Enzymatic detoxification of organophosphorus pesticides and related toxicants

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Millions of cases of pesticide intoxication occur yearly and represent a public health problem. In addition, pesticide poisoning is the preferred suicidal method in rural areas. The use of enzymes for the treatment of intoxication due to organophosphorus pesticides was proposed decades ago. Several enzymes are able to transform organophosphorus compounds such as pesticides and nerve agents. Some specific enzymatic treatments have been proposed, including direct enzyme injection, liposome and erythrocytes carriers, PEGylated preparations and extracorporeal enzymatic treatments. Nevertheless, no enzymatic treatments are currently available. In this work, the use of enzymes for treating of organophosphorus pesticide intoxication is critically reviewed and the remaining challenges are discussed.

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Introduction

Organophosphorus compounds (OPCs) are a diverse group of chemicals used in both domestic and industrial applications. Examples of organophosphates include insecticides (malathion, parathion), nerve gases (sarin), ophthalmic agents, anthelmintics, herbicides and other industrial chemicals. The first OPC was synthesized in the early 1800s through the reaction of alcohol with phosphoric acid.1) In 1872, the use of OPCs as insecticides was first explored2) In 1936, the German military developed chemical warfare agents (tabun, sarin, soman). A fourth agent, VX, was synthesized in England a decade later.3)

Since their discovery, the use of pesticides has benefited humanity by protecting and increasing agricultural production, not only of food but also raw materials, and protecting against infectious diseases such as malaria. Pesticides have been used to eliminate domestic and gardens pests and also as protective agents in many environments such as workplaces and schools. They are applied on the ground, in the air, and in divers products.4) During World War II, in 1941, organophosphates were reintroduced worldwide for pesticide purposes, as originally intended. The extensive use of pesticides has affected human health. There have been several massive cases of intoxication by organophosphorus compounds, such as the Jamaican ginger palsy incident in 1930, in which more than 30,000 people were affected.5) In 1995, a terrorist attack on the Tokyo subway using sarin resulted in more than 12 deaths and injuries to more than five thousand people.6) Nerve agents have also been used in battle, notably in Iraq in the 1980s.7) Most recently, sarin was used during Syrian Civil War in 2013, killing more than 635 people.8)

There are no reliable estimates as to how many people per year suffer pesticide-related health effects. Three decades ago, the World Health Organization9) estimated that three million cases of severe pesticide poisoning occurs each year, resulting in 220,000 deaths, occur each year. It is estimated that pesticide self-poisoning accounts for about one-third of the world’s suicides, conservatively estimating a plausible range of 234,000 to 326,000 deaths from pesticide self-poisoning each year worldwide.10)

Early efforts to reduce the environmental and health impacts of pesticides have been made. In the 1930s, Lange and Kruger synthesized phospshorofluoridates and examined their toxicity, which, in the 40s, inspired Schrader to synthesize safer OPs, resulting in the development of parathion. Parathion was used to control pests after WWII but it was banned due to its high toxicity. Since then, much safer ethoxy OPs have been developed and employed to control a broad range of pest insect species.

1. Toxic Effects

The primary mechanism of action of organophosphate pesticides (OPPs) is the inhibition of acetylcholinesterase (AChE). AChE is an enzyme that hydrolyzes the neurotransmitter acetylcholine into choline and acetic acid. AChE is found in the cen-
bral and peripheral nervous system, neuromuscular junctions, and red blood cells. AChE is involved in the termination of impulse transmission by the rapid hydrolysis of the neurotransmitter acetylcholine in numerous cholinergic pathways in the central and peripheral nervous systems. Enzyme inactivation, induced by organophosphorus pesticides, leads to acetylcholine accumulation, hyperstimulation of nicotinic and muscarinic receptors, and disrupted neurotransmission. Organophosphorus compounds inactivate AChE by phosphorylating the serine hydroxyl group located at the active site of the enzyme, establishing a covalent bond with AChE. Once inactivated, acetylcholine accumulates throughout the nervous system, resulting in the overstimulation of muscarinic and nicotinic receptors. Thus, clinical effects are manifested via the activation of the autonomic and central nervous systems and at nicotinic receptors on skeletal muscles.

Signs and symptoms of organophosphate poisoning are miosis; salivation; lacrimation; increased gastrointestinal motility, with fecal and urinary incontinence; nausea and vomiting; diaphoresis; anxiety; confusion; muscle fasciculation, cramping, and weakness; seizures that leads to severe respiratory distress; diaphragmatic failure. There have been reported late complications from acute or chronic exposure. Most cases presented with fatigue, paresthesia, and headache, as well as depression and chronic pain.

2. Current Pretreatment

Medical treatment for pesticide intoxications is difficult, with fatalities in more than 15% of cases. The available therapies and factors that should be considered have been reviewed. One century after the first use of organophosphorus compounds, there is still not a general protocol for treatment, with atropine, oximes, and diazepam being the most common.

In situations where the risk of exposure to OPPs has been established, pretreatments should be implemented for protection. Current prophylactic treatments consist of using AChE reversible inhibitors to hide the active site of AChE from the OPP. The most widely investigated is the carbamate pyridostigmine (the only FDA-approved substance for such pretreatment), a pseudoreversible inhibitor making a labile bond with the catalytic serine of AChE. This treatment maintains a sufficient amount of active AChE to preserve cholinergic transmission before intoxication and remain hidden from the OP during exposure. In this treatment, the dosage is extremely challenging. In addition, pyridostigmine does not readily cross the blood–brain barrier, leaving AChE of the central nervous system unprotected. Other prophylactic substance have been tested, such as physostigmine, tacrine, ranitidine and K-27. The most promising compound in this matter is the experimental oxime K-27, which is significantly more efficient than other tested cholinesterase inhibitors.

In the search of an AChE reversible inhibitor of the central nervous system, Huperzine A, a natural alkaloid, appears to have the greatest potential. However, this alkaloid is toxic at doses required for protection. A combination of isomers with lower toxicity could improve survival and reduce behavioral abnormalities.

3. Current Treatment

There are three FDA-approved chemotherapeutical agents in the therapy for OPP poisoning: atropine, oximes (pralidoxime or obidoxime), and benzodiazepines (e.g., diazepam). Initial management must focus on the adequate use of atropine. Atropine is a parasympatholytic and competitive antagonist of acetylcholine on muscarinic receptors. Although it does not act as an antidote, it is able to neutralize fasciculation, weakness, tachycardia, and respiratory arrest and alleviate bradycardia. Studies have shown that atropine (10 mg/Kg) before paraoxon could prevent a 2.08 mg/Kg (1.51–2.84 mg/Kg) LD50. Also, benactyzine has proven to have better effects on the central nervous system than atropine, furthermore, inhibiting sweating or impairing accommodation much less, making it more suitable for use in hostile environments.

When intoxication symptoms start, current recommendations for oxime treatment call for an initial dose of pralidoxime followed by an infusion. Exact doses have not been established. The nature and type of the OPP are important in determining the response to oxime therapy. Most compounds can be classified as either a dimethyl phosphoryl or diethyl phosphoryl compound. Human poisoning by OPPs bearing two methoxy groups (malathion, paraoxon-methyl, dimethoate) is considered to be rather resistant to oxime therapy. On the other hand, the potential therapeutic window for the administration of an AChE antidote is determined by the “aging process,” which is the inactive state of AChE (when it is attached to the OPP). If this occurs, there is a non-enzymatic removal of one alkyl side chain of the phosphoryl moiety, leaving a hydroxyl group in its place. Once the AChE is “aged,” regeneration is not possible. On the other hand, in mega-dose intoxication, even the optimal plasma concentration of oximes may not be able to cope with the fast reinhibition of reactivated AChE in the first few days following intoxication. The half-lives of aging dimethyl and diethyl phosphorylated AChE, as determined in isolated human red cells in vitro, are 3.7 and 33 hr, respectively. Therefore, the potential therapeutic window for the administration of the antidote, is recommended to be four times the half-life. Thus, the antidote works out to a maximum of 15 hr for the dimethyl phosphoryl group and 132 hr in the case of the diethyl phosphoryl group. For dimethylated compounds, one day after intoxication with dimethyl phosphoryl insecticides, virtually all AChE will be in the aged form so that oxime therapy will be useless by that time. Studies have shown better results with low doses of pralidoxime (< 2 g in a slow intravenous infusion). The World Health Organization recommends a 30 mg/kg dose of pralidoxime followed by infusions of >8 mg/hr. Other studies have shown that the administration of pralidoxime (90 mg/Kg) before paraoxon could prevent 4.50 mg/Kg (3.28–6.16 mg/Kg) LD50. Importantly, oximes are associated with multiple side
4. Enzyme-based Treatments for Organophosphorus Pesticide Intoxication

An alternative approach to AChE inhibitors is biocatalytic molecules that inactivate OPPs in the bloodstream or metabolize into less toxic metabolites before they can reach AChE at the physiological sites. The use of enzymes for treatment of OPP intoxication was first proposed by Cohen and Warringa.24) The enzymes for potential treatments (Table 1) can be divided according to their catalytic activity for OPP transformation in low, medium, and high activity. However, it is hard to compare the quantitative data on the transformation kinetics in the abundant data found in literature due to the heterogeneity of the reported values. Only a few papers report the enzymatic $k_{cat}$ and $K_M$ constants determined in rigorous procedures with purified enzyme preparations. Most of them report transformation rates in terms of pesticide molecules transformed by crude extracts or partially purified preparations referred to as total protein content. In some cases, microsomal preparations have been studied. On the other hand, the diversity of OPP chemical structures, enzyme affinities, and rates of transformations make it harder to compare the overall performance. Nevertheless, in Table 2 some $k_{cat}$ data are shown.

4.1. Low-activity enzymes

Numerous enzymes and proteins participate in natural defenses against OPPs and, in low-exposure doses, can be sufficiently protective. Low-activity enzymes, called "stoichiometric bioscavengers," are specific molecules that irreversibly bind to

| Pesticide   | Phosphotriesterase or organophosphorus hydrolase | Cytochromes P450 | Paraoxonase | Butyrylcholinesterase | Organophosphorus acid anhydrase | Diisopropylfluorophosphatase |
|-------------|--------------------------------------------------|------------------|-------------|------------------------|--------------------------------|-----------------------------|
| Cadusafos   | [35]                                             |                  |             |                        |                                |                             |
| Chlorpyrifos | [36–38]                                          | [39–44]          | [45]        | [46]                   | [47]                           |                             |
| Chlorpyrifosoxon | [48]                                              | [43]            | [45, 48–50] |                        |                                |                             |
| Coumaphos   | [35, 51, 52]                                     |                  |             |                        |                                |                             |
| Deltamethrin | [53]                                             |                  |             |                        |                                |                             |
| Demeton-S   | [54]                                             |                  |             |                        |                                |                             |
| Diazinon    | [35, 52]                                         | [55, 56–58]      | [50]        |                        |                                |                             |
| Diazoxon    | [48]                                             |                  |             |                        |                                |                             |
| Dichlorvos  |                                                  |                  |             |                        |                                |                             |
| Diisopropylfluorophosphate | [20]                     | [60]             | [61, 62]   | [59]                   | [63–65]                        | [66]                        |
| Dimethoate  |                                                  | [67]             |             |                        | [59]                           |                             |
| Dyfonate    | [35]                                             |                  |             |                        |                                |                             |
| Ethoprophos | [35]                                             |                  |             |                        |                                |                             |
| Fenamiphos  | [35]                                             |                  |             |                        |                                |                             |
| Fenitrothion | [35, 47]                                         |                  |             |                        |                                |                             |
| Malation    | [54]                                             | [65, 70]         |             |                        |                                |                             |
| Monocrotophos |                                                  | [71]            |             |                        | [59]                           |                             |
| Parathion   | [20, 35, 52, 72]                                 | [41, 44, 73]     |             |                        | [59]                           |                             |
| Paroxon     | [20, 35, 38, 47, 52, 61, 74–92]                  | [61, 73]         | [49, 50, 62, 65, 79, 93–101] | [69, 102, 103] | [63]                           | [74]                        |

Numbers in the brackets correspond to reference numbers.
OPPs in a mole-to-mole ratio. Human butyrylcholinesterase is the most advanced stoichiometric bioscavenger. Butyrylcholinesterase, also known as pseudocholinesterase, which is found in the liver and plasma, plays a role in protecting against low-dose exposure to OPPs, but ineffective for high-dose exposition. Protection in humans can be enhanced by the administration of exogenous butyrylcholinesterase. However, the administration of large amounts of enzymes displaying promiscuous activities might perturb certain metabolic processes or enhance immunogenic reaction. Therefore, using PEGylation or the inclusion of recombinant human and nonhuman enzymes and bacterial enzymes in nano-containers should prevent immune responses. A dose of 200 mg of human butyrylcholinesterase can protect a human against 2 times the median lethal dose of soman (2×LD50). Large doses can confer protection against up to 5.5×LD50 of soman or 8×LD50 of the warfare nerve agent VX. The required doses in humans are extremely expensive, so, actually, research is focused on the large-scale production of human butyrylcholinesterase. A recombinant human butyrylcholinesterase has been produced, but it has a much shorter half-life than does native human butyrylcholinesterase. Thus, efforts are also being made to expand its half-life by the addition of peptides, fusion to albumin, and PEGylation. The main goal of PEG-protein bioconjugates is to mitigate immune responses or adverse immunologically related responses associated with therapeutic protein products that affect their safety and efficacy. The PEGylation of biologically active molecules also results in the increased stability of proteins, increased circulation times, and reduced nonspecific binding to nontargeted or nondiseased areas. Thus, PEG conjugation is a rapidly evolving strategy for using therapeutic proteins and for drug delivery methods. In addition, the barrier properties of PEG can also protect therapeutic proteins from digestive proteolytic enzymes in the digestive system. PEGylated goat milk with recombinant human butyrylcholinesterase (72 mg/Kg) improved the half-life and allowed 100% survival of guinea pigs exposed to 2.5×LD50 with minimal signs of poisoning despite a 2 hr post-intoxication intramuscular injection dose. The dose of low-activity enzymes could be reduced by using a reactivator. The reactivation of human butyrylcholinesterase by the presence of oximes can slow, but not prevent, enzyme aging. Other approaches include butyrylcholinesterase modification by adding selective inhibitors, such as bambuterol or flavonoids.  

### 4.2. Medium-activity enzymes

Medium-activity enzymes are OPP-degrading catalysts with a relative high turnover, so the administration of a small dose of enzyme is thought to provide better protection than large doses of costly low-activity enzymes. Several human enzymes have been considered, including plasma paraoxonase-1 (PON-1), erythrocyte and liver prolidases, and regucalcin, a human liver senescence marker (SMP-30). Human paraoxonase-1 (hPON-1) is a calcium-dependent enzyme secreted by the liver and carried through the bloodstream that is associated with high-density lipoproteins. Mutations in the catalytic site have been made to hPON-1 to enhance its efficiency against OPPs. Significant activity improvements have been obtained with the H115W mutant by reducing a bulky group (Y71A) that enhanced 9-fold the hydrolysis of paraoxon, and by introducing a tryptophan (F347W) that reduces the Km by half against sarin. However, the catalytic efficiency is still limited by the lack of a proper hydrophobic environment. Additional chemical modifications, such as PEGylation, are required for immune acceptance and half-life modulation.

Another enzyme of interest is the regucalcin, also called human liver senescence marker (SMP-30), a promiscuous metal-containing (Zn2+ and Mg2+) lactonase enzyme that is structurally related to hPON-1 and diisopropyl-fluorophosphatase. Although the catalytic mechanism for OPP hydrolysis by regucalcin is still unknown, interest in studying this enzyme has begun. On the other hand, human prolidase from erythrocytes or the liver has been found to have catalytic properties against OPPs. A new variant of prolidase modified from the previously known thermophilic bacteria Pyrococcus horikoshii showed 8 times higher activity for diisopropyl-phosphorofluoridate transformation when compared to wild-type P. furiosus prolidase.

The monoxygenase family of cytochromes P450 (CYPs) is also able to transform OPPs. The role of CYPs on the metabolism of pesticides in humans has been studied extensively. CYPs in the human liver are generally involved in metabolizing pesticides, and, although a number of different isozymes are involved, CYP2B6 and CYP2C19 seem to be the most important. The polymorphism found in this group of enzymes determines susceptibility to the toxic effects of pesticides. Furthermore, it is well known that pesticides can act as inducers of CYP isozymes in human tissues. As in bacteria and fungi, CYPs are also found in plants and they all are able to transform a variety of pesticides. Recently, a variant of CYP from Bacillus megaterium has been assayed for pesticide transformation and the catalytic constant km for parathion transformation was of 10.9 min−1 with a Km of 59.4 μM.
The chemical nature of the products from the CYP-mediated transformation of several pesticides was determined. The main product from parathion transformation was p-nitrophenol, but a small amount of paraoxon was also found.131) 4.3. High-activity enzymes

Diisopropyl-fluorophosphatase is a Ca-dependent phosphotriesterase (similar to hPON-1) obtained from the squid Loligo vulgaris that has been found to be more effective at hydrolyzing diisopropyl-phosphorofluoridate than hPON-1 or regucalcin.114) Diisopropyl-phosphorofluoridate from murine erythrocytes showed promising advances, nevertheless its therapeutic use still needs further study.32) Chloroperoxidase, a fungal peroxidase from Caldariomyces fumago, was reportedly as able to transform organophosphorus pesticides containing the phosphorothioate group (P=S). However, the oxidized products were identified as oxon (P=O) derivatives where the sulfur atom from the thioate group is replaced by an oxygen atom.126,132) These oxon derivatives are known to be more toxic than the original pesticide.133) On the other hand, Versatile Peroxidase involved in lignin degradation was able to transform several pesticides mostly halogenated compounds, and no transformation of organophosphorus pesticide could be detected.134) Thus, the peroxidase capacity to transform pesticides could not be envisaged for OPP detoxification purposes.

Engineered cholineesterase displays poor catalytic properties, so efforts for a self-regenerable enzyme mutation have been carried out. The substitution of a glycine at position 117 for a histidine (G117H) in human recombinant choline esterase has proved to increase enzymatic activity. This variant is still useless for clinical purposes, as it does not reactivate fast enough, and the aging of the enzyme is not stopped.3,14) The encapsulation of human choline esterase in nanocarriers (polylysine/polyethylene oxide copolymers) has been capable of crossing the blood-brain barrier and remaining active for 72 hr in mice.135) Phosphotriesterases (PTEs), also called organophosphorus hydrolases (OPHs), are the most efficient enzymes thus far, and they could be very stable when PEGylated. PTE has shown an acceptable circulating half-life (a few days) and thermal stability.14) The PTE action is based on the bond cleavage between the P atom and the leaving group of OPP, resulting in less toxic

![Fig. 1. Different mechanisms of the enzymatic transformation of pesticides.](image-url)
and more polar metabolites; therefore, they are not accumulated in fatty tissues, and they are eliminated in urine.\(^{17}\) Initial studies showed that using PTE evenly with high doses of paraoxon (50 mg/Kg) could prevent symptoms and the important inactivation of acetylcholinesterase in the brain.\(^{136}\) A modified PTE expressed in E. coli resulted in 15,000-fold better hydrolytic properties when compared with wild PTE.\(^{137}\) Erythrocytes are the most studied PTE carrier cells. The Zn substitution for Co increased twofold the catalytic activity of PTE.\(^{139}\) The Co-PTE showed a \(k_{\text{cat}}\) of 526 sec\(^{-1}\) in parathion transformation as compared with wild Zn-PTE, with a \(k_{\text{cat}}\) of 254 sec\(^{-1}\). In addition to metal substitution, PEGylation increased the thermostability and stability against metal chelating agents of both metal phosphotriesterase preparations.\(^{138}\)

The use of murine erythrocytes has been proposed, since it has proven useful in other therapies, although successful encapsulation of PTE in erythrocytes was achieved through hypotonic dialysis, and \textit{in vitro} experiments have shown a high enzymatic hydrolysis (\(k_{\text{cat}}=2100\) sec\(^{-1}\)) of paraoxon.\(^{75,139}\) Recent studies showed better forms for erythrocyte PTE encapsulation with their fusion with liposomes and pretreatment with chlorpromazine, although the major problem seems to be the exposure of phosphatidylserine acting as a signal for removal (\textit{in vivo}) from circulation by macrophages in the liver and spleen.\(^{140}\) Structurally stabilized liposomes with PEG-derivatized phosphatidylethanolamine (PEG-PE) have also been studied as PTE carriers and as protection against an immune response. However, their clinical uses are limited due to their rapid degradation, and their use inside carrier cells has been proposed.\(^{20}\) Finally, a chimeric protein combining phosphotriesterase and a cellulose-binding domain was tested for paraoxon hydrolysis.\(^{141}\) The different enzymatic transformations of OPPs are schematized in Fig. 1.

### 5. Alternative Enzymatic Therapies

Alternative enzymatic therapies have been proposed. The direct administration of PTE was studied with exposure to different OPPs in mice.\(^{138}\) As expected, treatment with PTE increased the OPP-hydrolyzing activity in mouse serum by up to sixfold when measured 1 hr after administration, and the half-life of PTE in circulation was approximately 5 hr. PTE-pretreated animals tolerated even a 50-fold higher amount of OPP, as compared to untreated animals. Direct PTE treatment together with the administration of physostigmine, a compound used by various armed forces for pretreatment against nerve agent poisoning, was the most effective antidote against sarin intoxication. The LD\(_{50}\) value for sarin was increased 4.3-fold in mice receiving a combination of phosphotriesterase and physostigmine.\(^{136}\)

The use of free enzyme preparations as antidotes is limited due to their unfavorable physiological dispositions and potential immunological reactions. Enzyme bioconjugates with polyethylene glycol (PEG) have been produced to reduce the immunogenic reaction.\(^{138}\) PEGylation has also been used to enhance systemic stability; avoid enzymatic degradation; opsonize; for non-selective accumulation; and to maintain the solubility and activity of proteins and, in some cases, the enhancement of cellular uptake.\(^{142}\) In addition, the functionalization of PEGylated enzymes with specific ligands could enhance cell internalization, as recently demonstrated by PEGylated cytochrome P450 functionalized with folic acid.\(^{136}\)

The immunological response also can be overcome by encapsulating the enzyme within a protective nanoparticle, such as a liposome. PTE was encapsulated within sterically stabilized liposomes.\(^{20}\) The increase of paraoxon LD\(_{50}\) was 140 times, as when compared with that of untreated animals. Moreover, when stabilized liposomes containing PTE were administered together with atropine and pralidoxime, the LD\(_{50}\) increased 1000 times. Another alternative for protecting enzymatic treatment against immunological responses is PTE encapsulation in erythrocytes. Recombinant PTE annealed murine erythrocytes by hypotonic dialysis with subsequent rescaling and annealing and was employed as an enzyme carrier to antagonize the toxic effects of OPPs.\(^{139}\) Here again, mice treated with PTE erythrocytes and atropine and pralidoxime showed 1000 times higher paraoxon LD\(_{50}\).

Extracorporeal enzymatic treatment for OPP poisoning is another alternative. The first successful extracorporeal detoxification reported was after the Tokyo sarin attack in 1995, when a patient who showed no improvement after six hours of standard medical therapy underwent hemofiltration for 4 hr, followed by hemoperfusion with Hemosorba CH-350.\(^{59}\) However, this approach is questionable, as OPPs are very lipophilic, so their presence in the blood is minimal, serving only in the first few hours after poisoning; also equipment and skilled medical staff are needed, which, to have an international impact, is too costly, especially in developing countries. Recombinant PTE was immobilized onto a hollow-fiber reactor designed for extracorporeal blood circulation for post-exposure OPP detoxification.\(^{40}\)

### Conclusions

Enzymatic detoxification, doubtless, is an attractive alternative for treating OPP poisoning. However, further work is needed to engineer safer carriers for stabilizing the protein and for making the preparation stealthy for the immunological system. Nanotechnology is making possible great progress in biomedical fields. The design and production of nanoreactors based on a combination of the catalytic properties of enzymes and the unique characteristics on nanosized materials are, certainly, an opportunity to solve different challenges in biomedical applications. Recently, the use of nanoparticles for transporting therapeutic enzymes has been reviewed.\(^{143–145}\) These advancements, together with the improvement of catalytic activity of enzymes by molecular tools, seem to be a potential solution to contending with the large number of lethal OPP intoxications.

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