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Enzymes and bioscavengers for prophylaxis and treatment of organophosphate poisoning

Steven B. Bird[1], Andrew Dawson[2], David Ollis[3]

[1]University of Massachusetts Medical Center, Worcester, Massachusetts, USA, [2]South Asian Clinical Toxicology Research Collaboration (SACTRC), Faculty of Medicine, University of Peradeniya, Peradeniya, Sri Lanka and School of Population and Health, University of Newcastle, Newcastle, New South Wales, Australia, [3]Australia National University, Canberra, Australia

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1. ABSTRACT

Organophosphorus (OP) pesticide poisoning causes significant morbidity and mortality, particularly in the developing world, with upwards of 3 million people poisoned each year. Although OP poisoning is not common in developed countries, recently greater attention has been given to these chemicals because of their similarity to chemical warfare agents. Despite the agricultural use of OP pesticides for roughly 60 years, no new therapies have been developed since the 1960s. A promising field of novel antidotes for OP poisoning, OP hydrolases, has recently garnered increased support. These bacterial enzymes have demonstrated tremendous prophylactic and antidotal efficacy against a few different OP classes in animal models. These studies, as well as the limitations and challenges of therapeutic development of these enzymes, are discussed.

2. INTRODUCTION

Organophosphorus (OPs) compounds have been employed as pesticides for nearly 60 years (1). OP insecticide poisoning is a worldwide health problem, with approximately 3 million poisonings and 200,000 deaths annually (2). The brunt of this morbidity is borne by the developing world where OP pesticides are a common means of self-harm and successful suicide (Eddleston, 2000; Eddleston *et al.*, 1998; van der Hoek *et al.*, 1998). Although uncommonly used for self-harm, exposure to OPs in the US appears to be increasing. The Toxic Exposure and Surveillance System (TESS) database maintained by the American Association of Poison Control Centers has recorded increasing OP exposures and deaths over the last 10 years (Table 1).

Chemical nerve agents such as sarin, soma

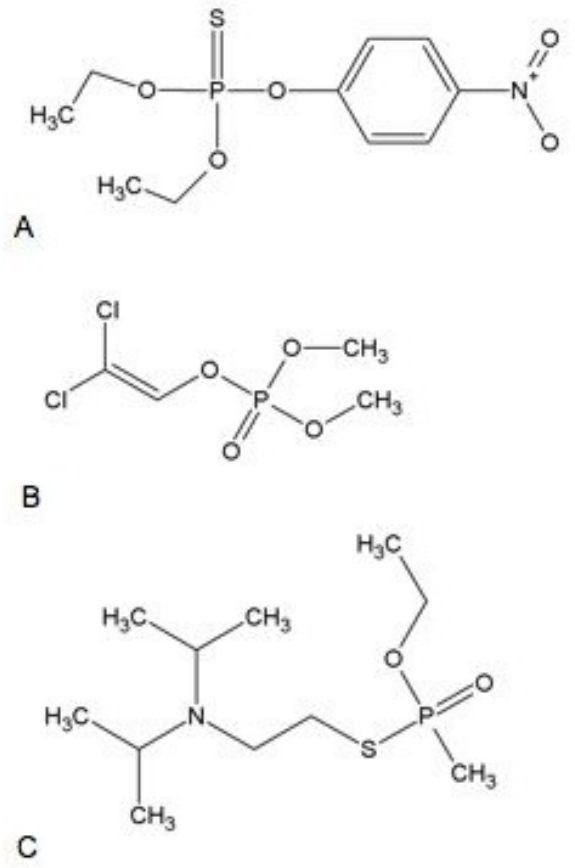

Figure 1. Chemical structures of representative acetylcholinesterase inhibitors. a) parathion; b) dichlorvos; c) VX.

tabun, and VX are also organophosphorus compounds with mechanisms of toxicity identical to the OP pesticides (Figure 1). However, the chemical nerve agents are more toxic than OP insecticides, producing symptoms at much lower doses. Despite calls for the prohibition of these chemical weapons, the Organization for the Prohibition of Chemical Weapons reports that as of April 2007 more than 71,000 metric tons of these agents still exist (www.opcw.org). Additionally, nerve agents are easily synthesized in crude laboratories and are readily accessible for terrorist use, such as occurred in Matsumoto and Tokyo, Japan, in 1994 and 1995, respectively (3). Increasing concerns about national security and terrorist actions has focused attention on both OP pesticides and nerve agents because of their threat for chemical warfare and their widespread availability as agricultural insecticides (4). These fears have led to an increased interest in methods of OP compound detoxification. This article will focus on the use of OP pesticide stoichiometric and catalytic bioscavengers for OP pesticide poisoning only, and not on the chemical warfare agents.

3. WHY NEW ANTIDOTES?

OPs function by inhibiting the action of AChE. The central phosphate has three ester linkages – one of which is a "good leaving group" that can be displaced by action of a catalytic serine with the consequent formation of a covalent bond. When bound to the protein the phosphate mimics the transition state formed during the formation of the covalent intermediate that is formed during the normal catalytic cycle of the enzyme. The formation of the acyl-intermediate and the modified catalytic serine is shown in Figure 2. Once formed, the phosphate in the OP-bound form of AChE can undergo hydrolysis (aging) to form a particularly stable entity that is not responsive to oxime therapy.

Four therapeutic goals exist when treating a patient poisoned with OP compounds: 1) decreasing the amount of OP present in, or on, a patient through the use of skin decontamination, gastric decontamination, or bioscavengers, (5); 2) reversing the effects of muscarinic stimulation via anti-muscarinic agents such as atropine, ipratroprium, or diphenhydramine (6-8); 3) regeneration of the inhibited acetylcholinesterase (AChE) via oxime use (9); and 4) mitigating central nervous system toxicity by use of benzodiazepines (10, 11). While the goals of treatment are straightforward, the management of severe OP poisoning remains challenging. Overall mortality after OP poisoning in the developing world is as high as 40%, and in the most sophisticated Western hospitals mortality approaches 10% (12).

Treatment with an oxime must be administered prior to aging of the AChE. The mode of action of the oxime is shown in Figure 3. However, even when administered before aging of AChE, the clinical benefits of oximes are not very significant. In fact, a few clinical trials (with methodologic flaws) have failed to show a benefit - and a trend towards harm - with oxime therapy in acute OP poisoning (13-16). Therefore, it is essential to develop new therapies for patients with severe OP poisoning.

Large quantities of OP pesticides are often ingested when used as a means of self-harm or suicide. It follows that the large ingestions lead to very high blood OP concentrations. From a cohort of patients with dimethoate poisoning in Sri Lanka, it is evident that patients with blood dimethoate concentrations above 750 μM always die despite maximal conventional therapy (Figure 4). It is likely that by decreasing blood OP concentrations via the degradation or functional inhibition of the OP, current standard care would allow a greater proportion of patients to survive. Towards this end, the most promising and exciting potential new therapy are catalytic and stoichiometric bioscavengers.

4. STRUCTURE AND KINETICS OF OP COMPOUNDS – IMPLICATIONS FOR ANTIDOTE DEVELOPMENT

Most OPs are lipophilic compounds, thereby promoting absorption after dermal exposure, which is the most common form of occupational exposure. OPs are also well absorbed from the pulmonary and gastrointestinal systems. Even though the majority of worldwide *exposures*

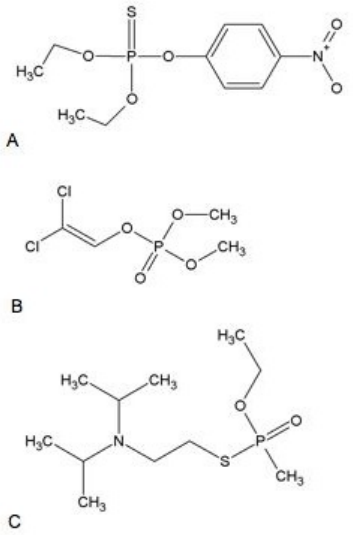

Figure 2. Acetylcholinesterase (AChE) reaction, inactivation and aging. Most OPs have a phosphate with three ester linkages - one of which is a leaving group that is displaced by action of a catalytic serine. When the phosphate is bound to AChE, it mimics the transition state that is formed during normal catalysis of the enzyme. Once formed, the phosphate in the OP-bound form of AChE can undergo hydrolysis (aging) to form a stable entity that is not responsive to 2-PAM.

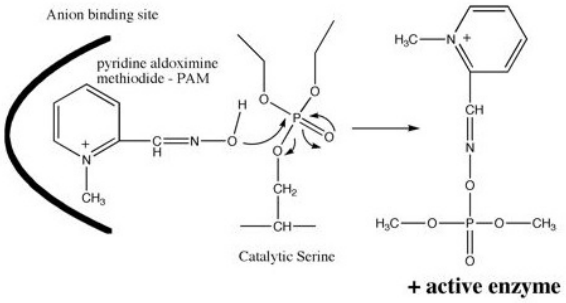

Figure 3. The mode of action of pralidoxime (2-PAM). 2-PAM is able to regenerate inhibited AChE if given before AChE is aged.

to OP pesticides occur through the dermal route, most severe *poisonings* occur after intentional OP ingestions for self-harm.

After adsorption OPs are distributed throughout the body, with the greatest amount in fat-rich tissues. These fat stores provide challenges to the treatment of some severe OP poisoning vicitims, as OP leaches out of the fat and back into circulation, where the OP continues to inhibit AChE. Because of this lipid reservoir of pesticide, it is not uncommon for patients to require pralidoxime therapy for several weeks after ingestion of highly lipophilic pesticides such as diazinon. This prolonged source of OP is another reason why enzymatic degradation of OPs is such a titillating therapeutic possibility. OP compounds generally do not accumulate *in vivo* due to degradation by non-specific reduction, desulpharation, oxidation, and hydrolysis (17).

In addition to their different lipophilicity, OPs may also be divided into groups depending on physiochemical properties such as what atom is doubly bound to the phosphorus (thion vs oxon); whether the two leaving groups are methyl or ethyl; and whether the OP is aromatic or aliphatic (Figure 1). The unique characteristics of the various OPs has recently received more attention as it is becoming increasingly evident that OPs should not be considered a single class of agents, but rather each OP has unique characteristics and properties that may make treatment of poisoned patients more challenging (18).

Oxon-OPs are generally much more potent AChE inhibitors than thion-OP compounds. *In vivo*, thions are oxidized and thereby metabolically activated to the oxon form by enzymes of the cytochrome P450 system and monooxygenases (19). Thus, the speed of onset of poisoning is greater with oxon-OPs than with thion-OPs. This is demonstrated in animal models of severe poisoning with dichlorvos and parathion (Figure 5). The need for metabolic activation of thion-OPs in part makes the use of OP degrading enzymes as therapeutic agents appealing. Improvements in survival could possibly be realized if a thion-OP could be degraded before its bioactivation to its potent oxon-OP metabolite.

Whether two methyl or two ethyl groups are present on the OP may also make a difference in the acute toxicity and response to treatment (20). In general, it appears that dimethyl-OPs irreversibly inhibit, or age, AChE more quickly than diethyl-OPs. This cholinesterase ageing renders the enzyme unresponsive to oxime therapy, and appears to be associated with worse patient outcomes (18, 20). The differential responsiveness of OP compounds makes the development of novel antidotes critically important, particularly for OPs that are not responsive to oximes.

Possible differences in the acute toxicity of aliphatic or aromatic OP pesticides have not been established. Nevertheless, larger or longer side chains and aromatic rings may confer disadvantageous structural and thermodynamic properties to current and future therapies. The multitude of physico-chemical properties of the many OP pesticides makes overgeneralizations treacherous, and new therapeutics a challenge to develop. Despite these difficulties, two unique classes of proteins are currently under investigation as therapies to acute OP poisoning: stoichiometric and catalytic bioscavengers.

5. STOICHIOMETRIC BIOSCAVENGERS FOR THE PROPHYLAXIS AND TREATMENT OF OP POISONING

An area of increased research has been the production of stoichiometric bioscavengers for AChE poisoning. This research primarily stems from the military need for a prophylactic way to prevent incapacitation and death of soldiers by chemical warfare agents. Although this

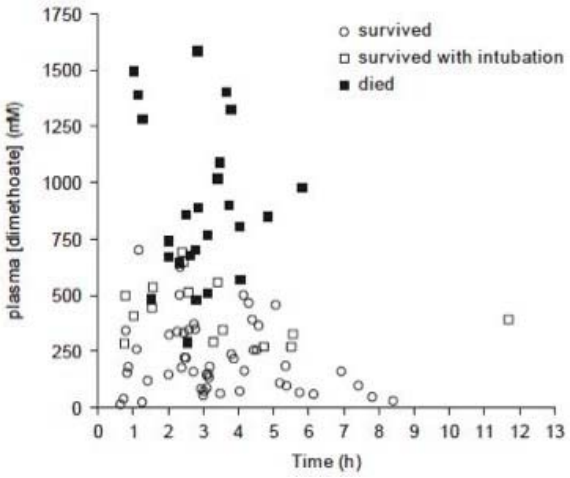

Figure 4. Nomogram of plasma dimethoate concentration vs time from hospital presentation. A plasma dimethoate concentration above 750 μM is fatal. (Used with permission of Oxford Journals from reference (74)).

Figure 5. Survival time in rat models of severe dichlorvos and parathion poisoning. Groups of 8 rats were given 3 x LD50 of dichlorvos or parathion by gavage. Dichlorvos (●), an oxon-OP, caused cholinergic signs by 3 minutes in all animals, and death by 13 minutes. Parathion (▲), a thion-OP that requires bioactivation to the active metabolite, paraoxon, first caused cholinergic signs at 11 minutes and death in all animals by 35 minutes.

review focuses on OP pesticides, the fascinating research into stoichiometric bioscavengers currently underway makes them worth mentioning.

Stoichiometric bioscavengers work like sponges, providing a sink to absorb the poison. Recent work has focused on purified human butyrlcholinesterase (BuChE) (21), recombinant human BuChE from transgenic goats (22), and recombinant AChE from plant sources (23).

Thus far, results with virtually all of the above methods have proven very encouraging. Lockridge *et al.* (21) developed a protocol for extracting human BuChE from plasma, with the ability to process up to 100 liters of human plasma at a time. After purification, the BuChE produced by their method had a mean residence time of 56 hours in mice and 93 hours in monkeys (21). Because the supply of human plasma is finite and concerns about transmitting infectious diseases with human blood products, Huang *et al* (22) sought to produce and express recombinant human BuChE in the milk of transgenic goats. Milk from the transgenic animals contained up to 5 grams per liter of active rBChE. The plasma half-life of the rBuChE after intravenous injection in guinea pigs of the was approximately 6.5 hours, but increased to roughly 44 hours after the additon of polyethylene glycol (PEG) moieties. Testing of similar rBuChE produced in transgenic mice was also found to inhibit nerve agents at the expected 1:1 molar ratio (22). Subsequent studies of rBuChE fused to human albumin resulted in an increased plasma half-life of 32 hours (from non-fused rBuChE half-life of 3 hours) in a pig model (24).

Recently an intriguing method of AChE (rather than BuChE) production has been described. Mor *et al* (23) have shown that tobacco plants (*Nicotiana benthamiana*) can be engineered to express a gene encoding the human acetylcholinesterase-R (AChE) isoform. The transgenic plants expressed AChE at >0.4% of total soluble protein. They were able to perform more than 400-fold purification that yielded a protein which was kinetically indistinguishable from human AChE. Subseqent prophylaxis studies (25) demonstrated a dose-dependent protection by intravenously injected AChE-R in mice challenged with a lethal dose of paraoxon. Importantly, the protection afforded was complete (without any clinical signs of cholinergic toxicity) at molar ratios of 0.5:1, and only mild signs of toxicity were evident at molar ratios of 0.2:1.

Although such prophylactic modalities may work for military applications, stoichiometric bioscavengers are unlikely to be effective for acute OP poisoning for several reasons. First, because stoichiometric bioscavengers simply bind the acetylcholinesterase inhibitors and do not enzymatically degrade them, stoichiometric bioscavengers are not as rapidly effective at clearing the toxin from circulation as an enzymatic bioscavenger. Second, because stoichiometric bioscavengers bind the acetylcholinesterase inhibitors in a 1:1 fashion, they would be useful only for the super-potent military nerve agents, but will not for the large suicidal ingestions commonly encountered in the developing world. To put this in perspective, an exposure to 36 mcg of the nerve agent VX or 450 mcg of sarin would require approximately 12 mg and 289 mg of BuChE, respectively, in order to be scavenged in 1:1 molar ratios. However, assuming a typical ingestion of 50 mL of 50% concentrate OP pesticide seen in the developing world, more than 5 kg of human BuChE would be needed in order to scavenge it (26). Third, the expense of human BuChE is prohibitive for wide scale use in the developing world. Thus, stoichiometric bioscavengers are not likely to be clinically useful or relevant for post-poisoning therapy of acute OP pesticide poisoning

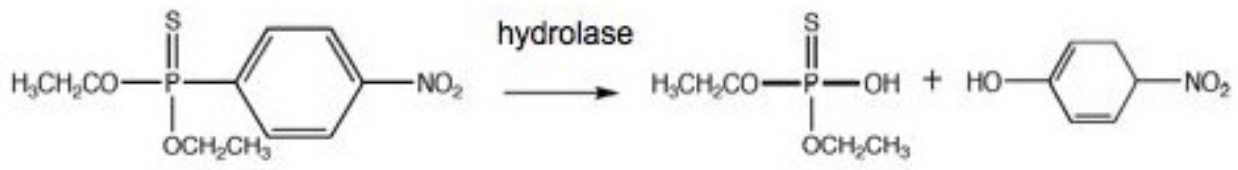

Figure 6. Schematic of parathion hydrolysis by an OP hydrolase. The products of hydrolysis do not inhibit acetylcholinesterase.

6. CATALYTIC BIOSCAVENGERS FOR THE TREATMENT OF OP POISONING

Several enzymatic systems have been discovered to be involved in the detoxification of OPs, including phosphotriesterases (PTE) and glutathione-S-transferases (19, 27). The resulting products of the hydrolysis are devoid of phosphorylating properties, and thus are not inhibitors of AChE (28-30) (Figure 6). Therefore, current research is directed towards the development of these detoxifying enzymes as antidotes to OP pesticide poisoning.

The most intensively studied PTEs in mammals are the paraoxonases, which hydrolyze the insecticide paraoxon. These proteins are synthesized in the liver and circulate in blood associated with high-density lipoproteins (31). The physiological role of mammalian serum paraoxonase seems to be related to the protection of low-density lipoproteins against lipid peroxidation (32). Mammalian serum paraoxonase is also able to hydrolyze the active metabolite of other OPs insecticides, such as chlorpyrifos-oxon (33) and diazinon-oxon, and some nerve agents such as sarin and soman (34, 35) Other PTEs such as DFPase, the enzyme capable of hydrolyzing iisopropylfluorophosphate (DFP), somanase, and other compounds are also found in mammals at very low concentrations (29).

Shih *et al.* demonstrated the involvement of paraoxonase in the detoxification of OP pesticides in experiments using genetically altered paraoxonase knockout mice (36). These knockout mice are extraordinarily sensitive to AChE inhibitors. Shih *et al.* found that dermal exposure of 1.5 mg/kg of chlorpyrifos–oxon had no effects on brain AChE of wild-type mice, whereas the same dose inhibited >80% of AChE activity in knockout animals 4 hours after poisoning (36). As further evidence of the importance of paraoxonase in the protection against OP pesticides, when the dose of chlorpyrifos-oxon was increased to 3 mg/kg, the brain AChE of wild-type animals was inhibited by 31%, whereas paraoxonase-knockout mice all died within 4 hours of poisoning (36).

Rabbit serum paraoxonase (PON) also has proven effective in the treatment of mice poisoned with OPs. A dermal exposure to 100 mg chlorpyrifos/kg body weight caused 60% and 80% inhibition of brain and erythrocyte AChE, respectively, 4 hours after treatment (37). Injection of 3.34 IU of rabbit serum paraoxonase IV, 30 minutes after the same chlorpyrifos exposure, was able to completely prevent the inhibition of brain AChE and to limit the inhibition of erythrocyte AChE to just 20% (37). A higher dose of chlorpyrifos (150 mg/kg) caused 75% inhibition of brain AChE and severe symptoms of poisoning within 2–3 hours of exposure. However, no inhibition of AChE and no signs of intoxication were detected in mice injected with 3.34 IU paraoxonase 30 minutes after dermal exposure to 150 mg chlorpyrifos/kg (37). Despite such promising results, paraoxonase maintains limited ability to hydrolyze a broad spectrum of OP substrates, thus limiting enthusiasm for continued development as an OP antidote.

Bacterial PTEs hydrolyze a large number of OP substrates including nerve agents (38) with higher efficacies than mammal PTEs. The enzymes from *Pseudomonas diminuta*, *Flavobacterium* sp. and *Alteromonas* sp. are the best characterized bacterial PTEs (39). Thus, bacterial PTEs appear to be the best candidates for use in the treatment of people poisoned with OP pesticides.

The most studied PTE is Oph. First isolated from *Pseudomonas* species (40), native Oph effectively hydrolyzes several different OPs *in vitro* and has been shown to improve animal survival with poisoning *in vivo* (41, 42). Other PTEs have been described, including a methyl-parathion degrading enzyme (mpd) from *Plesiomonas* sp. (43), a dimethoate-degrading ezyme from *Aspergillus niger* (44), and a chlorpyrifos-degrading enzyme from an *Enterobacter* strain (45).

Efforts to construct Oph mutants with improved efficacy and kinetics against both OP pesticides as well as the chemical nerve agents have been underway for over a decade (46). Several sources of exogenous PTEs have been utilized. However, PTE from *Pseudomonas diminuta*, and more recently the enzyme OpdA (isolated from *Agrobacterium radiobacter*) (27) seem to be the best candidates for therapeutic use because these enzymes display the widest substrate specificity. Table 2 summarizes studies evaluating various hydrolases for post-poisoning treatment of OP pesticide poisoning.

Using Oph from *P. diminuta,* Ashani *et al* (47) have suggested that a concentration of Oph as low as 1μg/mL of blood could result in a 100-fold decrease in the concentration of diethylphosphate pesticides within one blood-circulation time, thereby minimizing reinhibition of the reactivated AChE. This is especially important since many widely used OPs (e.g.: parathion, chlorpyrifos, and diazinon) are diethylphosphates. *In vitro* studies have also suggested that the combination of Oph treatment with an

Table 1. Number of OP pesticide exposures and deaths reported to US Poison Control Centers from 1997-2006

Year	# OP cases	# OP deaths
1997	88255	14
1998	86289	16
1999	78853	15
2000	86880	17
2001	90010	17
2002	96112	18
2003	97677	41
2004	102754	not reported
2005	101746	23
2006	96811	not reported

Compiled from references (64-73)

Table 2. Animals studies employing OP hydrolases for the post-poisoning treatment with OPs

Hydrolase source	Hydrolase name	OP Pesticide	Dose of OP Pesticide	Dose of hydrolase	Outcomes Measured	Reference
Pseudomonas diminuta	PTE	Paraoxon	0.7 mg/kg	1.5 IU	AChE inhibition	(50)
Pseudomonas diminuta	PTE	DFP	1.8 mg/kg	83 IU/g	AChE inhibition	(75)
Rabbit serum	PTE	Chlorpyrifos	150 mg/kg	3.3 IU	Signs of poisoning and AChE inhibition	(37)
Agrobacterium radiobacter	OpdA	Parathion	18 mg/kg	0.15 mg/kg	Survival to 4 and 24 hours	(49)

PTE = phosphotriesterase, DFP = diisopropyl fluorophosphate, One International Unit (IU) is the amount of enzyme that hydrolayzes 1 microM of OP pesticide/minute

oxime might significantly improve the therapeutic management of poisonings with diethylphosphates (47).

OpdA shares approximately 90% sequence homology with Oph. The 10% sequence difference, however, appears to confer some important kinetic and substrate specificity advantages to OpdA (48). OpdA is a metalloprotein that contains two metal ions in its active site. The mode of action of the enzyme is shown in Figure 2. The enzyme has broad specificity and will break down some common pesticides, such as paraoxon, at near a diffusion-limited rate while other compounds are broken down at more modest rates. OpdA possesses broad substrate specificity *in vitro*, and has been shown to prevent lethality in a post-poisoning rat model of parathion poisoning (49).

PTE from *P. diminuta* has an *in vivo* half-life in mice of approximate 5.5 hours after IV injection of 1.5 IU (50). In PTE-nontreated animals AChE was reduced by 60% at the paraoxon dose of 0.5 mg/kg, whereas in PTE-treated mice a reduction in AChE activity was not seen until a paraoxon dose of 2.0 mg/kg. PTE only prevented the decrease in brain AChE activity by paraoxon when it was administered before or immediately after the paraoxon. When mice were treated with 0.7 mg paraoxon/kg IP (a dose that causes 50% inhibition of brain AChE) and 1.5 IU of PTE IV immediately following the paraoxon, only 6% AChE inhibition was found 2 hours later. However, when the same PTE dose was delayed by 15, 30, or 60 minutes, AChE activity was inhibited by 31%, 44%, and 43%, respectively (50).Overall, PTE increased the serum paraoxon-hydrolyzing activity more than 5-fold. When administered by other routes (intraperitoneal or intramuscular), PTE demonstrated progressively less hydrolyzing activity. Therefore, the time elapsed between OP poisoning and treatment with PTE appears to be critical for the effectiveness of enzymatic therapy.

In fact, the time delay between poisoning and presentation for medical care is likely to be a major determinant in the clinical application of catalytic bioscavengers. Many patients in the developing world with OP poisoning arrive at a tertiary hospital more than 2 hours after ingestion (18). This time delay may place a patient out of the window of opportunity for maximal benefit from a bioscavenger. The amount of possible benefit will depend, of course, upon the exact OP ingested. Nevertheless, catalytic bioscavengers represent an area of opportunity with tremendous potential.

7. CATALYTIC BIOSCAVENGERS AS PROPHYLAXIS AGAINST OP POISONING

Most research with OP hydrolases has thus far focused on prophylaxis against OP poisoning. This is likely due to two reasons. First, the US military (which has been involved in much of the research) desires prophylactic treatment measures more so than post-poisoning therapeutics. Second, such initial proof-of-concept studies are critical to the development of therapeutic enzymes. Nevertheless, such a pre-poisoning treatment approach is not feasible for the hundreds of thousands of patients that die every year from OP poisoning.

The pretreatment with PTE purified from *Pseudomonas* sp. (without other adjunctive therapies) conferred protection to mice from the toxicity of paraoxon at doses 3.8–7.3 times the LD_{50} (51). The same dose of PTE offered protection against 2.9 times the LD_{50} of diethylfluorophosphate (DFP) (51). As expected, the increased protection was accompanied by an increase of the OP-hydrolytic activity in blood.

Ashani *et al.* also pretreated mice with PTE from *Pseudomonas* sp. prior to the administration of paraoxon (the active metabolite of parathion). They found that this prophylaxis conferred protection for up to 7 times the LD_{50} of the OP (51). In order to determine if the protection was

Table 3. Animal studies employing OP hydrolases for the prophylaxis against OP poisoning

Hydrolase source	Hydrolase Name	OP Pesticide	Dose of OP Pesticide	Dose of hydrolase	Outcomes Measured	Principal Result	Ref.
Agrobacterium radiobacter	OpdA	Dichlorvos	150 mg/kg	0.15 mg/kg	Survival to 4 and 24 hours	24-hour survival increased from 0% to 100% with single dose of OpdA	(49)
Alteromonas	OPA Anhydrolase	DFP	variable	20-30 IU in liposomes	Increase in LD50; clinical signs of OP poisoning	Approximately 2-fold increase in the DFP LD50 in mice; decreased clinical signs of OP poisoning	(76)
Flavobacterium	PTE	Paraoxon	91-170 mg/kg	139 IU in liposomes	Increase in LD50	139-fold increase in LD50	(53)
Pseuodomnas diminuta	OPA Anhydrolase	Paraoxon	1 mg/kg	16.8 IU	Signs of poisoning and AChE inhibition	PTE protected AChE against inhibition and decreased clinical signs of OP intoxication in mice	(41)
	OPA Anhydrolase	Paraoxon	3.8-7.3 x LD50	7-26 micrograms	Increase in LD50	Approx. 7-fold increase in the paraoxon LD50	(51)
	OPA Anhydrolase	DFP	2.9 x LD50	7-26 micrograms	Increase in LD50	Approx. 3-fold increase in the DFP LD50	(51)

One International Unit (IU) is the amount of enzyme that hydrolyzes 1 microM of OP pesticide/minute

isolated to paraoxon or if it could be accomplished with other OPs, they exposed mice to DFP after pretreatment with similar doses of PTE. Again they demonstrated protection of up to 2.9 times the LD_{50} of DFP (51).

Kaliste-Korhonen *et al.* examined the effect of paraoxon on brain AChE activity in mice treated with and without PTE from *Pseudomonas diminuta* (50). Animals that received 0.5 mg/kg of intraperitoneal paraoxon alone demonstrated brain AChE activity of just 39% of controls. However, in mice given PTE 10 minutes before paraoxon, the decrease in brain AChE was just 10%. Furthermore, PTE-treated mice did not show a significant decrease in brain AChE until a paraoxon dose of 2.0 mg/kg (50).

The beneficial effects on brain AChE are not limited to PTE from *Pseudomonas diminuta*. Tuovinen *et al.* examined the effects of paraoxonase on mouse brain AChE activity. They found that brain AChE in mice was inhibited by 64% 24 hours after the intraperitoneal injection of 1.0 mg paraoxon/kg (41). However, in mice treated with paraoxonase there was just 40% inhibition of brain AChE. Increasing the doses of paraoxonase by 10-fold and paraoxon 25-fold nearly completed protected brain AChE from inhibition 24 hours after dosing (41). Demonstrating that the injected paraoxonase contributes to serum hydrolyzing activity, Tuovinen *et al.* showed that four hours after injection the serum paraoxonase activity was up to 16 times higher in treated animals than in controls. Concomitant with the laboratory evidence of protection, the authors found that physical signs of poisoning or incapacitation were absent or minor in animals that received the paraoxonase. Further supporting the notion that no single ideal PTE has yet to be found (or is likely to be produced), Tuovinen *et al.* found that the protection afforded by PTE from *P.diminuta* against AChE inhibition by DFP was significantly lower than in the protection from paraoxon (41). This finding is likely explained by the fact that paraoxonase hydrolyzes DFP 100 times slower than paraoxon (52).

The duration of protection afforded by PTEs were amongst the earliest studies performed with the enzyme. When PTE was injected into mice, Kaliste-Korhonen *et al.* found that blood PTE activity returned to baseline levels by 24 hours after injection, with a satisfactory half-life of approximately 5.5 hours (50). Table 3 summarizes the experimental animal studies performed using various OP hydrolases for the prophylaxis against OP poisonings.

Further research focused on the ability to increase the serum half-life of the phosphotriesterase. Petrikovics investigated the encapsulation of a *Flavobacterium* sp. PTE within liposomes (53). When paraoxon was administered subcutaneously in mice, it exhibited an LD_{50} of 0.9 mg/kg. By administering 10 IU of PTE encapsulated in sterically stabilized liposomeswere one hour before paraoxon, the LD_{50} increased 139 times (53). With the concomitant administration of atropine, 2-PAM, and the PTE, the LD_{50} of paraoxon increased a remarkable 1,000-fold (53). While clinically irrelevant, such synergistic protection provides proof of concept of a multi-modality approach to severe OP poisoning.

Pei *et al.* used a different and interesting method to prolong the circulation time of an OP hydrolase (54). They incorporated PTE within resealed murine erythrocytes by means of hypotonic dialysis. When placed inside the erythrocyte, the PTE was shielded from degradation while maintaining its ability to effective hydrolyze paraoxon. Based upon other research using resealed erythrocytes, Pei *et al.* estimated that the PTE could effectively remain in circulation for up to 36 days (54).

8. IMMUNOLOGIC CONSIDERATIONS

Regardless if an OP poisoning antidote is a bioscavenger or a catalytic enzyme, safe dosing in test animals and in humans is of paramount importance. Concerns about immunologic response to foreign proteins

have lead many investigators to forego recombinant bacterial enzymes and focus on humanized enzymes or mammalian expression systems. However, when considering the immunologic implications of a therapy, it is important to consider the target population. For instance, as mentioned before, military personnel ideally would obtain prophylactic treatment against nerve agents. This requirement would necessitate repeated exposure to the bioscavenger or hydrolase, thus raising the spector of hypersenstivity reactions. OP-poisoned civilians, on the other hand, would likely need just one (or possibly a few) dose of an enzyme, thus mitigating concerns about repeat exposures. Supporting this contention, in a cohort of more than 1300 OP-poisoned patients (55), only a single patient had more than one poisoning episode (M. Eddleston, personal communication).

Perhaps the best analogies for the use of a recombinant bacterial OP hydrolase in humans are the use of streptokinase for acute myocardial infection and recombinant carboxypeptidase G2 for acute methotrexate overdose (56). Streptokinase is a metallo-enzyme derived from streptococcus sp. Clinical use over the last 20 years has demonstrated that while the risk of allergic reactions are real (generally less than 1%), they are usually mild and easily treated by standard medical therapies (57). More recently, carboxypeptidase G2 (a dimeric zinc-dependent peptidase produced by *Pseudomonas* sp.) has entered the marketplace for use in methotrexate overdose or when methotrexate elimination is prolonged due to renal failure. Although there is limited clinical experience with this enzyme, allergic reactions to the drug have not been reported (56). Thus, while immunologic concerns are valid, they should not prevent the continued development and testing of therapeutic enzymes for OP poisoning.

9. PHARMACOKINETIC CONSIDERATIONS

Pharmacokinetic specifics are another critical element of any novel antidote for acetylcholinesterase inhibitors. For any OP other than those with very low lipophilicity (e.g. dichlorvos), an enzyme ideally would remain in circulation long enough to hydrolyze OP pesticides as they redistribute from fat and other compartments such as brain and liver. To accomplish this, investigators have focused packaging the enzymes within red blood cell (RBC) ghosts (54) and liposomes (58). Other methods of increasing the half-life of enzymes include the addition of polyethylene glycol moieties (59) or conjugation to albumin (24).

When erythrocytes are dialyzed against hypotonic buffer in presence of PTE purified from the hepatopancreas of squid, the resealed cells encapsulate the enzyme and are able to hydrolyze OPs with similar properties to the free enzyme (60). Pei *et al* obtained similar results when PTEs isolated from bacterial sources were encapsulated in murine erythrocytes (54), with no apparent loss of activity after 2 weeks.

Taking a cue from liposomal amphotericin B, Petrikovics *et al.* have evaluated the prophylactic and post-poisoning efficacy of sterically stabilized liposomes, also referred to "stealth liposomes," in which to encapsulate Oph (53, 58). By using this biodegradable carrier to circumvent immune defenses - thus avoiding the phagocytic activity of the macrophages and the reticuloendothelial system (61) - Petrikovics *et al* were able to prolong the mean residence time of the enzyme from approximately 16 hours to more than 48 hours (62).

The use of stabilized liposomes instead of erythrocytes has several advantages. Liposomes are stable when stored for much longer than RBC ghosts. Importantly, liposomes also have much higher efficiencies than RBCs in the successful encapsulation of PTEs (80% versus 30%) and do not require prior blood typing, as must be conducted for the use of carrier erythrocytes (53). However, RBC-encapsulated enzymes do have one distinct advantage over liposomal enzymes: the mean residence time of RBC-encapsulated PTE can be as long as several weeks (63), thus making them more attractive for prophylactic use. Again, for acutely poisoned OP patients, there is likely to be little to a prolonged mean residence time for an enzymatic antidote.

10. CONCLUSION

The public health burden from OP exposures and poisoning worldwide necessitates that new and improved therapies are developed and brought to human clinical trials. Recent studies have shed light on the different responsiveness to conventional therapies (particularly oximes) that di-ethyl and di-methyl OP pesticides possess, making the use of catalytic or stoichiometric bioscavengers appealing.

While unarguably impressive, it is important to remember that virtually all prophylactic and post-poisoning studies in animals have employed different animals species, different OPs, different routes of poisoning and doses of OPs, and different combinations of adjunctive therapies. It is difficult therefore to directly compare studies' results.New cost-effective animal models that more closely mimic the physiology and responsiveness to human poisoning may be useful in future studies.

Enzymatic hydrolysis of acute OP pesticide poisoning is an exciting therapeutic possibility in the nascent stages of development. Currently, more interest and research is centered on the use of stoichiometric bioscavengers for both prophylaxis and treatment, primarily for the super-potent military nerve agents. Because of the large quantity OP pesticide typically ingested during a suicidal gesture in the developing world would necessitate a large amount of stoichiometric bioscavenger for treatment, research should focus on catalytic bioscavengers for post-poisoning clinical use.

The preliminary animal studies published thus far using catalytic bioscavengers are encouraging. Given the promising preliminary data and the tremendous morbidity and mortality caused by OP pesticides, we believe that human trials of catalytic bioscavengers are

justified as soon as the immunologic and pharmacokinetic aspects of candidate hydrolases are adequately addressed.

The design of catalytic bioscavenger mutants with enhanced activity against the most problematic OP pesticides in the agricultural areas of Southeast Asia, and increased governmental and industry-supported development of such mutants for clinical use is urgently needed.

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Send correspondence to: Steven B. Bird, University of Massachusetts Medical Center, Worcester, Massachusetts, USA, Tel: 508-421-1400, Fax: 508-421-1490, E-mail: steven.bird@umassmemorial.org