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Organophosphorus hydrolase as an *in vivo* catalytic nerve agent bioscavenger

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The use of proteins as a treatment for organophosphorus intoxication has been investigated since A. R. Main demonstrated protective efficacy against parathion with an exogenously administered arylesterase in the late 1950s. His experiments spurred over 60 years of research and progress in the development of enzymes as potential bioscavengers of nerve agents and pesticides. Efforts have been made to broaden the specificity of enzymes to make a universal scavenger that would protect against multiple compounds, and an understanding of the differential isomer toxicity of these compounds has provided the impetus for rational and random mutagenic approaches in the stereospecific design of enzymes. As improved candidate enzymes are continually developed, our understanding of the contributions of the catalytic parameters (k_{cat}, K_M and catalytic efficiency) to efficacy expands. In addition to the scavenging properties of the proteins, another important aspect of development is the pharmacokinetic profile of the drug product. Immunogenicity, absorption, distribution and elimination contribute significantly to the level of protection afforded by the protein. A review of the development of organophosphorus hydrolase (OPH) for use as *in vivo* catalytic bioscavengers is presented here. Copyright © 2012 John Wiley & Sons, Ltd.

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Introduction

Organophosphorus anticholinesterases (OPs) are among the most toxic substances known. The primary effect of exposure to these compounds is the inhibition of the carboxylic ester hydrolases acetylcholinesterase (AChE, E.C. 3.1.1.7) and butyrylcholinesterase (BuChE, E.C. 3.1.1.8). Although there is some debate over whether BuChE has a physiological role,[1,2] its ability to bind anticholinesterases makes it a natural bioscavenger that may serve to provide protection to synaptic AChE against acute OP neurotoxicity. While the classic view of AChE as a cholinolytic enzyme whose sole function is to terminate the cholinergic signal may be simplistic,[3,4] it is still considered that the primary effect of the inhibition of AChE by OPs is accumulation of acetylcholine at synaptic endplates, leading to the failure of autonomic and central nervous systems.[5] Depending on the OP compound, repeated, prolonged and/or low-dose exposure can cause neurotoxicity and delayed cholinergic toxicity; high-dose exposure can be fatal.[5,6] Some of the most toxic OP compounds are the chemical warfare agents (CWA), which include tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), VX and the Russian V-agent, VR. For all routes of exposure excepting dermal, CWAs have a median lethal dose (LD_{50}) in mammals in the μg/kg range. LD_{50}s for percutaneous exposure are in the mg/kg range,[7] with the exception of VX which has been reported to have an LD_{50} of 34 μg/kg in guinea pigs[8] and LD_{Lo} of 86 μg/kg in human.[7] OPs produce their acute toxic effects by irreversibly inhibiting the enzyme AChE, leading to a concentration increase of acetylcholine (ACh) in the cholinergic synapses of both the peripheral and central nervous systems. The physiological consequences of elevated ACh include an over-stimulation at neuromuscular junctions [9–11] and altered functioning of the respiratory centre.[12–14] A sufficiently high or rapid increase in ACh concentration can precipitate a cholinergic crisis, resulting in headache, respiratory depression, dimming of vision, muscle weakness and even seizures. Organophosphorus intoxication can be a life-threatening event, with death usually resulting from respiratory failure with secondary cardiovascular components.[7] The mechanism of OP toxicity suggests that an approach based on the reduction of the concentration of OP toxicant in the blood before it can reach its site of action should be effective at mitigating toxic exposures to mild symptoms and lower level exposures to inconsequential.

Current strategy for protection against OP intoxication

The conventional approach for treatment of OP intoxication involves efforts to counteract the effects of AChE inhibition. Cholinolytic drugs, such as atropine, are administered at the onset of signs of OP intoxication to antagonize the effects of the elevated acetylcholine levels that result from the inhibition of AChE. An oxime nucleophile is also given, which reacts with the inhibited phosphylated enzyme to displace the phosphoryl/phosphonyl group and restore normal activity.[15] The currently available oximes do not readily cross the blood–brain barrier,[16] and there is no universal oxime able to reactivate all OP-AChE conjugates efficiently.[17] As a consequence, central AChE is poorly reactivated

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and so acetylcholine accumulates in the central nervous system. Anticonvulsant drugs, such as diazepam, are administered to counteract the effects of this accumulation.

Reactivation of inhibited AChE presents an additional therapeutic challenge due to a time-dependent process known as 'aging'. Aging is a secondary reaction in which the phosphyl group bound to the inhibited enzyme is dealkylated. This process results in a phosphylated AChE that is refractory to either spontaneous or oxime-mediated reactivation.[18] In general, the rate of aging correlates with the structure of the alkoxy chain; the bulkiest branched chains promote rapid aging. However, recent studies have demonstrated that aging kinetics cannot be predicted unequivocally from the structure of the alkoxy group.[17] The half-time of aging of soman-phosphonylated human AChE is 1–3 min, [17,19] whereas that of the sarin-phosphonylated enzyme is about 5 h.[19,20] Tabun- and VX-inhibited enzymes do not age as rapidly, with half-times of more than 46 h.[21,22] Individuals considered at high risk for exposure to chemical agents – for example, certain military deployments – may be pretreated with pyridostigmine bromide. Pyridostigmine bromide is a spontaneously reactivating AChE inhibitor that reversibly binds the active site of peripheral AChE molecules, thus protecting a fraction of the enzyme from irreversible inhibition by the OP agent.[23]

This treatment regimen of an oxime for reactivation, atropine as an antagonist, a pretreatment carbamate and diazepam for seizures has been, with minor variations, the standard in the United States and in NATO countries for many years. While this regime can be effective in reducing immediate lethality, it does not prevent performance deficits, behavioral incapacitation, or permanent brain damage, all of which are potential outcomes of acute OP toxicity.[24]

Alternatives to conventional therapy: OP bioscavengers

Current treatment regimes for acute nerve agent poisoning attempt to therapeutically manage the toxic insult resulting from an OP exposure. Improved strategies are being developed which have the objective of avoiding the toxic insult altogether through the prophylactic administration of bioscavengers. This approach avoids the side effects and the requirement for rapid administration associated with current antidotes by prophylactically inactivating (through sequestration or hydrolysis) anticholinesterase agents before they can react with the target AChE. The challenges associated with this approach are similar to the use of any antidote as a prophylaxis: dose, timing and duration. The window of inactivation can be quite small – for example, estimated to be approximately 2 min in humans for soman[25] – so for situations involving acute exposure, the bioscavenger function must be available, rapid, and specific. In addition, the utility of bioscavengers would be enhanced if they can be developed or formulated to have a long vascular residence, to be safe in the absence of nerve agent and to be non-immunogenic.

Candidate bioscavenger proteins, in general, function either by stoichiometrically binding and sequestering the anticholinesterase or by catalytically hydrolyzing the OP substrate into biologically inactive products. The former category includes naturally occurring proteins that bind and/or react with nerve agents, including enzymes such as cholinesterases (ChEs) and carboxylesterases (CEs). Each of these biomolecules has the capacity to bind no more than one molecule of nerve agent per molecule of protein bioscavenger. This approach is limited in that the extent of protection is directly proportional to the concentration of unreacted, active bioscavenger in the bloodstream at the time of nerve agent exposure. Since the molecular weight of many protein scavengers under consideration (e.g. BuChE) is approximately 80 000 Da, and the molecular weight of the nerve agents is about 180 Da, the mass ratio of bioscavenger to nerve agent required for non-catalytic protection is significant. For example, to protect a 70 kg person against 1 $LD_{50\text{-dermal}}$ (0.14 mg/kg) of soman (182 Da) would require 3.7 g of human BuChE (hBuChE). In spite of this, hBuChE has been demonstrated in animal model to provide protection against lethal doses of both G- and V-agents,[26] and is the most advanced of the proposed bioscavengers.[27]

An alternative approach that has been the focus of much research activity is to either regenerate these stoichiometric bioscavengers through administration of reactivators after exposure, or through the creation of catalytic variants that are capable of reactivating spontaneously.[27–32] In either case, the objective is to reduce the amount of bioscavenger required by mimicking the non-consumed nature of a catalytic bioscavenger. Given the demonstrated success of hBuChE as a stoichiometric bioscavenger, it has naturally become the focus of these efforts. More than 60 variants of BuChE have been created for this purpose.[30,33,34] A recent series of studies has resulted in the best catalytic hBuChE to date. Using information from the crystal structure of AChE,[35] a homology model of BuChE was constructed.[36] Residues were identified in hBuChE that were analogous to those in the active site of the bacterial hydrolase organophosphorus acid anhydrolase (OPAA) and a catalytic variant of hBuChE, G117H E197Q, was designed and expressed.[37] Although this variant has the best catalytic rate yet for a hBuChE, it was not able to protect guinea pigs against OPs, even at supra-stoichiometric doses, because the on rate (k_1) is too slow for the catalysis to be biologically relevant.[38] Similar results have been reported for human carboxylesterase (hCE1).[39] Using the crystal structure of hCE1 in complex with nerve agent, a variant protein was designed that significantly increased rates of reactivation following exposure to sarin, soman, and cyclosarin. In spite of a nanomolar K_M with one of the substrates, this variant had a catalytic efficiency of only 8.86 x 10^2 M^{-1} s^{-1}. The redesigned hCE1 compares favorably to other mammalian enzymes that have been rationally engineered to improve hemi-substrate metabolism,[39] but will require substantial increases in catalytic efficiency in order to provide *in vivo* protection. It should be noted that, in each of these cases, the variants were transgenically expressed, effectively creating 'non-human' variants that, in addition to the catalytic insufficiencies, will likely suffer the same immunologic and pharmacokinetic fate as a foreign protein.

Natural catalytic OP bioscavengers

As early as 1956, it was demonstrated that an exogenous catalytic bioscavenger could protect rats against a subsequent exposure to multiple LD_{50} of the pesticide paraoxon.[40] In general, the use of catalytic bioscavengers is considered advantageous relative to their stoichiometric counterparts, because they can be administered in smaller quantities and yet still offer the same or greater extent of protection. A catalytic bioscavenger has the advantage of not being consumed in the process of detoxifying the nerve agent, so small amounts of enzyme can be sufficient to detoxify large amounts of nerve agent, assuming that the rate

of reaction is sufficiently fast. The half-time for reaction of a nerve agent with a bioscavenger can be calculated using some very conservative assumptions. Based on toxicity estimates in humans, the expected concentration of a nerve agent in the blood at an LD_{50} dose would be about 8×10^{-7} M.[41] The bimolecular rate constant for reaction of soman, for example, with AChE is ~9×10^7 M^{-1} min^{-1}.[42] If a bioscavenger were present in the vascular system at a concentration of 1 mg/ml (~1×10^{-5} M), then the rate constant for reaction of bioscavenger with toxicant would be pseudo first order and the $t_{1/2}$ for the reduction of toxicant would be ~3-7 x 10^{-4} min. Under these conditions, which assume perfect mixing and that both the bioscavenger and the toxicant remain in the blood system, the concentration of toxicant would be reduced to 0.1% of its initial concentration within 2–4 x 10^{-3} min. Where actual measurements have been made, a two LD_{50} dose of soman in guinea pigs was reduced approximately 1000-fold in 1.5 min.[43] These results support the contention that if a catalytic bioscavenger were present in circulation at the time of exposure, the reduction in toxicant concentration to a physiologically insignificant level would be very rapid, with no measurable inhibition of AChE. The need to administer a host of pharmacologically active drugs with a short duration of action at a precise time following exposure can potentially be eliminated. Further, with the development and use of appropriate bioscavenger(s), such an approach could afford protection against all of the current threat agents, including those that induce rapid aging of AChE and are refractory to the current atropine and oxime treatment regime.

Since bacterial-derived enzymes have no known mammalian homologues, they are logically considered as likely to be potent initiators of immune responses and undergo rapid clearance, and so are often deemed inappropriate for use as a bioscavenger in humans. Consequently, basic research efforts have mostly been directed towards human enzymes, and the obvious candidate for this effort is human paraoxonase (hPON1).[44] While hPON1 is an effective catalyst *in vitro* for numerous OPs [31,45,46] and some *in vivo* protection has been demonstrated,[47] it has been suggested that a 10- to 100-fold increase in the hPON1 catalytic efficiency (k_{cat}/K_M) would be needed to afford protection against the nerve agents (Table 1).[27] Directed evolution combined with high-throughput screening has proven to be as effective at creating relevant variants as an *in silico* design approach.[48–52] This has been demonstrated by the recent report in which, after five generations of gene shuffling combined with high throughput selection, an evolved PON1 variant was created that targets the most toxic enantiomer of a cyclosarin analog.[52] Similar to other human proteins, production, stabilization and pharmacokinetic challenges exist with large scale production of hPON1, whether from outdated plasma or transgenic systems. [53–56] To overcome these biotechnological challenges, gene delivery provides a novel and promising new approach for both stoichiometric and catalytic bioscavengers of human origin.[57,58]

Table 1. Kinetic properties of natural catalytic bioscavengers.

Enzyme	Substrate	K_M, mM	Kcat, s^{-1}	Kcat/ K_M (M^{-1} s^{-1})	Reference
hPON1	Paraoxon	0.5	12	2.4×10^4	[119]
	DFP	—	—	6.3×10^2	50
	GB	—	—	1.5×10^4	50
	GD	—	—	4.6×10^4	50
OPH	Paraoxon	0.12	7.8×10^3	6.5×10^7	[120]
	DFP	2.8	3.7×10^3	1.3×10^6	[120]
	GB	0.7	56.0	8×10^4	74
	GD	0.5	4.8	1×10^4	74
OPAA	Paraoxon	4.1	1.9	4.8×10^2	85, 86
	DFP	15.3	3.8×10^3	2.5×10^5	85, 86
	GB	1.6	441	2.8×10^5	85, 86
	GD	2.5	151	6.1×10^4	85, 86

A consequence of mutagenesis and transgenic expression of hPON1 is that these manipulations effectively make the enzyme and the resulting variants non-human, eliminating any advantages afforded by its human origin. As a result, hPON1 and its variants will suffer the same immunologic and pharmacokinetic fate as a foreign protein. This realization opens the door to the numerous OP hydrolyzing enzymes found in diverse environments and species throughout nature. One such example is the enzyme diisopropylfluorophosphatase (DFPase, EC 3.1.8.2) found in the squid *Loligo vulgaris*. [59] DFPase and PON1 are structurally similar, described as calcium dependent, six-bladed β-propeller structure.[60,61] However, DFPase has no arylesterase activity, but rather is a fluorophosphatase with good activity toward diisopropyl fluorophosphate (DFP) and the G-type nerve agents, tabun, sarin, soman, and cyclosarin. In spite of this somewhat narrow substrate profile, the enzyme is described as thermostabile and tolerant of organic solvents, has designed variants with enantioselectivity for the most toxic isomers of G-agents [62] and can be produced in large quantities.[63] These characteristics all make it a good target for continued development.

The most diverse source of OP hydrolyzing enzymes in nature is the microbial world. More than 65 microbial species, dispersed among the bacteria, archaea and fungi, have been reported to harbour enzymes that can be developed for OP detoxification.[64] The designations in the literature for these activities can be confusing, with each newly discovered activity often reported with a unique designation. There have been several reviews that discuss the organization of these enzymes and speculate on their origin and evolution.[64–67] For this review, the major groups within the phosphotriesterase family of proteins will be distinguished based on homology at the protein level.

Organophosphorous hydrolase (OPH)

This family is classified by the IUBMB as an aryltriphosphate dialkylphosphohydrolase (EC 3.1.8.1), and includes homologues of OPH encoded by the *opd* gene. OPH was originally cloned from *Sphingobium fuliginis* (formerly *Flavobacterium* sp. ATCC 27551) and *Brevundimonas diminuta* (formerly *Pseudomonas diminuta*). [68–71] (For purposes of this review, the strain designations as reported in the original reference will be used to avoid confusion.) This enzyme family also includes the OPDA enzyme from *Agrobacterium radiobacter*, which has 90% sequence identity with OPH.[72] OPHs are a member of the amidohydrolase superfamily,[66] and are homodimeric metalloproteins which adopt a TIM-barrel $(\alpha\beta)_8$ fold. This family of enzymes has the broadest intrinsic substrate specificity with hydrolytic activity toward the P-O (paraoxon), P-F (DFP, soman and sarin), P-S (demeton-S and VX) and P-CN (tabun) bonds;[73–77] secondary activities include a weak carboxylesterase activity and considerable lactonase activity.[78]

Organophosphorous acid anhydrolase (OPAA)

This family was originally classified as a diisopropyl-fluorophosphate fluorohydrolase (EC 3.1.8.2), and includes homologues of the OPAA encoded by the *opaA* gene originally isolated from

Alteromonas sp. [79,80] More recently, these enzymes are understood to be dipeptidases that hydrolyze dipeptides with prolyl residue at the carboxyl-terminal position. This is supported by the following: (1) OPAA has significant prolidase activity, with specificity for Xaa-Pro dipeptides;[80,81] (2) amino acid sequence alignments indicate similarity of OPAA with bacterial aminopeptidase P (APPro), proline dipeptidase, and prolidases;[80–82] and (3) OPAA and APPro both have a large C-terminal domain that folds into the 'pita bread' architecture first described for the methionine aminopeptidase from *E. coli*. [83] The C-domains of OPAA and APPro can be superimposed with a rmsd of ~2 A° for ~240 superimposed Cα positions and a 31% sequence identity. The active sites of both proteins are identical and superimposable.[84] Although the natural substrate of OPAA remains unknown, the fact that it possesses significant Xaa-Pro activity and shares significant structural homology with the peptidase family suggest that OP hydrolysis is an ancillary or promiscuous function of a generalist within the aminopeptidase family (EC 3.4.11). Like DFPase, the OPAAs have activity only against the phosphofluoridate OPs.[85,86]

Methyl parathionase (Mpd)

This enzyme is classified as an aryltriphosphate dialkylphosphohydrolase (EC 3.1.8.1), and includes homologues of the methyl parathion-degrading (*mpd*) gene that codes for the MPH of *Plesiomonas* sp. M6.[87] All MPHs reported to date have been isolated from agricultural soils or activated sludge collected from pesticide manufacturing units in China. The amino acid sequence of MPH is greater than 90% identical with those from *P. putida* and *Pseudomonas* sp. strain WBC-3; *mpd* lacks any sequence homology with the *opd* gene. The structure of MPH is a homodimer and the monomer structure is described as an αβ/βα sandwich typical of the metallo-hydrolase/oxidoreductase fold. A search for structural similarity revealed homology with the β-lactamase domain of a number of structures.[88] This suggests that OP hydrolysis is either an ancillary or promiscuous function of a generalist or a recently evolved specialist within the metallo-β-lactamase functionality.

PTE-like lactonases (PLL)

PLL is a family of enzymes that have been recently identified as putative phosphotriesterases on the basis of homology with OPH.[67,89] The PLLs have lactone hydrolysis as their main activity, and most have some level of phosphotriesterase activity as well. Lactones are natural compounds, ubiquitous in nature and important in cell signaling, growth and differentiation, while organophosphates are human-made chemicals introduced within the last 70 years. Thus, it has been suggested that lactones are the primitive/primary substrate for which phosphotriesterase enzymes evolved, and the organophosphatase activity arose as a secondary or promiscuous functionality.[67] A number of organisms have been identified with links to the PLL group, including some characterized as radiophiles, thermophiles, acidophiles and/or polyextremeophiles.[90] The investigated family members share the TIM barrel structure with OPH and the amidohydrolase superfamily, although with differences at the active site and a surface loop.[90] This family of lactonases offers the opportunity for development of enzymes with environmental stability due to the extremophile nature of their native hosts. However, while their lactonase specificity (k_{cat}/k_M) is significant ($10^4 - 10^6\ M^{-1}\ s^{-1}$), the reported paraoxonase activities can be slight ($10^{-1} - 10^3\ M^{-1}\ s^{-1}$), [69,91] making it doubtful they will play a substantial role in medical applications without significant catalytic improvements.

OPH as an *in vivo* bioscavenger: Defining the challenges

Over the past 20 years, native OPH has been demonstrated to serve as an effective bioscavenger against some of the most important OP pesticides and nerve agents. Ashani *et al*.[92] demonstrated that pretreatment with as little as 7.5 and 26 μg of OPH (approximately 0.25 and 0.9 mg/kg) from *Pseudomonas* sp. provided protection against multiple median lethal doses of diethyl p-nitrophenyl phosphate (paraoxon) and its P-F analogue, diethyl fluorophosphate (DFP) in mice. Subsequently, this observation was expanded to the nerve agent, tabun (GA), where as little as 0.6 mg/kg of OPH provided a protective ratio (PR, LD_{50} in protected animals divided by the LD_{50} in naïve mice) of approximately four.[77] Broomfield demonstrated that OPH from *P. diminuta* was able to afford protection in mice against soman toxicity and associated behavioral side effects.[93] Experimental animals that had been pretreated with OPH received an average of 0.03 mg/kg of soman over a period of 5 h with no signs of intoxication. In this study, soman was delivered incrementally, and all control mice died between 2–5 h after dosing was begun, at a mean lethal dose of 0.014 mg/kg (n = 5, avg weight = 27.48 g, OPH dose = 100 mg/kg).

In addition to the nerve agents, OP pesticides present a real threat to civilian and military personnel around the world. The potential of enzymes, specifically OPH, to serve as a catalytic bioscavenger for treatment or prevention of pesticide poisoning has been demonstrated with some of the most toxic OP pesticides. Untreated animals showed signs of severe poisoning at doses of paraoxon between 1 and 1.5 mg/kg, whereas OPH pretreated mice (168 U/animal, i.v.) tolerated paraoxon in doses up to 50 mg/kg, for a PR of 50 .[94] When OPH was used in combination with atropine and 2-PAM, Balb/c mice were protected against 780 × LD_{50} of paraoxon.[95] In both of these studies, OPH was applied as a pretreatment within 1 h of OP exposure. A recent study provides an interesting alternative in that the dosing of the OP toxicant, methyl parathion, was oral, and the bioscavenger was delivered post-exposure.[96] Ten minutes after poisoning, negative control rats received normal saline placebo intravenously, while rats in the treatment group were given a single dose (0.15 mg/kg) of the OPH homolog, OPDA. Seven of eight control rats developed muscarinic (salivation, urination, defecation) and nicotinic (fasciculations) signs within 30 min of poisoning, and had a median time to death of 79 min. All experimental rats survived to 4 h, but none survived to 24 h. For the first 16 h after poisoning, the rats exhibited no signs of cholinergic excess. Methyl parathion requires metabolism from the thion form to the biologically active oxon form and also distributes to fat. The lipophilicity provides a reservoir for leaching of methyl-parathion back into circulation over an extended period, one that likely exceeded the period within which OPDA was available. These studies illustrate strengths, as well as the challenges, of OPH and catalytic bioscavengers in general.

- Catalytic bioscavengers can provide more protection with less protein relative to their stoichiometric counterparts. In studies with tabun (K_M 0.101 mM, V_{max} 128 μmol/min/mg, k_{cat}/K_M 4.6 x $10^7\ M^{-1}\ min^{-1}$), paraoxon (K_M 0.035 mM, V_{max} 1387 μmol/min/mg, k_{cat}/K_M 1.4 x $10^9\ M^{-1}\ min^{-1}$) and DFP (K_M 0.25 mM, V_{max} 350 μmol/min/mg, k_{cat}/K_M 5.2 x $10^7\ M^{-1}\ min^{-1}$), the amount

of OPH required to provide protection was a fraction of what would be required for hBuChE. On average, approximately half a gram of hBuChE must be active in the blood stream for each milligram of OP to be neutralized. So, with a mean LD_{50} of just over 0.2 mg/kg tabun, and not less than a 1:1 stoichiometric requirement, a protective dose of BuChE would have required almost 27 mg/kg for a PR of 1. In contrast, a PR of 4 was achieved with a 0.6 mg/kg dose of the catalytic bioscavengers, OPH.

- The importance of matching catalytic capacity with the target OP is emphasized by the soman study. The protection against soman was demonstrated with incremental doses of the nerve agent over 5 h, which the author suggested was necessary to allow OPH time to hydrolyze the agent within the short time it was contained within the blood stream. This was attributed to insufficient catalytic rates (K_M 0.04 mM, V_{max} 15 μmol/min/mg, kcat/ K_M 1.6 x 10^6 M^{-1} min^{-1}).
- It is important to note these nerve agent studies can be assumed to have been performed with racemic agent, so the kinetics are an average of the rates with the individual stereoisomers. OPH has a preference for the P(+), or less toxic, stereoisomer of soman, so competition between the isomers can impede degradation of the most toxic isomers. In general, this is the case for all tested catalytic bioscavengers and OP agents: catalytic OP scavengers have a stereo-preference for the less toxic isomer(s).
- The significant protection of OPDA against methyl parathion poisoning for 16 h post-exposure suggest that a longer residence time of the bioscavenger in circulation would have successfully treated the animals against the 3 × LD_{50} exposure. In general, bioscavengers that are not of human origin and production are rapidly cleared from circulation. This is true of proteins for which the native host is non-human, as well as human proteins produced transgenically. This will be of less importance for those therapeutic applications in which a single administration of bioscavenger is required for rescue from OP poisoning. However, for a prophylactic or extended therapeutic application, successful formulation of the bioscavenger for extended vascular residence will be important.

OPH as an *in vivo* bioscavenger: Addressing the challenges

Substrate specificity

Since the original cloning and description of OPH more than 25 years ago, a large number of significant variants have been created to address stability or catalytic specificity requirements. As the methods available for enzyme improvement have developed, it has become increasingly possible to successfully re-design enzymes using either a rational or directed evolution approach. This is particularly true for enzymes such as OPH, which intrinsically have broad substrate specificity implying a plastic active site. An illustrative example is a variant that was tailored for increased stability and specificity against phosphonothioate OPs.[97] A possible drawback that is often attributed to OPH is a relative instability related to the formation of its bimetallic catalytic centre.[98–100] There are clear trade-offs in the stability of modifications that enhance catalytic activities. For example, a H254R H257L OPH variant has higher turnover numbers for the chemical warfare agents racemic VX and VR (Table 2). These increases are accompanied by a loss in stability in which the total Gibb's free energy for unfolding is 5.7 kcal/mol less than that of the wild-type enzyme. Using rational design, it was possible to restore the stability and maintain effective activities against racemic V-agent.[97] Unfortunately, when these enzymes were checked against the specific VX isomers, the specificity had been tailored almost exclusively for the less toxic isomer. Subsequently, OPH variants created using rational design successfully altered the stereospecificity toward VX. In one designed variant, a strong preference for the less toxic isomers was demonstrated, while a second variant demonstrated a reversal of the stereoselectivity and a catalytic preference for the most toxic VX isomer (Figure 1). To date, in spite of promising *in vitro* data, none of these designed or evolved enzymes have been able to afford *in vivo* protection against V-series agents. This emphasizes the challenges of designing enzymes as bioscavengers, substrate specificity and enantioselectivity without *in vivo* efficacy are simply interesting exercises in protein engineering.

A substantial body of work has detailed the toxicity of the individual isomers of the OP agents.[43,101–104] For the past decade, tailoring the stereospecificity of the catalytic bioscavengers has been the focus of several groups, and the efforts have produced remarkable success. In the directed evolution approach with hPON1 described earlier, five generations of mutagenesis yielded a single recombinant variant that can protect guinea pigs against multiple G-series agents.[52] A rational design approach implemented with the DFPase from *L. vulgaris* resulted in two quadruple mutants of DFPase with reversed enantioselectivity for G-type nerve agents.[62] Similarly, a rational approach implemented with OPH over a decade has resulted in variants of OPH designed and demonstrated to preferentially catalyze the P(−) isomers of GB.[105,106] Both the enantioselective hPON1 and OPH were achieved by reducing turnover of the less toxic enantiomer. So, although specificity for the more toxic enantiomer was achieved, the overall activity in both cases was low. In contrast, the DFPase variants demonstrated both enantioselectivity and an enhanced overall activity.[62]

There are two extremes with respect to the stereoisomers and catalytic efficiency. When the relative toxicity between isomers

Table 2. Conformational stabilities (k_{cal}/mol, determined by equilibrium urea denaturation and monitored by intrinsic tryptophan fluorescence) and kinetic constants of native OPH and variants.[97]

Enzyme		Paraoxon			Demeton-S			VX	VR
	ΔG°_{H2O}	k_{cat} s^{-1}	K_M (mM)	k_{cat}/K_M	k_{cat} s^{-1}	K_M (mM)	k_{cat}/K_M	turnover[b]	turnover[b]
H254, H257	25.3 ± 3.2	6900	0.1 ± 0.05	6.90x10^7	3.5	4.4 ± 1.7	7.95x10^2	14	12
H254R, H257L	19.6 ± 1.5	640	0.07 ± 0.01	9.14x10^6	50	6.2 ± 1.4	8.06x10^3	144	465
H254R,H257F	n.d.[a]	450	0.05 ± 0.02	9.00x10^6	34	7.6 ± 2.3	4.47x10^3	68	36

[a]n.d., not determined; this variant was incompletely denatured by either urea or guanidinium-Cl, and so the ΔG°_{H2O} could not be determined. The increase in stability was confirmed by proteolysis resistance, and temperature.

[b]μmol V-agent hydrolyzed/sec/μmol enzyme at 2 mM V-agent.

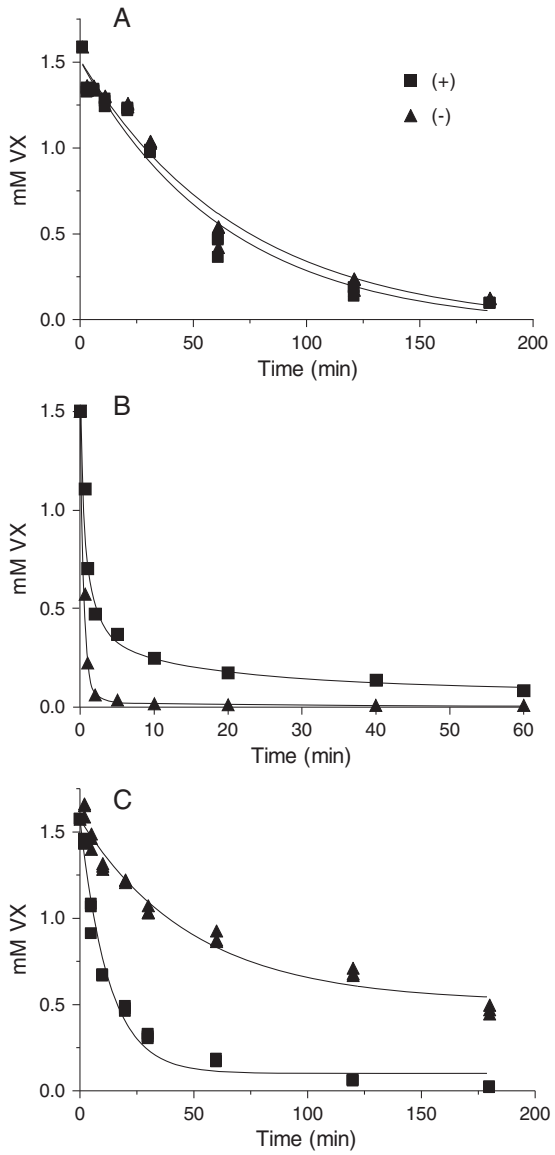

Figure 1. A series of rationally designed variants of OPH have successfully altered the native enzyme's stereospecificity toward VX. (A) The native OPH does not discriminate the VX isomers, hydrolyzing both at nearly identical rates. (B) In one designed variant, the rate of hydrolysis of both isomers was increased and a preference for the more toxic isomer was demonstrated. (C) A second variant demonstrated a reversal of the stereoselectivity and a catalytic preference for the least toxic VX isomer.

differs by several orders of magnitude, enzymes must have very good affinity for the more toxic isomer. If the bioscavenger is not able to distinguish the enantiomers, the less toxic isomer may functionally serve as a competitive inhibitor, preventing or slowing hydrolysis of the more toxic target. However, with substrates where isomer toxicity is more closely equivalent or in environments where racemization is supported, the bioscavenger needs to be less specific relative to its stereochemical preference, as a high enantioselectivity can prevent complete hydrolysis of the agent. Therefore, the goal is an enzyme selective for the more toxic stereoisomer, which would lead to a rapid decrease in toxicity, yet have broad enough selectivity to still be able to turn over the less toxic stereoisomer rapidly enough to ensure complete hydrolysis. This is the paradox that has kept bioscavengers from spanning the G- and V-series of nerve agents and may ultimately keep the ideal generalist variant, the 'silver bullet', out of reach for *in vivo* protection.

Pharmacokinetics

The pharmacokinetic parameters of the native OPH have been evaluated by a number of investigators following intravenous or intramuscular administration of the bioscavenger. Novikov *et al.* [107] demonstrated a dose independent clearing in guinea pigs at bioscavengers doses of 0.075 and 0.75 mg/kg. More than 90% of the bioscavenger activity was cleared from circulation within 4 h after administration via a carotid artery catheter, with a MRT of 1.1 ± 0.2 h (Table 3). Clearance was demonstrated to be coincident with protein elimination from circulation, rather than inhibition or loss of activity. These results are similar to those reported for the OPH homolog, OPDA.[96] Wistar rats were administered OPDA intravenously, and significant activity was apparent within 5 min, with peak enzymatic activity observed approximately 20 min after administration. In this case, clearance was observed to be dose dependent, with an *in vivo* half-life of 45 min at a dose of 0.15 mg/ kg, and 57.9 min at 1.5 mg/kg. Trovaslet-Leroy *et al.*[108] administered OPH (designated PTE) i.m. to rats at 4-week intervals. The T_{max} was reached at approximately 1.1 and 1.6 h, and the MRT was 2.2 and 3.2 h, for the first and second injection, respectively. The ability to deliver a bolus of catalytic activity that can begin immediately to serve as a bioscavenger of OP toxicants has obvious therapeutic implications. In industrialized countries, exposure of the civilian and military population from terrorist attacks, accidental industrial/agricultural exposure or release of OP pesticides, or as a secondary exposure resulting from a natural disaster are all scenarios that would potentially benefit from a safe, effective therapeutic bioscavenger.

Formulating for extended in vivo performance

To be useful in military scenarios, however, bioscavengers would need a vascular residence of sufficient length to serve as a pretreatment or prophylactic, which can be several days. In addition,

Table 3. Pharmacokinetic parameters of unmodified and PEGylated OPH enzymes.[107] Dose of OPH administered was 0.075 mg/kg (500–1000 U per animal).

	MW[a]	$t_{1/2}$-α, (h)	$t_{1/2}$-β (h)	AUC (U × h/mL)	CL (mL/h)	MRT (h)
Unmodified OPH		0.09 ± 0.07	0.86 ± 0.18	29.8 ± 4.9	24.6 ± 4.5	1.1 ± 0.2
OPH-PEG4	333	0.22 ± 0.30	1.28 ± 0.37	33.3 ± 7.0	10.7 ± 1.7	1.8 ± 0.5
OPH -PEG8	510	0.11 ± 0.05	2.52 ± 0.20	73.2 ± 7.7	7.1 ± 0.8	3.6 ± 0.3
OPH -PEG12	686	0.17 ± 0.07	3.89 ± 0.38	111.3 ± 30.3	4.5 ± 0.5	5.5 ± 0.5
OPH -PEG12[3]	2421	0.23 ± 0.09	32.49 ± 2.62	1173.8 ± 160	0.57 ± 0.03	46.7 ± 3.7

[a]MW of the PEG conjugate, in daltons.

there are those OP compounds that are sufficiently lipophilic that a successful therapeutic regime would potentially require multiple doses of the bioscavenger because of their slow release from fat stores. An alternative strategy would be to employ an enzyme carrier or encapsulation to present the bioscavenger in a manner that protects against the potential for adverse disposition and rapid clearing from the circulation. A variety of formulations and encapsulation of bacterial enzymes have been investigated for their impact on activity and *in vivo* efficacy; only a few have reported the pharmacokinetic parameters of the formulated enzyme. The first reported studies on *in vivo* protection using encapsulated OPH used erythrocytes, CRBCs, as the carrier.[109,110] Although CRBCs are no longer considered as a practical carrier system, these studies were successful in demonstrating that catalytic enzymes could be encapsulated within lipid membranes without being impeded in their scavenging function. With this recognition, researchers recruited liposomes as a delivery system for bioscavengers. Liposomes are small spherical systems that are synthesized from cholesterol and non-toxic phospholipids. Because they are natural materials, liposomes are considered attractive, harmless drug delivery carriers that can circulate in the blood stream for extended periods.[111] Other characteristics, such as their relatively easy preparation method, high encapsulation efficiency for small molecules, favorable pharmacokinetic properties and good *in vivo* tolerance make them an attractive candidate for use with bioscavengers. *In vivo* studies of OPH and OPAA encapsulated within liposomes[112–115] have demonstrated some promise as an intervention for organophosphorus intoxication (Table 4). When stabilized liposomes (SL) were employed to deliver OPH (SL-OPH) or OPAA (SL-OPAA) as the antagonist of the OP compounds, paraoxon (LD_{50} 0.85 mg/kg) or DFP (LD_{50} 4.55 mg/kg), SL-OPH demonstrated a 125 × LD_{50} when encapsulated. This was twice the protection afforded by the 2-PAM, atropine combination, and approximately 4× what was observed with the free enzyme. This was even more dramatic when SL-OPH was employed in combination with 2-PAM and/or atropine: the magnitude of prophylactic antidotal protection was 920 × LD_{50} and the therapeutic antidotal protection was 156 × LD_{50}.[115] Unfortunately, the encapsulation efficiency with OPH is less than 5%, and the purification process to separate encapsulated and free enzyme is difficult.

Table 4. Antidotal protection of mice by the encapsulated OPH and OPAA enzymes in dendritic polyer (DP) and liposome (SL). The encapsulated bioscavenger was administered 1 h prior to subcutaneous administration of the OP compound. [95,109,112]

OP compound	Antidotal System	LD_{50}, mg/kg	PR
DFP	Control	4.55	1.0
	OPAA	7.28	1.6
	DP – OPAA	12.3	2.7
	SL – OPAA	10.5	2.3
	2-PAM	5.5	1.2
	Atropine	8.2	1.8
	2-PAM + atropine	35.5	7.8
	2-PAM + atropine + DP-OPAA	111	24.6
	2-PAM + atropine + SL-OPAA	106	23.2
Paraoxon	Control	0.85	1.0
	OPH	28.5	33.5
	DP–OPH	81.2	95.5
	SL–OPH	106.4	125.2
	2-PAM	3.8	4.5
	Atropine	2.4	2.3
	2-PAM + atropine	51.9	61.1
	2-PAM + atropine + DP-OPH	666	780.0
	2-PAM + atropine + SL-OPH	782	920.0

More recently, dendritic polymers have garnered some attention as drug carriers, primarily due to their inertness relative to temperature, solvent, and pH. These polymers can be loaded with drugs, nucleic acid or protein in either the interior or attached to the surface groups. The *in vivo* efficacy of the nanostructures, DP-OPH and DP-OPAA, has been evaluated and DP-OPH provided a 95.5 × LD_{50} protection against paraoxon, while DP-OPAA demonstrated a more modest 2.7 × LD_{50} protection against DFP.[95] These studies demonstrate that catalytic bioscavengers can be encapsulated in a variety of systems without impacting their catalytic abilities. The impact of encapsulation, either with liposomes or dendrimers, on the stability, pharmacokinetics or immunogenicity of the bioscavengers was not evaluated, so it is not clear if these methods will address the safety or vascular residence requirements.

An alternative to encapsulation can be found in surface decoration through the covalent attachment of polymers such as polyethylene glycol (PEG). PEGylation has been shown to prolong circulation time in the blood, increase resistance to proteolytic digestion, reduce immunogenicity and antigenicity, and lower cytotoxicity.[116,117] In the case of proteins such as OPH, with molecular weight close to the exclusion limit of glomerular filtration (50–60 kDa), the use of relatively low-molecular weight PEGs has potential. Novikov *et al.* [107] performed a systematic study examining the effects of PEGylation on the biochemical and pharmacological characteristics of OPH. The enzyme was conjugated with linear and branched methyl-PEO_n-NHS esters of relatively small molecular mass from 333 to 2420 Da. PEGylated OPH displayed a decreased maximal catalytic rate, though substantial activity was maintained. Thermostability of the PEGylated enzymes defined a $t_{1/2}$ between 60 and 64 °C, compared to the unmodified OPH, which is approximately 67 °C.

The enzyme conjugates demonstrated a significant improvement of pharmacokinetic properties in animal studies. Unmodified enzyme had the fastest rate of elimination from circulation, with the mean residence time (MRT) increasing with increasing molecular weight of the PEG polymer (Figure 2). Although the shortest PEG polymer, PEG4, had a modest effect, increasing the circulated half-life from 51 to 80 min, when PEG8 and PEG12 were used for modification the circulation half-life, MRT and AUC more than doubled. This was evident by a decrease in the enzyme clearance, demonstrating that vascular residence of the conjugates correlates with the size of PEG conjugate. The intravascular residence was most enhanced when a branched PEG was used as a modifier. Paraoxonase activity and protein were above background in the plasma a week after administration, and the clearance was decreased more than 10 times relative to unmodified OPH, resulting in a 10x and 25x increase of residence time ($T_{1/2}$, MRT) and system availability (AUC).

Trovaslet-Leroy [108] performed similar studies using OPH conjugated with a 5 kDa methoxy-PEG. The catalytic properties of the PEGylated enzyme were not significantly modified compared to the native enzyme. Two doses of 34.5 μg/kg of both unmodified and PEG-OPH were administered i.m. to rats at a 4-week interval. The PEG-OPH had a Tmax of approximately 47.5 h. The activity level remained 50% elevated up to 80 h after

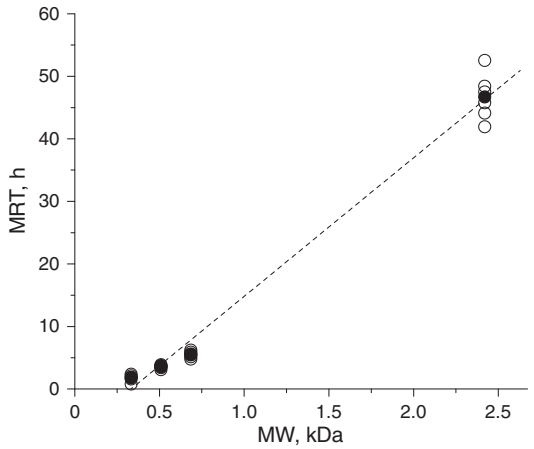

Figure 2. Illustration of the relationship between the mass of the PEG conjugate and the MRT of the bioscavenger. Four different amine-reactive PEGylation reagents were compared. Methyl-PEO_4-NHS (PEG4, MW 333.33 Da), methyl-PEO_8-NHS (PEG8, MW 504.54 Da), and methyl-PEO_{12}-NHS (PEG12, MW 685.71 Da) esters are linear molecules. The fourth reagent, (methyl-$PEO_{12})_3$-PEO_4-NHS ester (PEG12[3], MW 2420.8 Da), contains a PEO4 spacer between the NHS leaving group and three methyl-PEO12 branches. Open circles, replicates; closed circles, average.

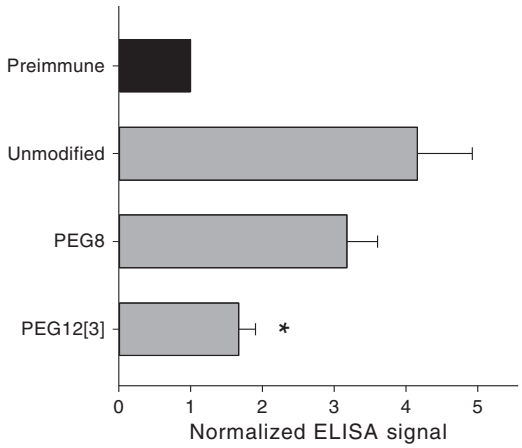

Figure 3. Immunogenicity of non-modified and PEGylated WT-OPH. The results of direct ELISA for plasma samples collected three weeks after intravenous administration of non-modified (gray) or PEGylated (light gray) enzymes.[107] The OD values at 405 nm in each of individual experiments were normalized to values of pre-immune plasma. Average values from 4 to 6 animals and standard errors are presented. Statistical significance of observed difference was estimated with ANOVA test; the values of $P < 0.05$ are labeled with asterisks.

administration, with an MRT of approximately 48 h. This is a virtually identical to the MRT that was reported for the branched PEG reagent.[107] (The branched PEG had a mass of approximately half that of the linear PEG reagent used in this study.)

Immunogenicity

Immunogenicity is one of the most important challenges in protein drug development.[118] Both Novikov[107] and Trovaslet-Leroy[108] reported evidence of immune response to the bioscavengers, although no animals displayed any sign of clinical toxicity or anaphylaxis. Novikov reported an increase in OPH-specific antibodies by all bioscavengers, both unmodified and PEGylated. The linear PEG moieties resulted in a slight reduction of anti-OPH IgG levels in plasma relative to the unmodified enzyme; the longer chains did not appear to result in improvement of OPH protection against the immune system. However, the branched PEG resulted in a significant decrease in anti-OPH antibody production (Figure 3). Trovaslet-Lery reported that the Tmax, peak levels and MRT were decreased about 30% and AUC was decreased 2.4-fold for conjugated PTE in the second administration compared to the first. This suggests that the enzyme was more rapidly eliminated, indicating that repeated use of the bioscavenger may result in diminished efficacy through immune-sequestration or elimination.

Conclusion

Currently, there is no single candidate that satisfies the requirements for an ideal bioscavenger. Candidate bioscavenger proteins, in general, function either by stoichiometrically binding and sequestering the OP toxicant or by catalytically hydrolyzing the compound into biologically inactive products. The stoichiometric proteins, such as butyrylcholinesterase, offer the advantage of exceptional affinity for both G- and V-series agents. However, since they have the capacity to bind no more than one molecule of nerve agent per molecule of protein bioscavenger, the extent of protection is directly proportional to the number of molecules of active bioscavenger in the bloodstream. With a high mass ratio, the amount of BuChE required to afford protection is unwieldy to deliver and costly to produce. This challenge can be addressed by the catalytic bioscavengers, such as hPON1. As a cardioprotective enzyme found in human serum, it offers the same safety and vascular residence advantages of hBuChE, unfortunately it's catalytic efficiency is not sufficient, and production is difficult. The challenge of production and catalytic efficiency can be addressed with an evolved variant, which can be produced transgenically in *E. coli*, making it a good candidate for engineering. However, it is no longer a human enzyme, so will have to overcome all the associated challenges: short circulating half-life and immunogenicity. If microbial enzymes gain acceptance as bioscavengers, the pool of OP hydrolyzing activities and homologues is rich and diverse. The bacterial OPHs are the most efficient of the known catalytic enzymes with all the organophosphorus compounds. Although the current panel of enzymes does not have sufficient catalytic capacity to be the desired generalist, some of them (such as OPH) have such an intrinsically broad substrate specificity that tailoring their activity is not only possible, but has been demonstrated. Variants of OPH have been created with increased stability, with specificity for the more toxic isomers of both G- and V-agents, and they are economical and easy to produce. Still, they are of non-human origin, with the associated immunogenicity and pharmacokinetic challenges. However, with the ability to produce these enzymes in a diversity of production systems, including large-scale fermentation, the economics for surface modification or formulation become acceptable. Almost two decades of encapsulation studies with OPH have demonstrated that it can be encapsulated in a variety of systems without impacting its catalytic abilities. Recent studies with surface modification using PEG conjugates demonstrated that this formulation can prolong circulation time in the blood, and reduce immunogenicity and antigenicity. There is no evidence of any cytotoxicity associated with OPH or PEGylated formulations. The mesophilic enzyme currently being investigated comes as close as any of the human proteins to fulfilling the requirements of the ideal bioscavenger, but there is an untapped reservoir of thermophilic and other extremophile sources to explore.

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