerous group of coral diterpenoids which have mild-to-strong cytotoxicity to the human tumor cell lines CCRF-CEM and DLD-1[59]. Other hopeful soft coral cytotoxic and cytostatic substances are eleutherobin[60,61] and sarcodictyin[62,63], which interfere with microtubulins by increasing polymerization. In addition, this natural product was shown to be a potent cancer cell inhibitor with an IC_{50} similar to that of paclitaxel (Taxol®) (10-15 nmol/L), and assays in the National Cancer Institute's 60 cell line panel showed a 100-fold greater potency over the mean cytotoxicity towards breast, renal, ovarian and lung cancer cell lines[64].

Secondary bioactive metabolites were also discovered in gorgonians. They are mainly steroids and terpenoids and several lipidic substances. As an example, strong and selective cytotoxicity was documented for the oxygenated lactones (menverins)[65] from *Menella verrucosa*. Furean sesquiterpenoids from *Acanthogorgia vegae* exhibited significant cytotoxicity toward the growth of A549, HT-29, KB, P-388 and P-388 cells[66]. For a summary of the most important anticancer molecules (Table 2).

CONCLUSION

Drug discoveries from marine invertebrates have enjoyed a renaissance. Currently, interest in evaluating marine invertebrate products, with the aim of obtaining new antitumor drugs with few side effects, is still growing. In many cases (particularly in sponges), the interesting materials (or the whole organisms) are difficult to obtain in sufficient amounts and researchers, therefore, have to start copying nature and preparing synthetic versions. Some of these therapeutics were approved in the United States and the European Union and many are in the final stages of clinical trials[67]. Not surprisingly, the outlook for the future is more promising then ever.

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S- Editor Yang XC **L- Editor** Webster JR **E- Editor** Yang XC

World Journal of Clinical Oncology

Online Submissions: http://www.wjgnet.com/2218-4333office
wjco@wjgnet.com
doi:10.5306/wjco.v2.i11.362

World J Clin Oncol 2011 November 10; 2(11): 362-366
ISSN 2218-4333 (online)

EDITORIAL

Bioactive substances with anti-neoplastic efficacy from marine invertebrates: *Bryozoa*, *Mollusca*, *Echinodermata* and *Urochordata*

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Received: July 13, 2011 Revised: October 10, 2011
Accepted: October 17, 2011
Published online: November 10, 2011

Abstract

The marine environment provides a rich source of natural products with potential therapeutic application. This has resulted in an increased rate of pharmaceutical agents being discovered in marine animals, particularly invertebrates. Our objective is to summarize the most promising compounds which have the best potential and may lead to use in clinical practice, show their biological activities and highlight the compounds currently being tested in clinical trials. In this paper, we focused on *Bryozoa, Mollusca, Echinodermata* and *Urochordata.*

Key words: Cancer; *Echinodermata;* Invertebrates; *Mollusca; Urochordata*

Peer reviewers: Murielle Mimeault, PhD, Department of Biochemistry and Molecular Biology, College of Medicine, Eppley Cancer Institute, 7052 DRC, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5870, United States; Kazuaki Chikamatsu, MD, PhD, Assistant Professor, Department of Otolaryngology-Head and Neck Surgery, University of Yamanashi, Faculty of Medicine, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan

Sima P, Vetvicka V. Bioactive substances with anti-neoplastic efficacy from marine invertebrates: *Bryozoa*, *Mollusca*, *Echinodermata* and *Urochordata*. *World J Clin Oncol* 2011; 2(11): 362-366
Available from: URL: http://www.wjgnet.com/2218-4333/full/v2/i11/362.htm DOI: http://dx.doi.org/10.5306/wjco.v2.i11.362

INTRODUCTION

Oceans contain the greatest known diversity of life, with 34 of the 36 phyla represented. It is not surprising that due to this diversity, a substantial number of biomedicinally potent molecules have been described, isolated and characterized. At the same time, despite decades of intensive research, cancer is still one of the most lethal diseases. Despite great achievements and decades of intensive, labor-consuming and expensive research, the incidence of various tumors and cancers is still increasing at an alarming rate. Based on the National Cancer Institute estimates, slightly less than one-in-two men and little more than one-in-three women in the United States are likely to contract cancer in their lifetime. In addition, the discovery of new anti-cancer drugs is painfully slow. In fact, very few fundamentally new anti-cancer drugs were introduced in last decade, thus leaving oncologists to rely on chemotherapeutic drugs developed in the fifties.

In the past 30 years, the role of natural products in drug discovery has undergone many changes. It is not suprising, therefore, that in the past few decades, marine animals (and plants) have been the focus of an intensive effort to identify new molecules with anti-cancer properties. Marine invertebrates contain metabolites of unprecedented molecular structures and activities. In addition, *de novo* synthesis and design of pharmacologically active substances can not replace millions of years of evolution. Despite the fact that only a very small number of marine animals have been investigated, more than 12 000 novel bioactive molecules have been discovered. Several

marine natural products are currently undergoing clinical trials and their success is encouraging. In this part of our review, we focus on the pharmacologically-effective molecules with potential anti-cancer abilities found in *Bryozoa, Mollusca, Echinodermata* and *Urochordata*.

BRYOZOA

Over 4000 living species of *Bryozoa* are known. Macrocyctic lactone bryostatin-1, a very promising anti-tumor metabolite with significant biological activities, was isolated from the bryozoan *Bugula neritina*[1]. It is a potent immunomodulator promoting hemato-, lympho-, and myelopoiesis, activates protein kinase C, and acts as an antagonist of tumor-promoting phorbol esters[2,3]. Moreover, it down regulates multi drug resistance gene 1 expression, influences *bcl-2* and *p53* gene expression, and induces apoptotic processes[4,5]. It also has strong anti-cancer activity and simultaneously enhances the activity of chemotherapeutics such as cisplatin, gemcitabine, paclitaxel, and vincristine[6,7]. Bryostatins are already in clinical use[8-10]. Lopanik *et al*[11], subsequently discovered that bryostatins are actually produced by a microbial symbiont (*Endobugula sertula*) which protects *Bugula* larvae from predators using these substances.

The alkaloids, pterocellins**,** were isolated from another bryozoan, *Pterocella vesiculosa*[12]. They possess cytotoxic activities against murine leukemia, human melanoma and breast cancer cell lines.

MOLLUSCA

The mollusks belong to the most successful evolutionary assemblage of animals. Malacologists estimate that there are up to 150 000 molluscan species living world-wide[13]. Contrary to the vast number of molluscan species and their relative accessibility, not many of their secondary bioactive metabolites have been investigated. Substances exerting anti-cancer activity are mainly peptides, dolabellanins and dolastatins, which are obtained from the albumen glands of the sea hare *Dolabella auricularia*[14-17]. The linear peptides, dolastatin 10 and depsipeptide dolastatin 15, have a strong cytotoxic impact on tumors through the inhibition of cell proliferation. They damage the microtubule cytoskeleton and induce apoptosis in neoplastic cells[18]. Some dolastatins have been selected for further clinical testing.

The cytotoxic substance aplyronine A, a structurally inseparable mixture of four diastereomers of two amino acid esters, and their congeners, aplyronines B-H, were isolated from another species of sea hare, *Aplysia kurodai*[19]. Recently, the bioactive properties of 33 cytotoxic substances including aplyronines and the alkaloids aplaminones isolated from *A. kurodai*, and dolastatin H, isodolastatin H, cyclodepsipeptides aurilide and doliculide, polyketides aurisides A and B, polypropionates auripyrones A and B, macrolides dolabelides A, C and D, and terpenoids auriol and auriculol from D. *Auricularia* were structurally and functionally characterized. Linear monoterpenes and steroids with significant anti-neoplastic activities were also described in the sea hare *Notarchus leachii cirrosus*. Many of these compounds exert promising anti-tumor activity, but they are only available in miniscule amounts[20]. Another macrolide substance-latrunculin A-was first discovered in the sponge *Negombata magnifca*[21]. It disrupts actin polymerization and binds to actin microfilaments, thereby impairing cellular migration and adhesion. It also suppresses tumor metastases and cellular viability (for review[22]). Other potentially cytostatic terpenoid derivatives have been discovered-*Hexabranchus sanguineus* and *Phyllidiella pustulosa*[23]. Further cytotoxic substances, the bistetrahydroisoquinolines, jorunnamycins A-C, were isolated from the nudibranch gastropod mollusk *Jorunna funebris*. These metabolites could also be accumulated from some sponges (e.g., *Euplacella sp*., *Haliclona sp*., *Oceanapia sp*., and *Xestospongia sp*.), which represent the main source of *Jorunna* nutrition. They are highly active against human colon, prostate and lung carcinoma cell lines[24].

The other peptide metabolite with anti-tumor activity is the dissipeptide, kahalalide F, which was isolated from the mollusk *Elysia rubefescens*. It induces cytotoxicity by blocking the G1 phase of cell cycle and has selectivity against cell lines derived from solid tumors like prostate, breast, and colon cancer[25]. Various bioactive peptides, e.g., angiotensin-converting enzyme inhibitory peptides, anti-fungal and anti-cancer peptides[26] were also discovered in oysters. Wang *et al*[27] treated tumor-bearing mice with oyster peptides and documented a significant inhibition of tumor growth accompanied by an increase in NK cell activity.

An important and very hopeful group of anti-cancer drug candidates are the hexacyclic pyrrole alkaloids, the lamellarins, which were first isolated in 1985 from prosobranch mollusks of the genus *Lamellaria*[28]. Over 38 lamellarins denominated A-Z and α-γ were discovered. It was shown that these substances are effective inhibitors of a number of so-called disease-relevant protein kinases such as cyclin-dependent protein kinases, glycogen synthase kinase 3, serine/threonine kinase Pim-1, and specificity to both the tyrosine phosphorylation regulated kinase 1A, and casein kinase 1, which are involved in cancer cell proliferation. Baunbæk *et al*[29] shown that 22 lamellarins inhibit 6 kinases which are essential for transition from G1 to G2 phase and induce cell cycle arrest and cell death. Investigations into the therapeutic effects of these substances and their artificial analogues is ongoing and promises to acquire new, less toxic, but still effective compounds.

Echinodermata

Deuterostomian invertebrates-the echinoderms-comprise about 6000 species. The main secondary bioactive metabolites are the saponins. Sulfated glycosides belonging chemically to asterosaponons are regularosides and novaeguinosides from the starfish *Culcita novaeguineae*. These

Table 1 The most important bioactive substances with anti-neoplastic effects isolated from *Bryozoa*, *Mollusca*, and *deuterostomian* invertebrates

Component	Species	Ref.
Bryostatin-1	*Bugula neritina* (bryozoan)	[1]
Pterocellins	*Pterocella vesiculosa* (bryozoan)	[12]
Dolabellanins	*Dolabella auricularia* (mollusc)	[14]
Dolastatins	*Dolabella auricularia* (mollusc)	[17]
Aplyronine A	*Aplysia kurodai* (mollusc)	[19]
Jorunnamycins A-C	*Jorunna funebris* (mollusc)	[24]
Kahalalide F	*Elysia rubefescens* (mollusc)	[25]
Lamellarins	*Lamellaria sp./Didemnum sp.* (mollusc/tunicate)	[28,39]
Intercedensides	*Mensamaria intercedents* (echinoderm)	[23]
Philinopgeneins	*Pentacta quadrangularis* (echinoderm)	[23]
Colochirosid	*Colochirous anceps* (echinoderm)	[23]
Ritterazine A	*Ritterella tokioka* (tunicate)	[33]
Ecteinascidins	*Ecteinascidia turbinate* (tunicate)	[34]
Cystodytins	*Cystodytes dellechiajei* (tunicate)	[38]
Didemnins	*Tridemnum solidum* (tunicate)	[41]
Ascidiacyclamide	*Lissoclinum patella* (tunicate)	[44]
Cephalostatin 1	*Cephalodiscus gilchristi* (hemichordate)	[50]

exert only marginal cytotoxicty. On the other hand, triterpene glycosides, the intercedensides from sea cucumber *Mensamaria intercedents,* the triterpenoids, philinopgeneins and their analogues philinopsides from *Pentacta quadrangularis*, and the pentalycosid analogue, colochirosid, from *Colochirous anceps* have strong cytotoxicity against human tumor cancer cells. Same of them also exert potent anti-angiogenic activity and therefore could represent promising new drugs[23].

UROCHORDATA

There are approximately 4000 species of urochordates, commonly known as the tunicates. Molecules with cytotoxic and cancerostatic properties were found in these deuterostomian animals[30-32]. A cytotoxic steroidal alkaloid, ritterazine A, was isolated from the tunicate *Ritterella tokioka*[33]. A group of pharmacologically active metabolites with a strong antitumor effect, the tetrahydroisoquinolone alkaloids ecteinascidins from *Ecteinascidia turbinata* were identified[34]. These substances have been under evaluation by the National Cancer Institute for 15 years[35]. In particular, ecteinascidin 743 exhibits numerous bioactive effects in comparison to other ecteinascidins. Similar to the above mentioned kahalalide F, it interferes with cell cycle blocking G2 phase inducing apoptosis, and interacts with the cytoskeletal microtubules as with spongian, coelenterate, and molluscan derivatives[36] (see above). Ecteinascidin 743 has strong antineoplastic potential and has been in preclinical and clinical trials since 2000[37]. It was shown to have activity against melanoma cell lines, and carcinomas of breast, colon, ovarian, neural and lung origin[32].

The alkaloids, just as in some mollusks, corals and sponges, represent the main group of urochordate metabolites with cytotoxic and antineoplastic activity. The tetracyclic alkaloids, cystodytins**,** from tunicate *Cystodytes dellechiajei* were the first pyridoacridine alkaloids, interesting levorotatory compounds, discovered in tunicates[38]. These compounds showed potent cytotoxicity against murine lymphoma cells and human epidermoid carcinoma cells *in vitro*.

Additionally, important tunicate molecules with antineoplastic activity against many mammalian tumor cell lines are the polyaromatic alkaloids belonging to the family of lamellarins described previously in the prosobranch mollusks *Lamellaria sp.* (see above), which were isolated from several species of the tunicate genus *Didemnum*[39,40]. A family of cyclic dispeptide derivatives called didemnins with potent antineoplastic properties was isolated from *Tridemnum solidum*, from which didemnin B exerted the highest degree of antitumor activity[41] and was included in clinical trials as early as 1988[42]. Because of its toxic side-effects, it was discarded from further clinical examinations. A similar molecule to didemnin, the aplidine (dehydrodermin B), was extracted from the tunicate *Aplidium albicans*[43]. Aplidine interfered, similar to kahalalid F and ecteinascidins, with the cell cycle, however, its cytotoxicity against tumors is based on inhibition of the enzyme ornithine descarboxylase which is required for tumor growth[36]. It also exerts inhibiting activity on HIF-1. Clinical phase II studies confirmed its cytostatic activity against acute lymphoid and myeloid leukemia.

From the point of view of anticancer activity, lipophilic cyclic peptides like ascidiacyclamide, ulithiacyclamide, several patellamids from *Lissoclinum patella*[44,45], and some polyunsaturated amino alcohols such as crucigasterins from *Pseudodistoma crucigaster*[46] are interesting with regard to therapeutic development. Equally the polyketide, palmerolide A, from the tunicate *Synoicum adareanum*, which was found to be particularly active against melanoma cells[47], is an attractive substance for the construction of new synthetic derivatives[48].

The bi-steriodal substance, cephalostatin 1, which activates the apoptosis signals, was recently discovered in a representative of a unique phylum of marine deuterostomian invertebrates, the hemichordate *Cephalodiscus gilchristi*[49]. It inactivates the antiapoptotic mitochondrial protein bcl-2[49] and activates caspase-4, an endoplasmic reticulum stress response and induces apoptosis[50]. These effects strongly suggest that cephalostatin 1 may be useful in the development of a drug to treat drug-resistant cancers.

CONCLUSION

The most important bioactive substances with anti-neoplastic effects isolated from these phyla are summarized in Table 1. In general, these substances manifest one or more anti-cancer mechanisms, including induction of apoptosis, enhancement of the effects of chemotherape-

utic drugs, direct cytotoxicity, inhibition of proliferation, impaired cell migration, suppression of metastases, gene regulation, or anti-angiogenesis. Despite the extensive effort and enormous amounts of money used in the development of new types of drugs, significant progress in cancer treatment remains elusive. The use of plants as a source of new drugs resulted in few clinically important drugs, but in recent years, more attention has been focused on marine organisms. Readers seeking additional data should read these excellent articles[41,51-55]. From the data in both sections of this work, it is clear that the world's oceans will play an important role in the future control of cancer treatment. Although some of the molecules isolated from marine invertebrates are already used for cancer treatment in the United States and the European Union, substantial efforts are still necessary to further advance clinical applications and to fulfill the potential offered by marine invertebrates.

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S- Editor Yang XC **L- Editor** Webster JR **E- Editor** Yang XC

World Journal of Clinical Oncology

Online Submissions: http://www.wjgnet.com/2218-4333office
wjco@wjgnet.com
doi:10.5306/wjco.v2.i11.367

World J Clin Oncol 2011 November 10; 2(11): 367-376
ISSN 2218-4333 (online)

REVIEW

Epidermal growth factor receptor and K-Ras in non-small cell lung cancer-molecular pathways involved and targeted therapies

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Supported by Portuguese Oncology Institute – Research Center
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Received: June 8, 2011 Revised: October 10, 2011
Accepted: October 17, 2011
Published online: November 10, 2011

Abstract

Lung cancer is currently the leading cause of cancer death in Western nations. Non-small cell lung cancer (NSCLC) represents 80% of all lung cancers, and adenocarcinoma is the predominant histological type. Despite the intensive research carried out on this field and therapeutic advances, the overall prognosis of these patients remains unsatisfactory, with a 5-year overall survival rate of less than 15%. Nowadays, pharmacogenetics and pharmacogenomics represent the key to successful treatment. Recent studies suggest the existence of two distinct molecular pathways in the carcinogenesis of lung adenocarcinoma: one associated with smoking and activation of the *K-Ras* oncogene and the other not associated with smoking and activation of the epidermal growth factor receptor (EGFR). The *K-ras* mutation is mainly responsible for primary resistance to new molecules which inhibit tyrosine kinase EGFR (erlotinib and gefitinib) and most of the *EGFR* mutations are responsible for increased tumor sensitivity to these drugs. This article aims to conduct a systematic review of the literature regarding the molecular pathways involving the EGFR, *K-Ras* and EGFR targeted therapies in NSCLC tumor behavior.

Key words: Epidermal growth factor receptor; K-Ras; Non-small-cell lung carcinoma; Pharmacogenomics; p21ras proto-oncogene proteins

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de Mello RA, Marques DS, Medeiros R, Araujo AM. Epidermal growth factor receptor and K-Ras in non-small cell lung cancer -molecular pathways involved and targeted therapies. *World J Clin Oncol* 2011; 2(11): 367-376 Available from: URL: http://www.wjgnet.com/2218-4333/full/v2/i11/367.htm DOI: http://dx.doi.org/10.5306/wjco.v2.i11.367

INTRODUCTION

Recently, it was estimated that about 11 million people presently have cancer worldwide[1,2]. In the United States, lung cancer (LC) is the main cause of cancer death, in both genders, and it has a global incidence of about 70 cases per 100 000 inhabitants[3-5]. In Europe, LC incidence is about 52.5 cases per 100 000 inhabitants (82.5/100 000 in males and 23.9/100 000 in females) and mortality is approximately 48.7/100 000 (77/100 000 in males and 23.9/100 000 in females)[3,6]. Smoking status was demonstrated in previous reports to be an important

prognostic factor due to its influence on overall survival (OS) regardless of the treatment received[7]. Histology[8, 9], co-morbidity using the Charlson score[10] and admission performance status[11] also have an impact on OS and patient outcome.

Non-small cell lung cancer (NSCLC) corresponds to 80%-85% of LC and, although there is progression in the development of new chemotherapeutics, NSCLC prognosis remains unsatisfactory with a 5-year OS of less than 15%[12,13]. Surgery is the best curative therapeutic approach in the early stages (Ⅰ and Ⅱ). However, even in these patients, the mean 5-year OS is less than 70%. Thus, most NSCLC patients will be candidates for adjuvant, neoadjuvant or palliative chemotherapy and/or radiotherapy at any time of the disease evolution. Although patients with metastatic disease benefit from standard chemotherapy, its impact on OS is not more than two months. This is why knowledge of the molecular pathways involved in cancer progression is very important[1, 6,14-16].

The aim of this study was to conduct a systematic review of the literature regarding the molecular pathways involving the epidermal growth factor receptor (EGFR) and *K-Ras* in NSCLC behavior and to address some issues on EGFR targeted therapies.

NSCLC PHARMACOGENOMIC CONTRIBUTION

Molecular pathway model

In the last few years, knowledge about molecular mechanisms and cellular transformation in association with cancer behavior has increased[17-19]. More interest has been generated since the development of specific targeted therapies against the processes involved in the carcinogenesis of many types of cancers[20-22]. During the 1990s it was discovered that the EGFR[23] played an important role in tumoral biology and behavior[14]. As summarized in Figure 1, EGFR stimulation activates intracellular signaling and cascades that influence cellular proliferation and mobilization, angiogenesis and other mechanisms. Normal cells are influenced by external factors, in tumor cells it was found that the activation of cell proliferation mediated by this receptor would no longer need external stimuli, but act independently and autonomously[14, 24]. In the case of NSCLC, it was shown that the overexpression of this receptor, as well as specific somatic mutations occurred in their intracellular domain with tyrosine kinase activity (between exons 18 and 21), which may influence prognosis, being significantly related to stage, survival and chemotherapy response [14, 25, 26]. These data led to the development and study of various substances, including monoclonal antibodies directed to the extracellular domain of EGFR (e.g., cetuximab, Erbitux ®) and small molecules that inhibit the tyrosine kinase intracellular domain (tyrosine kinase inhibitors, TKIs) of EGFR (e.g., gefitinib and erlotinib)[14,26-30]. Preliminary results of randomized clinical trials conducted with these TKIs have shown that their use in patients with advanced disease is effective, significantly increasing the survival of these patients, especially if they harbor mutations in the EGFR which are more frequently found in a subgroup of non-smoking, female patients, of Asian ethnicity and with adenocarcinoma histological sub-type (especially in the presence of bronchioloalveolar carcinoma). Some of these results were so impressive that this phenomenon was designated, the Lazarus effect, and led to the approval, in the United States and Europe, of erlotinib for the second- and third-line treatment of NSCLC patients; and gefitinib in Europe, for patients harboring the *EGFR* mutation[25,27-29,31].

Other molecular biomarkers have been investigated in NSCLC, such as *COX-2*, *p53* and *K-Ras*[1,32]. Among these biomarkers, *K-Ras* was shown to be important in NSCLC carcinogenesis. This biomarker is mutated in about 20% to 40% of these tumors[26,33-35] and over 95% of the mutations described are located at codons 12 and 13, rarely at codons 59 and 61[33]. Several environmental factors are associated with the *K-Ras* mutation, such as smoking (there is a relationship between the number of cigarettes smoked and the prevalence of mutations) and exposure to asbestos[33,36]. Mutation of *K-Ras* appears to be an early phenomenon in NSCLC carcinogenesis and it is often associated with other molecular aberrations such as *p53* mutation, *p16* methylation, *Bcl-2*, *RASSF1* inactivation and increased expression of several growth factors, among them vascular endothelial growth factor, thereby promoting cell proliferation, suppression of apoptosis and angiogenesis[26,33]. Although there is no consensus between the studies, *K-Ras* mutation seems to be the main poor prognostic factor in LC adenocarcinoma patients in stage Ⅰ, and possibly stage Ⅱ, associated with significantly lower survival rates regardless of other involved factors such as the number of treatment regimens[26,33,35]. Similar conclusions can be drawn from the analysis of studies regarding its predictive value in response to currently recommended treatments, targeted chemotherapy and radiation therapy; furthermore, it may be a factor in resistance to therapy in the early stages (Ⅰ and Ⅱ), but not in advanced stages[26,33].

Several studies have found an inverse association between *K-Ras* mutations and mutations in the EGFR tyrosine-kinase domain[20,33]. These data suggest the possible existence of two distinct molecular pathways in lung carcinogenesis: one associated with smoking and activation of *K-Ras;* and another not associated with smoking and activation of EGFR[28,29,36,37]. When combined, *K-Ras* mutation[38] is mainly responsible for primary resistance to new molecules which inhibit tyrosine kinase EGFR (e.g. erlotinib and gefitinib)[20,25,26,33,39,40].

EPIDERMAL GROWTH FACTOR RECEPTOR

C-erbB family

EGFR (or ErbB1) is a transmembrane glycoprotein encoded by a gene located on chromosome 7 (*7p12.1-12.3*).

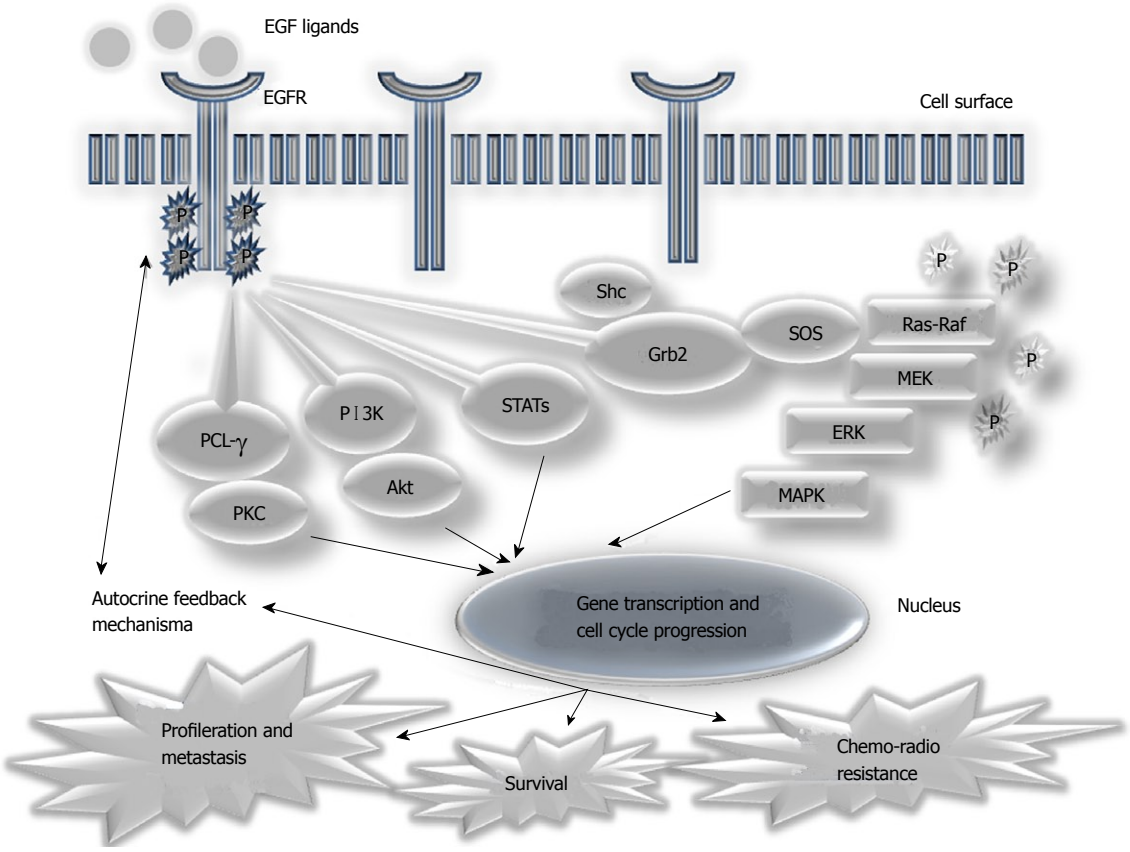

Figure 1 Molecular mechanisms through the Epidermal Growth Factor Receptor pathway. This figure shows EGFR ligands binding with their receptor and triggering mechanisms toward downstream intracellular signaling throughout the PLCγ, PI3K, Ras/Raf/MEK/ERK, STATs pathways leading to proliferation, metastasis, autocrine feedback, and survival (Adapted from reference 7 with permission). EGFR: Epidermal growth factor receptor; Shc: Src homologous and collagen protein; PCL-γ: Phospholipase Cγ; PI3K: Phosphatidylinositol 3-kinase; STATs: Signal transducer and activator of transcriptions; PKC: Protein kinase C; Grb2: Growth factor receptor bound protein 2; SOS: Guanine nucleotide exchange factor sos; MAPK:mitogen-activated protein kinase

It comprises 1186 amino acids (a.a.) and 26 exons[41]. Exons 1-14 encode the extracellular domain, exon 15 encodes the transmembrane region and exons 16-26 the intracellular domain. This glycoprotein belongs to the ErbB receptor family, which also consists of: ErbB2 (HER2/*neu*), ErbB3 (HER3) and ErbB4 (HER4). Each of these proteins is structurally composed of an extracellular domain, a hydrophobic transmembrane domain and an intracellular domain with intrinsic tyrosine kinase (TK) activity (except ErbB3). These receptors exist as inactive monomers, being activated by their interaction, through the extracellular domain, with growth factors of the EGF family. The binding of ErbB receptor molecules to one of these ligands leads to its interaction with other monomers of the same family (receptor dimerization). This dimerization can occur between two identical receptors (homodimerization, e.g., ErbB1-ErbB1) or between two different receptors (heterodimerization, e.g., ErbB1-ErbB3). The stimulation caused by a specific ligand triggers a unique pattern of dimerization, which is also specific to the tissue/tumor in which the phenomenon occurs. Dimerization of the receptors leads to their autophosphorylation with activation of TK and activation of a cascade of intracellular biochemical processes that regulate such diverse activities, like proliferation, differentiation, apoptosis and cell migration[14,40] as shown in Figure 1.

Regulating the activity of tyrosine kinase

Usually, TK activity is regulated by the conformational state of the catalytic domain of the molecule. The conformation of the catalytic domain, either active or inactive, governs the ability of a kinase to transfer phosphate from adenosine triphosphate to a peptic substrate, thereby regulating the intracellular signaling pathways. There are several mechanisms that regulate this balance of active-inactive protein kinase at the atomic level. First, a.a. residues should be properly oriented in order to facilitate the transfer of phosphate and, second, the peptide substrate binding site should not be occluded. There are two important regions of the catalytic domain able to regulate these mechanisms according to their spatial orientation: the activation loop and the helix-C[41].

In the active conformational state, the activation handle extends outside the catalytic cleft of the molecule in order to allow the substrate to bind to it, while the catalytic glutamate residue (C-helix) forms ionic interactions with a lysine residue that coordinate α and β phosphates of ATP. In the inactive conformation, the activation loop

changes its conformation drastically and hinders bonding of the peptide substrate to the catalytic domain of the molecule, while the C-helix wheel drags the residue of glutamate clear of the lysine residue[41].

Current approaches in EGFR assessment

Mutations occurring in these TK molecule catalytic domains lead to conformational changes that promote permanent active status independent of external factors. The most common, accounting for about 85% of the mutations described, include deletions in exon 19 and substitution of a.a. leucine-858 by arginine in exon 21. These mutations increase the sensitivity to TKIs, probably by promoting conformational changes of the molecule so that the fit of the TK catalytic cleft simulates the inactive conformational status[25,41].

On the other hand, the substitution of threonine-790 by methionine in exon 20 is a factor in resistance to TK, probably because the conformational change of the molecule caused by it does not allow the same type of effect observed for other mutations. This mutation may be acquired, having been described in patients with progressive disease after effective treatment with erlotinib and gefitinib, or innate, resulting in increased susceptibility to LC and primary resistance to molecules that inhibit TK[25,39-42].

EGFR amplification and/or over-expression are also predictors of response to TKI treatment[43]. The EGFR over-expression accounts for about 43%-83% of NSCLC, being more common in squamous cell carcinoma (70%), followed by adenocarcinoma (50%) and to a lesser extent, in large cell carcinoma. This phenomenon is very rare in small cell lung cancer patients[14,39,42].

Intracellular signaling pathways

There are four main intracellular signaling pathways involved in the activation of EGFR (Figure 1): Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, phospholipase Cγ (PLCγ), protein kinase C and signal transducer and activator of transcription (STAT)[14,42]. Activation of PI3K leads to activation of Akt. This is translocated to the cell nucleus and mediates the transcription of many genes while other cytosolic proteins are activated simultaneously (such as mTOR and Bad), resulting in the ultimate expression of several anti-apoptotic proteins. PLCγ hydrolyzes phosphatidylinositol 4, 5-bisphosphate into diacylglycerol and inositol triphosphate with subsequent activation of protein kinase C, resulting in cell cycle progression. STAT proteins are translocated to the active nucleus and regulate transcription of genes essential for survival and proliferation, mediating cellular transformation and progression to carcinoma[33,42]. The Ras/MAPK pathway is described in detail below.

Ⅳ–*K-RAS*

Ras family

The Ras family (Rat sarcoma viral oncogene), or p21ras (so designated by the molecular weight common to the various elements that constitute the 21Kd), belongs to the super-family of small guanosine triphosphatases (GTPases) and is composed of several members (the most studied are *H-Ras*, *K-Ras* and *N-ras*)[33,44-50].

Unlike the classic G proteins which are heterotrimeric, they remain in the form of monomeric units of connection (similar to the α subunit of classic G proteins), functioning as a small switch that toggles between the inactive monomer bound to guanosine diphosphate (GDP) and actively linked to guanosine triphosphate (GTP)[33,44,47,49,50]. Different stimuli from the cell surface, mediated by several types of transmembrane receptors, activate these proteins, leading to a cascade of intracellular biochemical processes that regulate such diverse activities as proliferation, differentiation, apoptosis and cell migration[33, 45-50]. The most studied members of this family share high structural and functional homology (although encoded by different genes); they are expressed in all tissues (there are variations in the level of subtypes expressed); and they are implicated in the carcinogenesis of various types of tumors[33,44-50].

Activation of K-Ras protein through the EGFR pathway

Stimulation of EGFR protein activates both *K-Ras* 4B and *H-Ras* in different ways. These are preferentially located in areas of dense cell membrane (e.g., *K-Ras* 4B), and this is the Ras protein primarily activated[35]. The binding of EGF to its receptor causes dimerization of α and β subunits and subsequent activation of this intrinsic TK receptor, which autophosphorylates the tyrosine residues and thus allows the receptor to bind to proteins such as Shc adaptation or Grb2. These proteins serve as the link between the EGFR and a set of proteins that are able to stimulate the dissociation of guanine (GNEFs - Guanine Nucleotide Exchange Factors). GNEFs promote the dissociation of polymer Ras-GDP, releasing the Ras to bind to GTP and thus causing its activation[33,44,46,48]. In this particular case, the first class of GNEFs to be activated is the Son of sevenless (Sos), which promotes the dissociation and activation of *K-Ras* 4B at the inner surface of the cell membrane. Secondly, through mechanisms that involve the formation of diacylglycerol , a second class of GNEFs, also called RasGRPs, promote the activation of H-Ras in the Golgi apparatus, by mechanisms not yet completely clarified[44,46].

Generally, the Ras-GTP complex is active only transiently, since each Ras molecule has intrinsic GTPase activity that, when stimulated by GAPs, hydrolyzes rapidly to Ras-GTP binding and promotes its return to inactive baseline status and binds to GDP (GAPs increase the activity of intrinsic Ras-GTPase proteins approximately 10 000 times)[33,44-50].

Once activated, Ras protein promotes the initiation of several distinct signaling cascades, which are reflected at the nuclear transcription of several genes and the production of factors that induce proliferation, differentiation, migration, apoptosis/cell anti-apoptosis and angio-

Table 1 Summary of studies which assessed the current main epidermal growth factor receptor targeted drugs for non small cell lung cancer

Study	Molecule	Place of study	EGFR positive selected mutations	No. of patients	Clinical stage	Response rate (%)	Median OS (mo)	Median PFS (mo)
Kris *et al*, 2003	Gefitinib	United States	No	221	ⅢB and Ⅳ	22	6-7	-
Perez-Soler *et al*, 2004	Gefitinib	United States	No	57	ⅢB and Ⅳ	12.3	8.4	-
Maemondo *et al*, 2010	Gefitinib	Asia	Yes	230	ⅢB and Ⅳ	73.7	30.5	10.8
Mok *et al*, 2009	Gefitinib	Asia	No	609	Ⅲ and Ⅳ	71.2	18.6	5.7
Mitsudomi *et al*, 2010	Gefitinib	Japan	yes	177	ⅢB and Ⅳ	62.1	30.9	9.2
Shepherd *et al*, 2005	Erlotinib	America, Europe and Asia	No	731	ⅢB and Ⅳ	8.9	6.7	2.2
Herbst *et al*, 2005	Erlotinib	United States	No	526	ⅢB and Ⅳ	30	10.6	6
Capuzzo *et al*, 2010	Erlotinib	Italy	yes	437	ⅢB and Ⅳ	11.9	12.3	12.3

EGFR: Epidermal growth factor receptor; OS: Overall survival; PFS: Progression-free survival.

genesis by VEGF secretion[33,44-51]. In the case of *K-Ras* 4B, the main intracellular signaling pathways activated are the Raf/MEK/ERK (which promotes cell proliferation) and PI3K/Akt (which promotes cell survival by inhibiting apoptosis)[33,44,46,48]. A comparison of the molecular structure of the active form (bound to GTP) and inactive form (bound to GDP) of the Ras protein showed that the transition from an active to inactive form is accompanied by changes in the conformation of the molecule into two regions, designated switch I (residues 30 to 38, the same effectors region in the center) and switch II (residues 60-76). The blockade of residues 63 to 73 using a directed antibody inhibits change of the protein binding of Ras GTP to GDP, proving that this region of the molecule is essential for conformational change of the molecule that allows the passage of the inactive and active state[32,48,52].

As previously mentioned, Ras protein is rapidly inactivated after initial stimulation, remaining as the inactive form most of the time[45]. However, when mutations occur, especially at codons 12, 13, 59, 61, 63, 116, 117, 119 or 146, its structure is altered by binding sites for guanine, affecting its normal function. The effects of these mutations can be translated either in a reduction of the activity of oncoprotein GTPases, blocking it into the active form bound to GTP (especially if they involve the a.a. 12, 13, 59, 61 and 63) or in decreased binding affinity and increasing the change in GDP by GTP attachment (especially if they involve the a.a. 116, 117, 119 and 146)[35]. The inefficient deactivation of oncoprotein is intensified by the fact that GAPs have reduced ability to promote the return to the disabled state (Ras-GDP). All mutations thus facilitate accumulation of the active form (Ras-GTP), contributing to the malignant cell phenotypic change[44,48,53].

The modified molecule is independent of stimulation by the activation of cell membrane receptors. Thus, it is understandable that patients with NSCLC who have a mutated *K-Ras* do not respond to treatment with TKI[25]. Brugger *et al*[54] demonstrated that *K-Ras* works as a prognostic factor for reduced progression-free survival (PFS) regardless of the treatment for advanced NSCLC.

The search for new targeted therapies able to inactivate *K-Ras* has led to the discovery of farnesyltransferase inhibitors[42]. These inhibitors act at a protein level by blocking *K-Ras* farnesylation and preventing their anchor to the cell membrane. The *K-Ras* molecule, thus trapped in the cytoplasm, is not able to activate effectors of intracellular signaling pathways. Several farnesyltransferase inhibitors have been investigated in LC with unsatisfactory results. Tipifarnib, although capable of effectively blocking this enzyme, did not result in clinical response. Moreover, preliminary clinical trials conducted with lonafarbin (in combination with paclitaxel in patients with NSCLC resistant to taxanes) showed that this combination therapy was effective in controlling some 50% of patients and is currently under further Phase Ⅲ clinical trials[42].

V-CURRENT AND FUTURE ANTI-EGFR TARGETED THERAPIES

Over the few last decades, the platinum-based treatment of NSCLC has remained unsatisfactory[55]. Many researchers have tried to identify biomarkers of response to chemotherapy such as ERCC1 (excision repair cross complementing 1) and platinum response[56], ribonucleotide reductase subunit M1 (RRM1) and gemcitabine resistance[57], *K-Ras* mutation and EGFR status[38,58]. At the moment, some studies such as those shown in Table 1, have demonstrated that personalized therapy through the EGFR pathway have the potential to improve the survival of advanced NSCLC patients[22,27,59-65]. However, the results are not linear. Many factors influence OS, PFS and response rate, such as *EGFR* mutation status, clinical TNM stage, gender and ethnicity[66].

Erlotinib: current trends

Since 2005, based on the study by Shepherd *et al*[27], erlotinib has been used for second/third-line therapy[63] to prolong survival in refractory NSCLC IIIb and IV patients irrespective of EGFR status. This drug showed

improvements in response rate, OS and 1-year-survival when added to carboplatin and paclitaxel in a recent evaluation[67]. Furthermore, as showed in the TRIBUNE phase Ⅲ trial, it was first studied in United States, Asia and Europe, but did not have consistent results following approval by the US Food and Drug Administration (FDA) and European Medicine Agency (EMEA) in first-line chemotherapy patients[27,30,61,62,68]. The genetic mutation of EGFR in exons 19 and 21 of chromosome 7 demonstrated an association with response to erlotinib [40, 68]. Therefore, erlotinib is approved for the treatment of patients with refractory disease. In April 2010, based on the SATURN phase Ⅲ trial conducted by Capuzzo *et al*[63], erlotinib was also approved for maintenance treatment in patients with locally advanced or metastatic NSCLC without progression after four chemotherapy cycles in the first-line setting. In 2011, an American Society of Clinical Oncology provisional opinion panel started to consider erlotinib as first-line therapy for advanced NSCLC[69]. A recent Chinese phase 3 trial (OPTIMAL, CTONG 0802), enrolled 83 patients with advanced NSCLC and mutations of the EGFR gene (exon 19 and 21) from 22 centers in China[70]. This study was conducted in order to compare the efficacy and tolerability of the TKI erlotinib *versus* standard chemotherapy, in this case gemcitabine plus carboplatin. They showed significantly higher PFS in patients treated with erlotinib compared with those treated with standard chemotherapy: 13.1 mo (95% CI 10.58-16.53) *vs* 4.6 months (95% CI 4.21-5.42). Furthermore, grade Ⅲ and Ⅳ toxicities, mainly neutropenia and thrombocytopenia, were more frequent in the chemotherapy arm. Thus, these findings suggested that erlotinib might be an important agent in the first-line treatment of advanced NSCLC patients with positive EGFR mutations (mainly in exon 21). Based on these results, erlotinib was approved in Europe in September 2011, as first-line therapy in patients with locally advanced or metastatic NSCLC harboring EGFR activating mutations. Currently, erlotinib is also recommended as second- and third-line therapy in a subset of advanced NSCLC patients irrespective of their EGFR status, due to its impressive results described in the above studies[27,63,70].

Gefitinib

Gefitinib did not initially show significant clinical benefit on OS, PSF and tumor response in Western patients with NSCLC IIIB and IV stages[59,61]. The iressa survival evaluation in lung cancer (ISEL) trial showed disappointing results when no improvement in OS was observed in patients treated with gefitinib in either the overall or adenocarcinoma population[60]. However, others studies (IPASS) reported the superiority of gefitinib when compared with platinum-taxane-based therapy protocols mainly in patients with *EGFR* mutations, adenocarcinoma histology, nonsmokers or former light smokers in East Asia[64,65]. Subsequently, it was also confirmed that EGFR mutations had a predictive role in the response of lung adenocarcinoma to gefitinib as compared with carboplatin-paclitaxel treatment[43,64,70,71]. These findings resulted in the approval of gefitinib by EMEA for use in the first-line treatment of patients with advanced metastatic NSCLC *EGFR* mutation positive tumors[65].

The role of MET targeted inhibition

Recently, the MET proto-oncogene was discovered which encodes for the high affinity cell surface receptor for hepatocyte growth factor (HGF) and also control the main steps of carcinogenesis: cell growth, invasion, proliferation and apoptosis[72,74]. Thus, MET inhibitors emerged as a promising new class of targeted drugs in patients with MET-mediated resistance to EGFR inhibitors[66,74]. Nowadays, dual MET-EGFR multi-target TKI therapies may be considered a good approach for MET-mediated resistance to EGFR inhibitors to improve NSCLC patient outcome. In 2010, a recent multi-target of MET, VEGFR2 and RET, called XL 184, in association with erlotinib in NSCLC *EGFR T790M* and MET amplified patients was presented at the American Society of Clinical Oncology annual meeting as a promising choice in these patients[75]. Another drug, ARQ197, which is a selective non ATP competitive inhibitor of c-MET, when combined with erlotinib in the second/third-line treatment of EGFR inhibition *naïve* NSCLC patients showed increased PSF, mainly among patients with non-squamous histology, *K-Ras* mutations, and EGFR wild-type status[73]. Other drugs targeting MET pathways, such as AMG102[74], a monoclonal antibody against HGF, and MetMab (Genentech)[77], a human recombinant agonist of the HGF-Met signaling pathway, are still in phase I studies and show promise in the treatment of NSCLC patients[76-78].

EML4-ALK mutation and the role of crizotinib in EGFR TKI resistant patients

In the last few years, the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) fusion gene has been identified by Fluorecence in Situ Hybridization (FISH) Assay as an oncogene in about 11.3% of patients with NSCLC[79]. These patients are resistant to EGFR TKI therapies and should be directed to ALK-targeted agents[80]. FISH and real time polymerase chain reaction represent two primary methods to assess ALK fusions[81]. Recently, crizotinib, was identified as a potent inhibitor of ALK and MET tyrosine kinases[82,83]. It was demonstrated in previous studies that this drug was well tolerated and resulted in important tumor shrinkage in NSCLC EML-4ALK positive patients [84, 85]. Crizotinib has been shown to significantly control disease when used in NSCLC patients with EML4-ALK mutation fusion who were refractory to EGFR TKI treatment[82,86]. However, some patients have resistance to crizotinib and other EML4-ALK inhibitors are in development[87]. Other studies[88] showed that EML4-ALK mutation prolonged PSF in patients treated with premetrexed. Thus, this should be considered in trials involving patients treated with this drug. Recently, it was reported

that crizotinib does not cross the blood brain barrier and thus its cerebrospinal fluid levels are insufficient to control brain metastases[89]. Further studies are warranted to assess this situation.

CONCLUSION

In recent decades, therapeutic advances in LC studies, with the use of combined platinum-based chemotherapy strategies, radiotherapy or surgery, have not been completely satisfactory in terms of overall survival; and thus the prognosis associated with this disease remains very poor[54,90-95]. The need to find new targeted agents has renewed interest in the study and understanding of the molecular pathways involved in lung carcinogenesis[3,9,17,18,58,91,92], and several targeted therapeutic molecules have been synthesized[22,96-99]. However, it has also became evident that there are multiple pathogenic mechanisms in lung cancer working in parallel or with several loops of activation/inhibition, thus therapeutic exploration with the goal of disease control can not be based on the study of a single mechanism[22,97-99]. Through experience with molecules such as gefitinib and erlotinib, it is now understood that the benefit of EGFR TKIs depend on several biological characteristics in individual patients[30,59,100-103]. The study of new targeted agents and their combination in order to optimize therapy should therefore take into account the individual characteristics of each patient[55,58]. This is currently a promising field of cancer research in which genetics, tumor molecular biology and clinical experience interact to achieve more effective combination therapies adjusted to the patient profile.

ACKNOWLEDGMENTS

We would like to thank Dr. Miguel Nunes from Department of Informatics of Faculty of Medicine of the University of Porto, Porto, Portugal and Professor. Daniel H. Pozza, Department of Experimental Biology, Faculty of Medicine of the University of Porto, for his help in technical support.

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World J Clin Oncol 2011 November 10; 2(11): I
ISSN 2218-4333 (online)

Acknowledgments to reviewers of *World Journal of Clinical Oncology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Clinical Oncology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Clin Oncol 2011 November 10; 2(11): I
ISSN 2218-4333 (online)

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January 15, 2011
Current Trends in Breast Cancer: Updates From the 2010 San Antonio Breast Cancer Symposium, Dallas, TX, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA, United States

January 21-23, 2011
8th Meeting of the EAU Section of Oncological Urology, London, England, United Kingdom

January 27-28, 2011
2nd National Conference: Recent Advances in Renal and Bladder Cancer, London, United Kingdom

January 27-28, 2011
8th Annual Cancer Drugs Research & Development, San Diego, CA, United States

February 10-12, 2011
17th Annual NOCR Meeting, Las Vegas, NV, United States

February 19-22, 2011
Scripps Cancer Center's 31st Annual Conference: Clinical Hematology and Oncology, San Diego, CA, United States

February 24-26, 2011
European Multidisciplinary Conference in Thoracic Oncology (Lung 2011-EMCTO), Lugano, Switzerland

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7th European Congress on Hematologic Malignancies: From Clinical Science to Clinical Practice, Budapest, Hungary

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64th Society of Surgical Oncology Annual Cancer Symposium 2011, San Antonio, TX, United States

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8th Annual Oncology Nursing Advanced Practice: Innovation through Practice, San Diego, CA, United States

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9th International Symposium on Targeted Anticancer Therapies, Paris, France

March 09-13, 2011
16th National Comprehensive Cancer Network Annual Conference (NCCN 2011), Hollywood, FL, United States

March 11-12, 2011
12th European Congress: Perspectives in Lung Cancer, Torino, Italy

March 14-18, 2011
Oncology Imaging Update in Costa Rica, Guanacaste, Costa Rica

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International Cancer Prevention Update Symposium, New York, United States

March 18-22, 2011
Vienna, Austria 26th Annual EAU Congress

April 02-06, 2011
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Asian Oncology Summit 2011, Hong Kong, China

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Cancer Survivorship Conference, Minneapolis, MN, United States

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47th American Society of Clinical Oncology Annual Meeting, Chicago, IL, United States

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Hallmarks and Horizons of Cancer, Lausanne, Switzerland

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World J Clin Oncol 2011 November 10; 2(11): I-V
ISSN 2218-4333 (online)

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Name of journal

World Journal of Clinical Oncology

ISSN

ISSN 2218-4333 (online)

Indexed and Abstracted in

PubMed Central, PubMed, Digital Object Identifer, and Directory of Open Access Journals.

Published by

Baishideng Publishing Group Co., Limited

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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