



Toxic effects of pesticide mixtures at a molecular level: Their relevance to human health

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ABSTRACT

Pesticides almost always occur in mixtures with other ones. The toxicological effects of low-dose pesticide mixtures on the human health are largely unknown, although there are growing concerns about their safety. The combined toxicological effects of two or more components of a pesticide mixture can take one of three forms: independent, dose addition or interaction. Not all mixtures of pesticides with similar chemical structures produce additive effects; thus, if they act on multiple sites their mixtures may produce different toxic effects. The additive approach also fails when evaluating mixtures that involve a secondary chemical that changes the toxicokinetics of the pesticide as a result of its increased activation or decreased detoxification, which is followed by an enhanced or reduced toxicity, respectively. This review addresses a number of toxicological interactions of pesticide mixtures at a molecular level. Examples of such interactions include the postulated mechanisms for the potentiation of pyrethroid, carbaryl and triazine herbicides toxicity by organophosphates; how the toxicity of some organophosphates can be potentiated by other organophosphates or by previous exposure to organochlorines; the synergism between pyrethroid and carbamate compounds and the antagonism between triazine herbicides and prochloraz. Particular interactions are also addressed, such as those of pesticides acting as endocrine disruptors, the cumulative toxicity of organophosphates and organochlorines resulting in estrogenic effects and the promotion of organophosphate-induced delayed polyneuropathy.

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1. Introduction

Pesticides are unique, intrinsically toxic chemicals designed to be deliberately spread into the environment to kill off pests. They are comprised of many different categories of chemicals whose toxicity is increasingly reduced as less toxic compounds benefit from stricter regulations. Approximately 5.2 billion pounds were used worldwide in 2006 and a similar amount in 2007 (US-EPA, 2011), but only 1% of this amount reaches the target pests at lethal doses (Gavrilescu, 2005). Herbicides account for the largest portion of that amount, followed by other pesticides, insecticides and fungicides (US-EPA, 2011).

Exposure to pesticides can occur through multiple pathways (e.g. food, drinking water, residential, occupational) and routes

(oral, inhalation, dermal). Although the contribution of a given route or pathway to overall exposure depends on the pesticide, it is the totality of exposure, by multiple routes and multiple pathways, what determines the risk (EFSA, 2008). The type and severity of adverse health effects of pesticides are determined by the individual chemical category, the dose and the duration of exposure and the exposure route.

Because of their intrinsic toxicity and limited species selectivity, pesticides exhibit undesirable harmful effects on sensitive non-target organisms such as humans and wildlife populations (Hernández et al., 2011a). Given that humans are much larger than the target species for pesticides, they are expected to be unaffected by small amounts of these compounds. However, pesticides are indeed toxic to humans not only at high doses, responsible for acute poisonings, but even in low doses, as are mixtures of pesticides (Tsatsakis et al., 2009; Zeliger, 2011). Long-term exposures may lead to an array of health effects including cancer and neurodegenerative diseases (Bassil et al., 2007; Kanavouras et al., 2011; Parrón et al., 2011), reproductive and developmental toxicity (Hanke and Jurewicz, 2004) and respiratory effects (Hernández et al., 2011b). It

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is now well established that exposure to pesticides during critical periods of development can present lasting adverse effects in early development and later in life, particularly the developing brain and the endocrine system are both very sensitive targets (London et al., 2012).

Since pesticides are often applied in mixtures to crops, their residues can be found in foods and drinking water. However, mixtures of pesticides are common not only in the human food supply but also in the aquatic environment, including surface waters that support aquatic life (Laetz et al., 2009). In fact, more than 50% of all streams tested in the United States contained five or more pesticides (Zeliger, 2011). If streams and groundwaters are used as a source of drinking water, and the previous treatment fails to eliminate pesticide residues, humans can be exposed to mixtures of pesticides and their degradates.

Despite many papers describe the toxic effects of pesticide mixtures, relatively little information is available on the nature of interactions that may occur between the constituents of a pesticide mixture, especially when they deviate from additivity.

2. Toxicological interactions of pesticides

Pesticide interactions include agent-to-agent interactions, toxicokinetic and toxicodynamic interactions. Thus, to make science-based judgments about these interactions it is necessary to have a good understanding of the chemical reactivity, the toxicokinetics (including metabolic pathways) and the mechanisms of action of each compound (IGHRC, 2009).

Exposure to multiple pesticides may cause changes in the toxicokinetics of the individual compounds, thus modifying the predicted toxicity. Toxicokinetic interactions are the result of one pesticide altering the absorption, distribution, metabolism or elimination of others (Reffstrup et al., 2010) and can occur at all dose levels, but the effects may not be measurable at low doses. The most likely effect of these interactions is to alter the relationship between the external dose and the corresponding level of a pesticide at its target site, leading to an alteration in the threshold for effects (IGHRC, 2009).

Toxicodynamic interactions requires that a sufficient amount of a pesticide reach a target tissue and causes some perturbation of normal physiology and that a sufficient amount of a second toxicant also reach the same tissue and causes a second perturbation which either exacerbates (potentiates) or compensates for (antagonizing) the effects of the first compound. The key requirement for toxicodynamic interactions is that the dose levels for each pesticide are sufficient to have an effect (i.e., above the threshold of effect). When dose levels are below thresholds of effect, no toxicodynamic interactions are expected to arise (IGHRC, 2009).

There are two main principles describing how individual pesticides in a mixture affect one another: the concept of additivity and interaction. Additivity expectations can be derived from the concepts of dose addition and independent action, which assume that chemicals act by the same or different modes of action, respectively (Silins and Högberg, 2011). In this situation, also termed “non-interaction”, the toxicity of a mixture resembles the effects expected to occur when all mixture components act without enhancing or diminishing their effects. By contrast, interaction arises when the observed mixture effects deviate from what was expected. In this case, one or several pesticides may have interacted with each other, e.g. by facilitating or diminishing their uptake, transport, metabolism or excretion. Interaction is the term for synergisms (mixture effects greater than expected) and antagonisms (Kortenkamp et al., 2009).

The combined toxicological effects of two or more components of a pesticide mixture can take one of three forms: independent, dose addition or interaction.

2.1. Independent action

Independent action, also referred to as response or effect addition, occurs when the toxicological effects of the individual pesticides in a mixture have different mechanisms or modes of action. The effects of such a combination will be the sum of the effects of each compound when given alone, reflecting that they do not influence each other's action, and therefore neither additivity nor potentiating interactions are generally found (Boobis et al., 2008; COT, 2002). When this is applied to pesticide residues that have different modes of action, and each component is below any threshold of effect, the default assumption is that the combined exposure will not have any toxicological effect in consumers, given that the individual exposures do not exceed the respective reference values (Boobis et al., 2008).

2.2. Dose-addition

The generally held view is that dose addition should be assumed for mixtures of pesticides that produce the same toxic effect by affecting the same target organ via the same mechanism of toxicity over the whole dose range (COT, 2002; IGHRC, 2009). Two pesticides act via a common mechanism of toxicity if they cause the same critical effect and act on the same molecular target or tissue or on the same biochemical mechanism of action, possibly sharing a common toxic intermediate (US-EPA, 1999).

The combined effect of pesticides that have similar chemical structures and/or modes of toxic action can be predicted by an additive toxicity approach that assumes that the cumulative toxicity of the mixture can be estimated from the sum of the individual toxic potencies of each individual compound (Lydy et al., 2004; Hernández et al., 2011a). This is the case of organophosphates (OP) pesticides, (di)thiocarbamates or chloroacetanilides. In concurrent multiple OP exposure, a summation of the inhibitory effects of individual compounds on acetylcholinesterase (AChE) activity is usually observed. This also applies to OPs and *N*-methylcarbamates (NMC), two different classes of insecticides that share a common mode of toxic action: inhibition of AChE. According to acute oral toxicity (LD_{50}), the combined effects of two insecticides have been reported to be additive for OPs plus OPs and for OPs plus NMC in most cases (Sun et al., 2000).

Additive effects may be observed when a mixture of two pesticides, each below the no observed effect, produces a predicted toxic effect when the sum of their concentrations is greater than the threshold level for toxic action (Zeliger, 2011). Given that food residues of pesticides are generally found at exposure levels far below their respective NOAEL, they are not expected to cause more than an additive effect.

2.3. Interaction

Chemical interactions represent a deviation from simple additivity because individual compounds affect toxicity of one another, resulting in more or less than an additive effect (Rider and LeBlanc, 2005; Silins and Högberg, 2011). Thus, the combined effects of two or more pesticides is either greater (supra-additive, potentiating, synergistic) or less (infraadditive, inhibitive, antagonistic) than that predicted on the basis of dose-addition or response-addition (Boobis et al., 2008). Interaction does not occur at doses that are at or below the NOAEL of pesticides in a mixture; however, when exposures exceed their respective NOAELs, both toxicodynamic and

toxicokinetic interactions resulting in synergy or antagonism can occur (Moser et al., 2006).

2.3.1. Potentiation

Potentiation is observed when the effect of a pesticide is enhanced by the presence of one or more other chemicals (either pesticides or not) that are only slightly active. Evidence for potentiation has been reported when exposure to the mixture exceeded the threshold of effect for some or all of the components, although it is not scientifically valid to extrapolate this assumption to much lower dose levels (COT, 2002). Potentiation may occur in mixtures that involve a secondary chemical that changes the toxicokinetics of the pesticide as a result of its increased activation or decreased detoxification, ultimately leading to an enhanced or reduced toxicity (Hodgson and Rose, 2007; Lydy et al., 2004). If the dose levels of compounds in the mixture are large enough to cause enzyme induction, this could lead to potentiation of pesticides requiring metabolic activation.

Piperonyl butoxide (PBO) is a pesticide synergist, especially for PYRs and rotenone, because it is a potent CYP450 and non-specific esterase inhibitor. PBO does not have, by itself, pesticidal properties; however, when added to insecticide mixtures, typically pyrethrin, pyrethroid and carbamate insecticides, their potency increase considerably (Tozzi, 1998). CYP450 and esterases act as major detoxification pathways for many pests, and so inhibiting them allow higher unmetabolized systemic concentrations of the active insecticide to remain within the target animal for a longer period (Androustopoulos et al., 2009; Moores et al., 2009).

2.3.2. Synergism

Synergism is observed when the effect of exposure to a pesticide mixture is much greater than that expected from an additive effect and responses cannot be predicted by the known toxicology of the each individual compound (Zeliger, 2011). One of the compounds in the mixture changes the body's response resulting in either a greater response than would be observed for an additive effect (quantitative response) or in the attack on a different target organ that is not predicted (qualitative effect) (Zeliger, 2011). Frawley et al. (1957) documented for the first time a pesticide synergy when observed that the simultaneous administration of malathion and EPN (ethyl 4-nitrophenyl phenylphosphonothioate) resulted in a 10-fold synergistic effect in rats and a 50-fold synergistic effect in dogs for the acute toxicity.

Certain insecticide combinations show a clear pattern of synergism at low, environmentally relevant concentrations (Laetz et al., 2009). Mixtures of five OP pesticides (chlorpyrifos, diazinon, dimethoate, acephate and malathion) have been reported to produce greater than additive effects when administered to laboratory animals (Moser et al., 2006). Not all mixtures of pesticides with similar chemical structures always produce additive effects; thus, different toxic effects can be observed if they act on multiple sites. Despite the similarities in their chemical structure, PYRs act on multiple sites and their mixtures produce different toxic effects (Zeliger, 2011).

Exposures to mixtures of different classes of pesticides produce effects that are often difficult to anticipate. Permethrin and the carbamate propoxur elicited greater than additive toxicity due to the complementary modes of toxic action of PYR and NMC, two insecticide classes that act on different components of nerve impulse transmission (Lydy et al., 2004). Diazinon (an OP) and benomyl (a benzimidazole fungicide), which individually fail to exhibit genotoxicity, are genotoxic when administered together (Zeliger, 2011).

2.3.3. Antagonism

Antagonism occurs when two pesticides interfere with each other's effect. The result is a reduction in the effect predicted for the

individual compounds which do not need to be structurally similar (Zeliger, 2011). One chemical may stimulate the metabolism of a second one or somehow interfere with its absorption. The following are examples of pesticide mixtures that produce antagonisms: DDT and parathion, because DDT induces and parathion inhibits CYP450 activity; prochloraz and triazine herbicides, because prochloraz exerts an inhibitory effect on aromatase (CYP19) whereas triazine herbicides stimulate this enzyme activity involved in the conversion of testosterone to estradiol (Fig. 1). PBO may also antagonize the toxicity of OPs, as has been demonstrated by the progressive shift to the right of the concentration-response curves for malathion and parathion (Rider and LeBlanc, 2005).

A simulation of the interaction threshold for the joint toxicity of two OP pesticides (parathion and chlorpyrifos) has been developed in rats using physiologically based-toxicokinetic models (PBTK) that take into consideration CYP450 bioactivation and AChE binding sites, that is toxicokinetic and toxicodynamic interactions, respectively (El-Masri et al., 2004). The simulations showed an interaction threshold below which additivity was observed, but above this threshold antagonism occurred by enzymatic competitive inhibition (El-Masri, 2007).

3. Examples of toxicity of pesticide mixtures at molecular level

The toxicological effects of low-dose pesticide mixtures on the human health are largely unknown, although the general public is increasingly concerned about their safety and potential toxic effects (Dialyna et al., 2004; Hernández et al., 2011a; Tsatsakis et al., 2011). Toxicity studies involving pesticide mixtures have resulted in a full spectrum of responses in which the complexity of the interactions depends on differences in the chemical properties and modes of toxic action of the pesticides.

3.1. Potentiation of malathion toxicity by isomalathion

In some cases, concurrent multiple OP exposure may lead to a potentiating effect, based on the interference exerted by one compound on the detoxification process of the others through metabolic inhibition. Such an effect has been observed in Pakistani applicators after handling of malathion formulations that had deteriorated during storage in a tropical climate with a significant increase in the isomalathion content (Baker et al., 1978; Aldridge et al., 1979). The same enhanced toxicity was observed in Belgium after people were exposed to pure malathion that was converted to a highly toxic mixture of malathion and isomalathion after more than 5 years of storage (Dive et al., 1994). Isomalathion acted as a potentiator of the malathion toxicity by inhibiting the carboxylesterase responsible for the detoxication of the insecticide (Maroni et al., 2000).

Mammalian carboxylesterases (CEs), enzymes widely distributed in the body, particularly in the liver, gastrointestinal tract and the brain (Jansen et al., 2009) are involved in the rapid degradation of malathion to monoacid and diacid derivatives. This reaction efficiently competes with the CYP450-catalyzed formation of malaaxon, which is also detoxified by CEs rendering malaaxon mono- and dicarboxylic acids (Buratti et al., 2005; Wielgomas and Krechniak, 2007) (Fig. 2).

3.2. Potentiation of PYR toxicity by anticholinesterase insecticides

The interaction effects of simultaneous OPs and PYR exposure is relatively well known, since formulations containing both classes of insecticides are available on the market. CYP450-activated OPs decrease the organism's ability to detoxify PYRs due to esterases inhibition, so greater than additive toxicity is often observed.

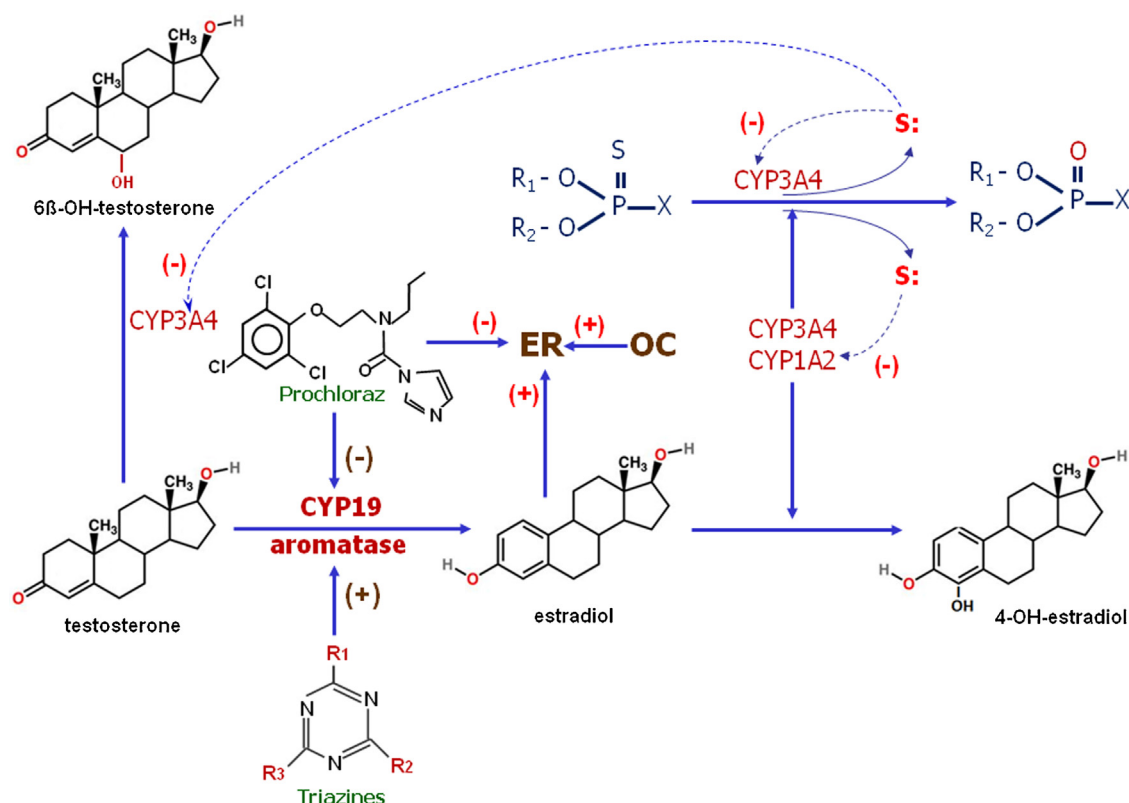


Fig. 1. Organophosphorus chemicals (OPs) are potent inhibitors of the human metabolism of both testosterone and estradiol. These compounds are activated by a P450-catalyzed desulfuration reaction that releases highly reactive sulfur atoms that immediately bind to the heme iron of P450 and inhibits its activity. Testosterone is converted to estradiol by CYP19 (aromatase) that can be either inhibited by the fungicide prochloraz or induced by triazines herbicides. Prochloraz also antagonizes the estrogen receptor (ER). Conversely, organochlorine pesticides (OC) can interact with the ER receptor thus eliciting a hormonal effect.

Coexposure to α -cypermethrin and chlorpyrifos inhibits the carboxylesterase-mediated hydrolysis of α -cypermethrin, leading to an increased tissue concentration of this compound and a decreased urinary excretion of 3-phenoxybenzoic acid (3-PBA), the major metabolite of PYRs (Wielgomas and Krechniak, 2007). Similarly, chlorpyrifos oxon (the toxic metabolite of chlorpyrifos) strongly and irreversibly inhibits permethrin hydrolysis at low concentrations, whereas carbaryl inhibits such hydrolysis although with less potency than chlorpyrifos oxon (Choi et al., 2004), indicating that it also potentiates permethrin toxicity (Hodgson and Rose, 2007) (Fig. 3).

3.3. Potentiation of OPs toxicity by organochlorine insecticides

The CYP450 family of enzymes is important for both the activation and detoxification of OPs. The relative rates at which organophosphorothioates (OPTs) insecticides are activated and detoxified may be an important determinant of toxicity. Independently of the chemical structure, bioactivation of OPTs such as malathion, chlorpyrifos, diazinon, azinphos-methyl and parathion to the very highly toxic oxon forms is carried out by the same CYP450 systems (Buratti et al., 2005). These insecticides feature a thio-phosphate moiety that is subjected to oxidative desulfuration in which the CYP450s remove the sulfur atom attached to the phosphorus and insert atomic oxygen (Fig. 4). This results in covalent binding of sulfur to CYP, a reaction that occurs preferentially at cysteine residues leading to an irreversible inactivation of the enzymes that catalyze the reactions. This mechanism-based inhibition of CYP450 is a suicide reaction for the P450 carrying out the reaction (Hodgson and Rose, 2007; Kyle et al., 2012).

At low OPT concentration, oxon derivatives formation is mainly catalyzed by CYP1A2 and to a lesser extent by CYP2B6, whereas CYP3A4 is relevant only at high OPT levels (Buratti et al., 2005). Individuals with low CYP2C19 activity, responsible for the oxidative cleavage of OPT, but high CYP1A2 and CYP2B6 activities might be more susceptible to OPT toxicity (Hodgson and Rose, 2007). Given that organochlorine pesticides, such as endosulfan- α , are CYP3A4 inducers, previous or concurrent utilization of these compounds can potentiate OPs toxicity (Casabar et al., 2006), particularly when the OPs occur at high concentrations (see Fig. 4). DDT is also able to induce CYP2B and CYP3A in rat liver (Smith, 2011).

3.4. Potentiation of carbaryl toxicity by OPs

An increased sensitivity to the NMC pesticide carbaryl following pretreatment with malathion or chlorpyrifos has been attributed to the OPT-induced suicide inhibition of CYP450 ultimately resulting in inhibition of carbaryl metabolism (Hodgson and Rose, 2007; Johnston, 1995). CYP1A2 has the greatest ability to form 5-hydroxycarbaryl, while CYP3A4 is the most active in generation of 4-hydroxycarbaryl (Fig. 5). The production of carbaryl methylol is primarily the result of metabolism by CYP2B6 (Tang et al., 2002). Therefore, the suicide reaction of the CYP450s involved in the oxidative desulfuration of OPT insecticides will prevent carbaryl from being detoxified, remaining only the hydrolysis step catalyzed by esterases yielding α -naphthol. However, such esterases can be inhibited by the oxon generated in the desulfuration step and thus any pathway of carbaryl metabolism is completely blocked, leading to a potentiation effect beyond the expected additive anticholinesterase effect (Fig. 5).

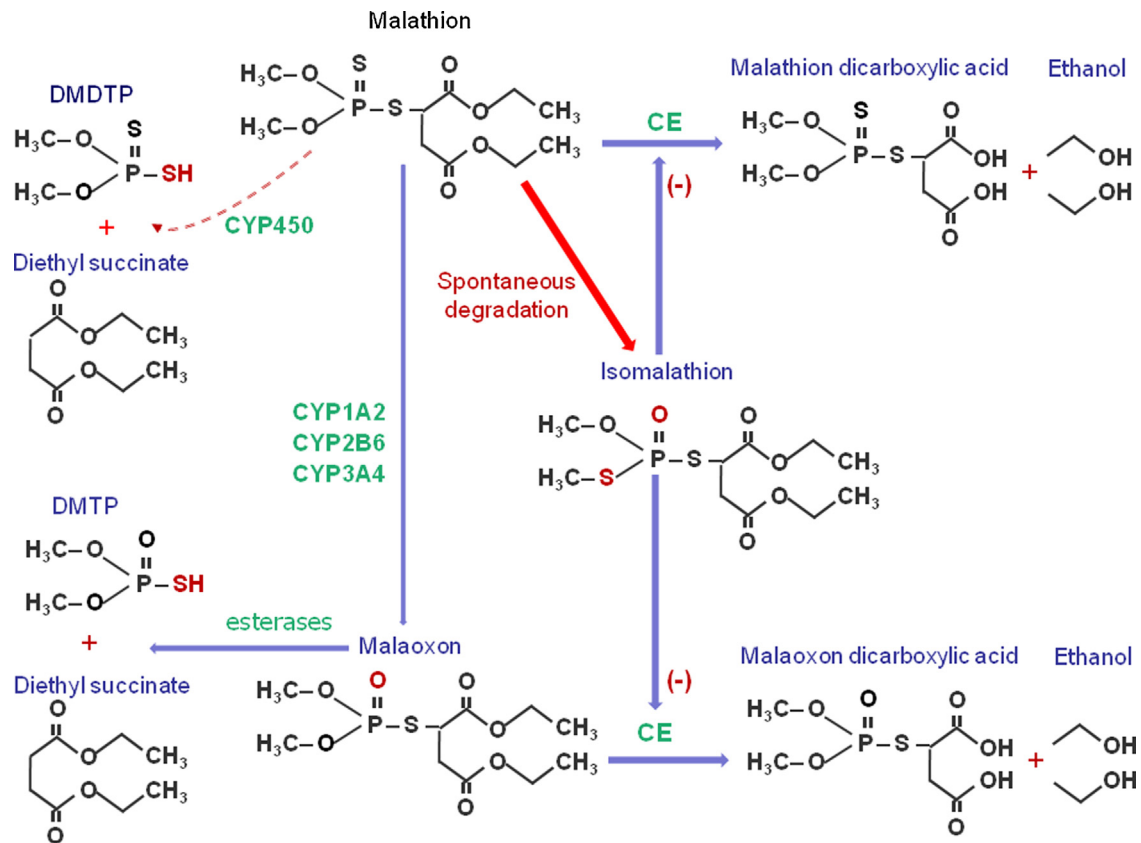


Fig. 2. The OP insecticide malathion can be spontaneously degraded to a highly toxic mixture of malathion and isomalathion after protracted storage. Isomalathion acted as a potentiator of the malathion toxicity by inhibiting the carboxylesterase (CE) responsible for the detoxification of the insecticide. CEs are enzymes involved in the rapid degradation of malathion to monoacid and diacid derivatives, a reaction that efficiently competes with the CYP450-catalyzed formation of malaaxon, the biologically active metabolite, which in turn can also be degraded by CEs yielding similar metabolites or by other esterases to render dimethylthiophosphate (DMTP) and a detoxification product. On the other hand, malathion can be degraded to dimethyl dithiophosphate (DMDTP) and a non-toxic phosphoric metabolite, an ester cleavage mediated by CYP450s isoforms.

3.5. Potentiation of OP toxicity by triazine herbicides

As previously mentioned, many OP insecticides are bioactivated by the CYP450 system to an oxon form within the target organism, which further inhibits the activity of AChE, a key enzyme in the breakdown of the neurotransmitter acetylcholine. Recent studies have demonstrated the potentiating effects of triazine herbicides, such as atrazine (the most commonly used triazine herbicide), to the toxicity of a variety of OP insecticides (Trimble and Lydy, 2006). Triazine herbicides may stimulate CYP450 activity thus increasing the rate of bioactivation of OPs to the oxon form. This interaction results in the potentiation of the cholinesterase inhibiting property of OPs (Belden and Lydy, 2001; Miota et al., 2000). In insects, atrazine has been shown to induce not only CYP450 but also general esterase activity, which may increase or decrease the toxicity of insecticides depending upon whether the subsequent metabolite is more or less toxic than the parent compound (Pape-Lindstrom and Lydy, 1997).

3.6. Promotion of organophosphate-induced delayed polyneuropathy

OP-induced delayed polyneuropathy (OPIDP) is a rare toxicity caused by certain OPs compounds whose molecular target is considered to be neuropathy target esterase (NTE), a nervous system enzyme that possesses phospholipase activity for lysophosphatidylcholine. Neuropathic OP compounds cause OPIDP when

>70% of NTE inhibition is achieved and the residue left attached to the NTE undergoes the "aging" reaction that consist in the loss of an alkyl group bound to the enzyme. However there exist other compounds (such as other OPs, sulphonyl halides, carbamates, thiocarbamates and phosphinates) which inhibit NTE without undergoing the "aging" reaction and thus protect from OPIDP when given before a neuropathic OP. Interestingly, when these compounds are given after neuropathic OPs an aggravation of the clinical expression of OPIDP is observed, which is referred to as promotion of OPIDP, a sort of potentiating effect that seems to be caused by esterase inhibitors (Gambalunga et al., 2010; Jokanovic et al., 2002; Lotti, 2002). The above mentioned compounds can also promote a peripheral neuropathy even in the context of a pre-existing subclinical neuropathy. This refers to the aggravation of traumatic and toxic morphological neuropathies, which seem to be caused by esterase inhibitors and NTE as a modulator (Holisaz et al., 2007).

3.7. Endocrine disruptor chemicals

Some pesticides have the potential of modulating the neuroendocrine system in humans by producing agonism or antagonism of estrogen receptor (ER) binding (Fig. 1). Pesticides such as vinclozolin, *p,p'*-DDE, fenitrothion and procymidone are androgen receptor (AR) antagonists, whereas others such as *p,p'*-DDT and methoxychlor are ER agonists (Kojima et al., 2003). For certain endocrine disruptors, dose-additivity may occur even if they do not

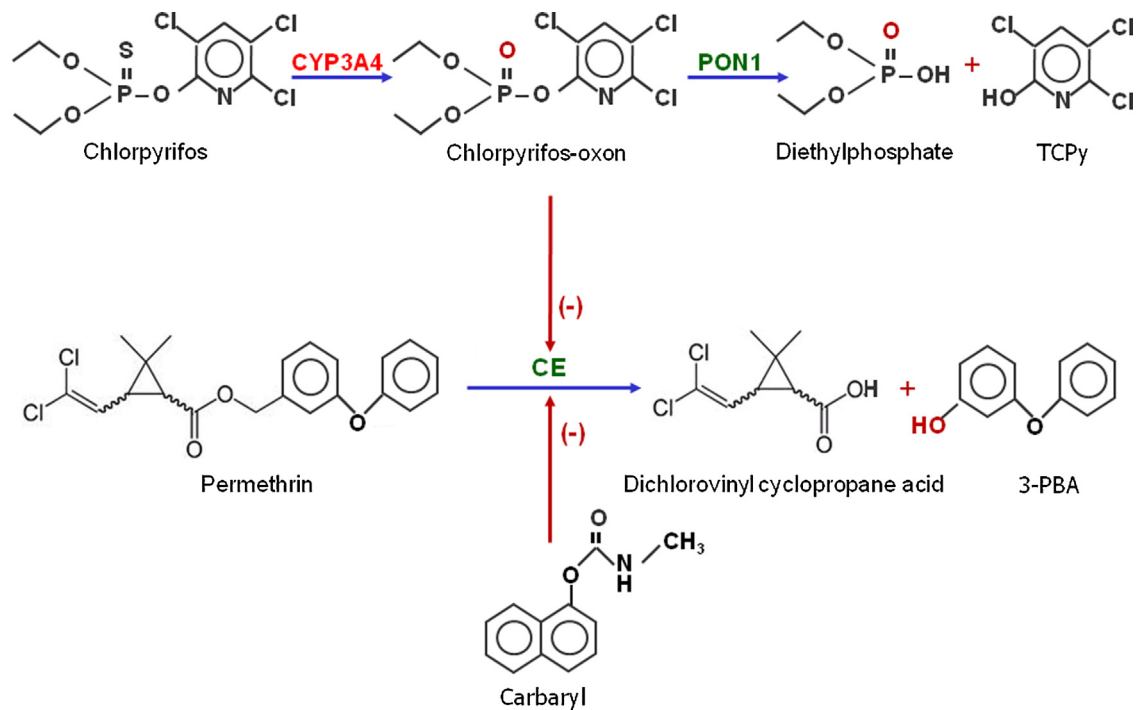


Fig. 3. The pyrethroid insecticide permethrin is hydrolyzed by carboxylesterases (CE) yielding inactive metabolites, one of them is phenoxybenzyl alcohol that is further metabolized to phenoxybenzoic acid (PBA), a common metabolite useful for biomonitoring pyrethroids exposure. Permethrin metabolite can be inhibited by the insecticides chlorpyrifos and carbaryl, although the former compound needs to be activated to chlorpyrifos oxon, which is a strong inhibitor of CEs. The oxon form is then detoxified to diethylphosphate and trichloropyridinol (TCPy) by paraoxonase-1 (PON1). Thus, both chlorpyrifos oxon and carbaryl effectively inhibit the initial hydrolysis of permethrin thus potentiating its insecticidal activity.

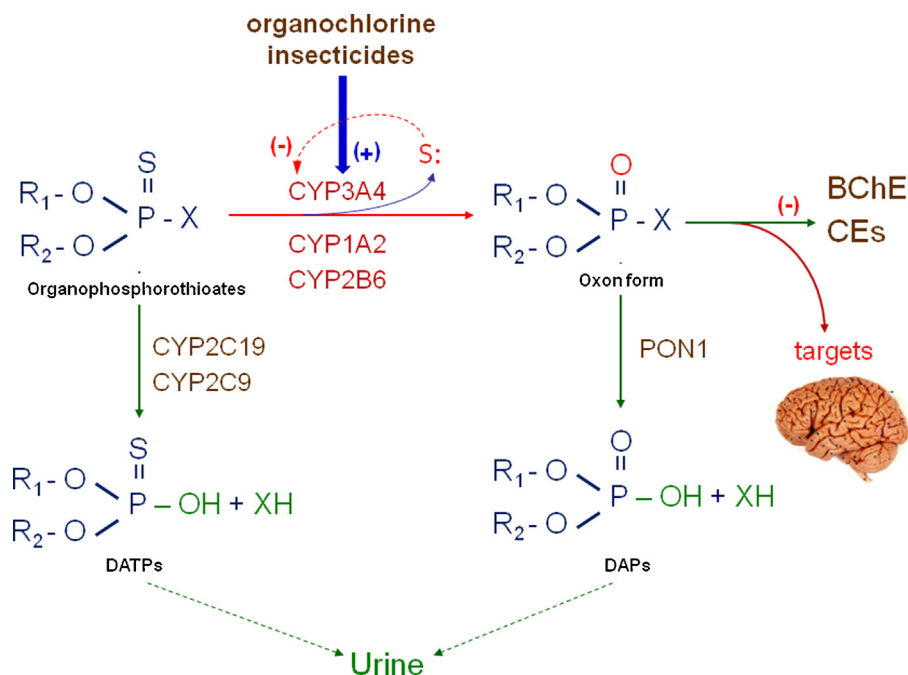


Fig. 4. Specific CYP450s may both bioactivate the parent organophosphorothioates (OPTs) to highly toxic oxon forms and concurrently detoxify them by dearylation to form dialkylthiophosphates (DATPs), inactive metabolites. CYP2C9 or CYP2C19 are the isoforms responsible for the dearylation reaction. Bioactivation of OPTs is also carried out by CYP450 systems. At low concentrations, oxon derivatives formation is catalyzed by CYP1A2 and CYP2B6, whereas CYP3A4 is relevant only at high concentrations. The oxon form is a strong esterase inhibitor that can be effectively scavenged by pseudocholinesterase (BChE) and carboxylesterases (CEs), and the remaining free (non-detoxified) amount may reach target organs where inhibit acetylcholinesterase. The oxon can be alternatively hydrolyzed by paraoxonase-1 (PON1) yielding dialkylphosphates (DAPs), inactive metabolites that are excreted through the urine. OPT insecticides feature a thio-phosphate moiety that is subjected to oxidative desulfuration in which the CYP450s remove the sulfur atom attached to the phosphorus and insert atomic oxygen. This is a suicide reaction for the P450 carrying out the reaction. Given that organochlorine pesticides, such as endosulfan- α and DDT, are CYP3A4 inducers, prior exposure to these compounds can potentiate OPs toxicity, particularly when these compounds occur at high concentrations.

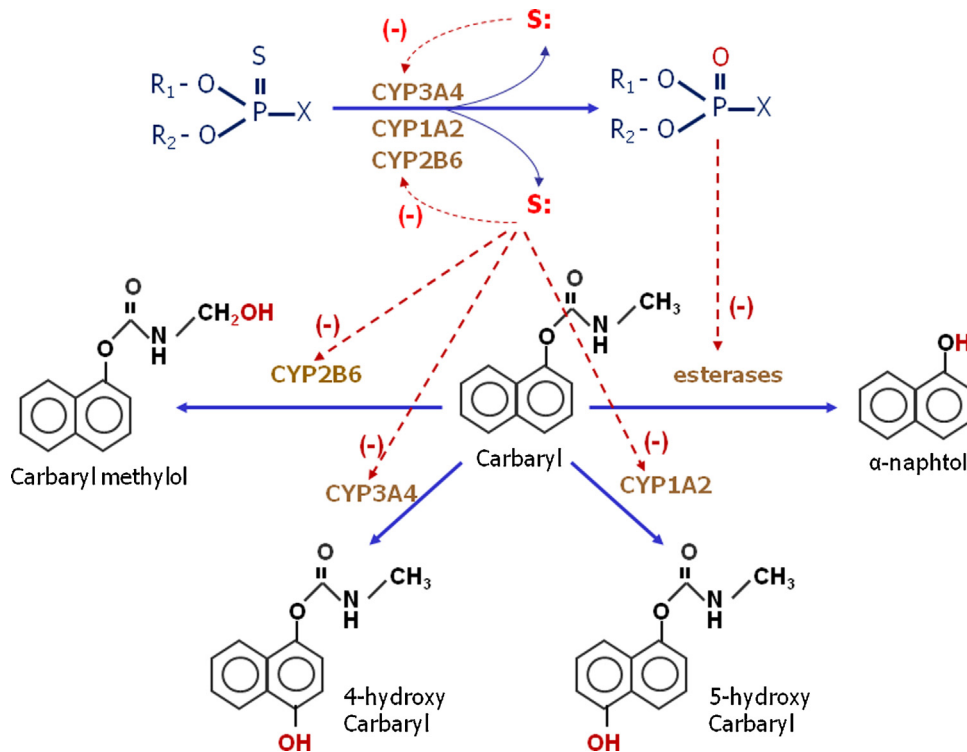


Fig. 5. OPs insecticides inhibit the metabolism of carbaryl, a widely used anticholinesterase carbamate insecticide. Carbaryl undergoes oxidative metabolism forming either ring or side-chain hydroxylated metabolites (4-hydroxycarbaryl and 5-hydroxycarbaryl or carbaryl methylol, respectively). The different CYP450 isoforms involved in these hydroxylations that can be previously inhibited by the reactive sulfur released from the oxidative desulfuration of OPs, which is mediated by the same CYP450s. Another detoxification pathway for carbaryl is the hydrolysis catalyzed by non-specific esterases such as carboxylesterases (CEs) resulting in the formation of α -naphthol and methyl carbamic acid. However, this reaction can also be effectively inhibited by the oxon form of OPs generated during the oxidation of the parent compounds.

share a common molecular target (Kortenkamp, 2007). Thus, combinations of compounds with different mechanisms of toxic action (i.e., steroid biosynthesis or antagonism of the AR) exhibit dose additivity (Boobis et al., 2008). Organochlorine pesticides may act as endocrine disruptors through more than one mechanism, including their agonistic or antagonistic effects on different receptors. They have the potential for additive rather than synergistic effects, suggesting that a toxic equivalency factor approach is appropriate (Kelce and Wilson, 2001; Li et al., 2008). Despite the assumption of additivity has been applied to environmental endocrine active substances, more than additive and less than additive effects have been observed (Kelce and Wilson, 2001).

3.8. Cumulative toxicity of OP and organochlorines resulting in estrogenic effects

Metabolic interactions of pesticides are not limited to other pesticides and have also been reported with endogenous substrates, such as testosterone and estradiol (Hodgson and Rose, 2007). OPs are potent irreversible inhibitors of the human microsomal metabolism of testosterone, by interfering with CYP3A4, and estradiol, by inhibiting CYP3A4 and CYP1A2 function (Usmani et al., 2006) (Fig. 1). This metabolic interference may partially account for the endocrine disruption effect attributed to OPs. Inhibition of steroid hormone metabolism by OPs compounds containing the P=S moiety is assumed to be due to the generation of reactive sulfur that binds irreversibly to the CYP450s (Hodgson and Rose, 2007). Concurrent exposure of OP with organochlorine pesticides, which are known ER agonists, may produce cumulative toxicity due to a dose-addition approach. These findings suggest that low concentrations of OPs insecticides have the potential to inhibit

enzymes important in normal sexual development (Hodgson, 2011).

3.9. Synergism between PYRs and carbamate compounds

The synergism between insecticide mixtures may not only occur by enzymatic competition between components but also through complex physiological processes involving target sites, such as sodium channels, AChE, muscarinic receptors, etc. (Corbel et al., 2006). The following sequence of events may occur after the application of propoxur in combination with permethrin. Permethrin increases the release of acetylcholine within the synaptic cleft by affecting voltage-dependent sodium channels. At the same time, the propoxur-induced inhibition of AChE increases acetylcholine concentration and the excess of non-hydrolyzed acetylcholine activates presynaptic muscarinic receptors involved in the negative feedback mechanism. This leads to a decrease in the subsequent release of acetylcholine resulting in depression of the compound excitatory postsynaptic potential.

Gulf War Syndrome has been reported to be related to the synergistic health effects of the combination of the insect repellent *N,N*-diethyl-*m*-toluamide (DEET) and permethrin with pyridostigmine bromide, a drug taken prophylactically to counteract toxic gas warfare agents (Abou-Donia et al., 1996). The concurrent administration of any two compounds of these three chemicals resulted in greater neurotoxicity than that resulting from treatment with any individual compound. Neurotoxicity was further enhanced by the concurrent administration of all three compounds. However, when the chemicals were administered alone, even at doses three times the level Gulf War veterans received, no effects were observed. Animals exposed to the three chemicals in combination experienced neurological deficits similar to the

symptoms of the soldiers (Abou-Donia et al., 1996). Brain damage has been observed after prolonged exposure to the combination of the three chemicals under stress conditions, very likely because of stress-induced disruption of the blood–brain barrier and further neuronal death (Abdel-Rahman et al., 2004).

3.10. Antagonism between triazine herbicides and prochloraz

The commonly used 2-chloro-s-triazine herbicides are able to induce aromatase activity (CYP19), the rate-limiting enzyme of the conversion of androgens to estrogens that plays a key role in the biosynthesis of steroid hormones. Higher than normal CYP19 activity may account for some of the reported hormonal disrupting and tumor promoting properties of these herbicides (Sanderson et al., 2000).

The fungicide prochloraz elicits multiple mechanisms of action as it antagonizes both ER and AR, agonizes the Ah receptor and disrupts steroidogenesis by inhibiting aromatase activity (Fig. 1). The molecular basis of this inhibition is the presence of an imidazole moiety that interacts strongly with the iron atom of CYP19. The binding is fairly unspecific and thus imidazole fungicides also inhibit the activities of a broad spectrum of other CYP450-dependent enzymes (Vinggaard et al., 2005). Therefore, concurrent exposure to these two pesticides (triazines and prochloraz) might counteract their respective effects on aromatase.

4. Summary and implications for human health

Human exposure to low-dose pesticide mixtures may occur from environmental or nutritional sources (foods and drinking water) and may have a long-lasting and negative health impact in the long-term, some being connected with the increase of chronic degenerative diseases, neurodevelopmental deficits and cancer in humans from western countries. The interaction between several different types of pesticides may result in multiple responses, depending on differences in the chemical properties and modes of toxic action of each compound. For a better understanding of the toxicity of pesticide mixtures it is necessary to have enough knowledge on the chemical reactivity, the toxicokinetics, metabolic pathways and the mechanisms of action of each compound. In addition, epidemiological studies, physiologically based-toxicokinetic and toxicodynamic models, statistical modeling and inference, and computational (*in silico*) toxicology approaches can be also used for the evaluation of toxicological interactions. Predicting risk from exposure to pesticide mixtures is complex, as these compounds can interact in terms of both toxicokinetics and toxicodynamics, causing greater or lower effects than when found alone or than simply the sum of the individual effect of each pesticide.

For some pesticide mixtures the health effects may be more or less than what would be expected from simply adding the effects of the individual components, raising concerns about their potential impact on the health. If each component has different modes of action and is below any threshold of effect, the combined exposure will not have any toxicological effect. When pesticides have the same mechanism of action, their combined effect is the sum of the potency-corrected doses of each individual compound (i.e., a summation of the inhibitory effects of individual compounds on AChE activity in case of concurrent multiple OP exposure or when OPs and NMC are found together). However, not all mixtures of pesticides with similar chemical structures produce additive effects; thus, if they act on multiple sites they can elicit different toxic effects, with some mixtures having the potential of producing greater toxicity than would be predicted based on the potencies of the individual compounds (synergism). Synergistic effects could also occur when exposures are above dose thresholds.

Metabolic interactions of pesticides involve inhibition or induction of detoxifying enzymes and can occur not only between two or more of these compounds but also between pesticides and endogenous substrates. Potentiation may occur when one of the components of the mixture changes the toxicokinetics of the pesticide as a result of its increased activation or decreased detoxification, leading to an enhanced or reduced toxicity, respectively.

Antagonism may occur when two pesticides interfere with each other's effect or when one of them stimulate the metabolism of the other, resulting in a reduction in the effect predicted for the individual compounds.

Assessment of pesticide exposure to humans is generally based on measurement of non-specific metabolites in urine and hair samples, which enables the assessment of the type of exposure and to associate this exposure to relevant health issues (Kavvalakis and Tsatsakis, 2012; Tsatsakis et al., 2008). According to biomonitoring studies, more than half of adults and children have detectable concentrations of pesticide residues in their bodies (Zeliger, 2011). Pesticide interactions may occur when doses of exposure are above the NOAEL of each compound. Thus, either toxicokinetic or toxicodynamic interactions may occur, resulting in potentiation, synergism or antagonism. Nevertheless, as pesticide residues are found at concentrations far below their respective NOAEL, they are expected to cause additive effects.

In the European Union and the U.S., legislation has been laid down regulating the presence of pesticide residues in food products by setting maximum residue levels (MRL) of individual pesticides. As long as the individual residues do not exceed the MRLs, the presence of multiple residues in one sample as such is not a reason to be considered as not compliant with the MRL legislation (EFSA, 2011). Although international regulations do not consider harmful health effects of pesticides interactions at their low (<MRL) concentrations, in the near future the combined toxicological effects of low dose pesticide mixtures will have to find regulatory forms for a more comprehensive protection of human health. A number of toxicological challenges will need to be overcome before this vision can be fully implemented.

Conflict of interest

Authors declare that they do not have any conflict of interest.

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