Stoichiometric and Catalytic Scavengers as Protection against Nerve Agent Toxicity

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ABSTRACT

The use of a human protein such as plasma-derived butyrylcholinesterase (huBuChE) to neutralize the toxic effects of nerve agents in vivo has previously been shown to provide both survival in guinea pigs and non-human primates and protection against decreased cognitive function in guinea pigs following exposure to a cumulative challenge of 5.5 LD_{50} of soman. Subsequently, a recombinant form of human butyrylcholinesterase (r-huBuChE) has become available. This material was biochemically similar to plasma-derived huBuChE in in vitro nerve agent inhibition assays. Pharmacokinetic analysis of a polyethylene glycol coated (pegylated) form of r-huBuChE revealed rapid bioavailability and a half-life ($t_{1/2}$) which resembled that of plasma-derived huBuChE. When guinea pigs were administered pegylated r-huBuChE 18 h prior to exposure (sc) to 5.5 LD_{50} VX or soman, 100% survival was observed. These data supported the decision to select both plasma-derived and recombinant forms of BuChE for advanced development and transition to clinical trials. Efforts are now focused on identification of a catalytic protein capable of hydrolyzing the standard threat nerve agents. Work to date has focused on paraoxonase-1 (PON1), a naturally occurring human serum enzyme. Using rational design several amino acids involved in substrate binding have been identified, and site-directed mutations have revealed that residue H115 plays an important role in determining substrate specificity. The effect of these mutations on the ability of PON1 to catalyze the hydrolysis of nerve agents was investigated. In addition, the stereospecificity of PON1 for the catalytic hydrolysis of soman has been examined. The enzyme exhibits a slight stereospecificity for the C+P+ isomer of soman, consisting of both a lower K_M (higher

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affinity) and higher k_{cat} (more rapid hydrolysis) than for other soman isomers. Current studies are directed toward the identification of a PON1 mutant with enhanced catalytic activity for nerve agents as a next generation bioscavenger.

1.0 BACKGROUND

While the current therapies for nerve agents are effective in preventing lethality, they do not prevent performance deficits, behavioral incapacitation, loss of consciousness, or the potential for permanent brain damage (Maynard and Beswick 1992). In an attempt to identify alternative approaches to protect against organophosphorus (OP) poisoning, efforts have focused on identifying human proteins that can remain stable in circulation for long periods of time (Dacre 1984; Ballantyne and Marrs 1992; Maynard and Beswick 1992) while acting as biological scavengers for OP compounds. Biological scavenger function must be very rapid, irreversible and specific. Ideally, the scavenger should have a prolonged circulation time, be biologically innocuous in the absence of OP compounds, and not present an antigenic challenge to the immune system. Theoretically, this approach would avoid the side effects commonly associated with current therapies (de Candole et al. 1953; Stewart 1959; Grob 1963; Stewart and Anderson 1968; Bajgar et al. 1971; Brimblecombe 1977; Somani et al. 1992; Taylor 2001; Mesulam et al. 2002). As a prophylactic, human proteins can provide protection by inactivating (through sequestration or hydrolysis) OP compounds before they can react with the target acetylcholinesterase (AChE). The time frame for this inactivation must occur before endogenous AChE is affected (approximately two minutes after an OP exposure in humans (Heffron and Hobbiger 1979)). For these reasons, efforts to identify candidate bioscavengers focused initially on plasma-derived human butyrylcholinesterase (HuBuChE) (Lenz et al. 2005; Saxena et al. 2005; Sun et al. 2005; Doctor and Saxena 2005; Lenz et al 2007), with additional efforts focused on recombinant human butyrylcholinesterase (r-HuBuChE) (Cerasoli et al. 2005a, Welsh et al. 2006, Huang et al. 2007).

2.0 STOICHIOMETRIC BIOSCAVENGERS

A number of different enzymes capable of reacting with OP inhibitors but not catalyzing their hydrolysis have been tested over the past 15 years for their ability to provide protection against OP poisoning in vivo beginning with the work of Wolfe et al. (1987), who first reported the use of exogenously administered fetal bovine serum-derived acetylcholinesterase (FBS-AChE) as a bioscavenger in mice. Broomfield and co-workers (1991) reported that equine butyrylcholinesterase (eq-BuChE) alone afforded complete protection against a 2 x LD_{50} challenge dose of soman in rhesus monkeys and against 3 to 4 x LD_{50} doses when atropine was administered (post-exposure). Protection against a single LD_{50} dose of sarin was also demonstrated. There were no fatalities in any of these cases. When animals were assessed for behavioral deficits using a serial probe recognition (SPR) task, they all returned to baseline performance within nine hours after soman exposure (Castro et al. 1994). Similarly, Maxwell et al. (1992) reported that in experiments with rhesus monkeys no performance decrements (assessed by SPR task) were observed in animals treated with FBS-AChE alone when compared with animals pre-treated with FBS-AChE followed by soman challenges at either 1.5 or 2.5 x LD_{50}. Wolfe et al. (1992) assessed the ability of pre-treatment with either FBS-AChE or eq-BuChE to protect rhesus monkeys against multiple LD_{50} doses of soman. The animals that received FBS-AChE as a pre-treatment were protected against a cumulative exposure of 5 x LD_{50} of soman and showed no decrement in the primate equilibrium platform (PEP) task.

The ultimate goal of scavenger molecule research is to generate a means to protect humans from the toxic effects of nerve agents. In an effort to minimize any physiological, immunological or psychological side effects of scavenger use in humans, more recent research efforts focused primarily on the use of HuBuChE. In a series of studies, Ashani and co-workers (1991) examined the scavenger properties of FBS-AChE and

particularly plasma-derived HuBuChE in mice, rats and rhesus monkeys against several different nerve agents as well as other OP compounds (Raveh et al.1993; 1997). Following administration of exogenous cholinesterase, there was a linear correlation between the concentration of cholinesterase in the blood and the level of protection against OP poisoning. It was also observed that a scavenger had to be present before exposure to the OP compound, because the nerve agent must be scavenged within one blood circulation time period (Raveh et al. 1993), which is about seven minutes in humans. In the final paper of this series, the authors reported similar protective results against a 3.3 x LD_{50} dose of soman or a 2.1 x LD_{50} dose of VX in rhesus monkeys (Raveh et al. 1997). Considerable protection against soman-induced behavioral deficits in a spatial discrimination task was also observed. More recently, Lenz et al. (2005) determined the pharmacokinetics and efficacy of HuBuChE in guinea pigs and cynomolgus monkeys against multiple LD_{50} challenges of nerve agents. The half-time for elimination of the plasma-derived HuBuChE was about 73 hours for guinea pigs or cynomolgus monkeys, respectively. Guinea pigs were protected against a cumulative 5.5 x LD_{50} dose of either soman or VX (Table 1). At necropsy, seven or fourteen days after surviving the nerve agent challenge, all tissues appeared normal upon light microscopic examination. Cynomolgus monkeys were protected against a cumulative challenge of 5.5 x LD_{50} of soman as well. Of the six animals challenged (Table 1), one died after the final challenge dose of soman (total dose of 5.5 x LD_{50} within four hours) and one was euthanized 48 hours after the final dose of soman. The remaining animals displayed neither short-term signs of poisoning nor signs of lasting consequences as revealed by blood chemistry examinations and long-term (> 20 months) observations. These studies were complimented by the work of Saxena et al. (2005), who measured the in vitro stability of plasma-derived HuBuChE. When plasma-derived HuBuChE was administered to mice or rhesus monkeys at a dose in 10-fold excess of that needed for protection against a 5 x LD_{50} challenge of soman, no deficits on a variety of behavioral tasks were observed (Sun et al. 2005). Likewise, 14 days after guinea pigs were administered 60 mg/kg of HuBuChE (~10-fold higher than the assumed mg/kg human dosage for protection against a 5 x LD_{50} dose of soman), no changes were observed upon examination of histopathological, hematological, or serum chemistry parameters (Doctor and Saxena 2005).

The supply of plasma-derived HuBuChE is dependent on the availability of outdated human blood, which is in turn dependent on the extent of donor participation and the fluctuating need for blood in response to natural disasters or unforeseen medical emergencies. To identify a more reliable source of HuBuChE, research efforts were focused on the development of recombinant expression systems. If successful, such efforts would enable a constant supply of material at reproducible purity and activity, thus eliminating a dependence on whole blood supply. r-HuBuChE material purified from the milk of transgenic goats (Cerasoli et al. 2005b, Huang et al. 2007; Lenz et al. 2007) has been the most extensively studied. Goat-milk-derived r-HuBuChE has a different glycosylation pattern than that of the plasma-derived material (Garcia et al. 2005). This difference in glycosylation patterns is presumed to account for the fact that goat-milk-derived r-HuBuChE has a different pharmacokinetic profile, with a much shorter circulatory half-life, than plasma-derived HuBuChE. To enhance its biological residence time, goat-milk-derived r-HuBuChE was modified to include polyethylene glycol adducts (pegylated). The pegylated enzyme had a pharmacokinetic profile very similar to that of the plasma-derived BuChE (Cerasoli et al. 2005a: Huang et al. 2007), suggesting that differences in pharmacokinetics between plasma purified and recombinant enzymes can be addressed using in vitro post-translational modifications (Chilukuri et al. 2005). Studies with r-HuBuChE from transgenic goat milk have yielded efficacy results in guinea pigs (Table 2) that were very similar to those described previously with plasma-derived material, i.e., complete protection against 5.5 x LD_{50} of GD or VX (Cerasoli et al. 2005a). These preliminary results suggest that safe and effective recombinant stoichiometric bioscavengers can be developed, potentially providing a source for sufficient material to supply both the entire military force and possible domestic users such as first responders, emergency medical personnel, and agricultural workers that may be occupationally exposed to oxon-type OP pesticides, e.g., paraoxon, chlorpyrifos-oxon.

3.0 CATALYTIC BIOSCAVENGERS

While stoichiometric scavengers afford good protection as long as they reside at high levels in circulation, they are molecules of high relative molecular weight (~86 kDa per binding site for BuChE), and a comparatively large quantity is required to neutralize an equimolar amount of nerve agent. A catalytic scavenger, even one with the same high molecular weight, could be administered in smaller quantities to produce the same or greater extent of protection against OP poisoning. A catalytic scavenger has the advantage of not being consumed in the process of detoxifying the nerve agent, so it would be available to protect against multiple exposures to either high or low doses of OP inhibitors. To date, enzymes with catalytic anti-OP activity have been identified from a variety of sources, such as the organophosphorus anhydride hydrolase (OPAH) from Pseudomonas diminuta (Serdar and Gibson 1985; Broomfield 1992; 1993), the prolidase from Alteromonas haloplanktis (Cheng et al. 1996), and human serum paraoxonase 1 (HuPON1) (Gan et al. 1991; Josse et al. 1999; Josse et al. 2001; Masson et al. 1998; Tuovinen et al. 1999). Theoretically, a functional catalytic scavenger must have both a lower K_M (inversely proportional to the strength of binding of a substrate to the enzyme) and a higher k_{cat} (turnover number) for nerve agents than have been found to date among these naturally occurring catalytic enzymes. The HuPON1 enzyme has been identified as having a similar potential to that of the bacterial OPAH for affording protection but without the potential complication of inducing an immune response. It is important to note that in the case of HuPON1, the native enzyme was capable of catalytically hydrolyzing nerve agents, albeit with poor catalytic efficiency, i.e., modest affinity and a slow rate of turnover. Since OP compounds are "accidental" substrates for PON (Masson et al. 1998; Tuovinen et al. 1999), it is likely that improvement in activity can be realized through protein engineering. Unfortunately, protein engineering requires both knowledge of the three-dimensional structure of the enzyme and a detailed understanding of active site residues, which are both poorly understood at present.

Recent works by Josse et al.(1999 ; 2001) Harel et al. (2004a; 2004b), Aharoni et al. (2004), and Yeung et al. (2004; 2005) have partially addressed these complications regarding development of HuPON1 as a nerve agent bioscavenger. Josse et al. (1999, 2001) have postulated that, based both on site-directed mutations of amino acids believed to be at or near the active site of HuPON1 and on limited sequence homology with a DFPase, the molecule is a six-fold beta propeller (Figure 1). Meanwhile, Harel et al. (2004a) and Aharoni et al. (2004), using a bacterially expressed mouse-rat-rabbit-human chimera of PON1 obtained through gene shuffling experiments, confirmed the postulated structure through X-ray crystallographic studies. Subsequently, Yeung et al. (2004) have carried out numerous site-directed mutation studies to identify and ‘map’ amino acid residues critical for binding and/or catalytic activity (Table 3). More recent efforts have revealed a subtle but appreciable degree of stereospecificity in the hydrolysis of soman by native HuPON1, with the least toxic soman stereoisomer (C+P+) being hydrolyzed approximately six times more efficiently than the most toxic one (C-P-; Yeung et al. 2007). The observed stereospecificity is due primarily to preferential binding rather than enhanced turnover of the C+P+ soman stereoisomer by HuPON1 (Table 4).

The capacity of wild-type and mutant HuPON1 enzymes to hydrolyze the structurally isomeric nerve agents VX and VR, which both contain thiol leaving groups, was examined by Yeung et al. (submitted) using a modified Ellman assay (Ellman et al. 1961). For wild type HuPON1 an approximate five-fold difference in binding affinity (K_M) was observed between the two V-agents, with an accompanying seven- to eight-fold difference in the rates of turnover (Figure 2, Tables 5 and 6). Though the wild-type HuPON1 enzyme binds VX with less affinity than VR, the k_{cat} value suggests that once bound, VX is quickly hydrolyzed. Interestingly, the opposite appears to be the case for VR hydrolysis, where the substrate is easily bound, but the rate of turnover is much lower. Despite the differences in both binding affinity and turnover rates, the catalytic efficiency (k_{cat}/K_M) of wild-type HuPON1 for the two V-agents differs by less than two-fold.

When a variety of mutants of VX were made, based on examination of the structural models of PON1, it was found that in addition to the importance of residue S193 in the hydrolysis of soman (GD), mutating S193 to A or G also affected the kinetics of hydrolysis for VX and VR with a more pronounced effect observed in the S193A mutant. A three- to four-fold enhancement relative to wild-type HuPON1 in both turnover rate and catalytic efficiency was observed for VX and VR hydrolysis by the S193A mutant. Likewise, enhanced turnover of both V-agents was also detected in the R214Q mutant. In contrast, the double mutant S193A/R214Q did not display enhanced hydrolysis of either VX or VR; rather, the k_{cat} and k_{cat}/K_M values for this double mutant were restored to wild-type levels. These results suggest that multiple mutations that individually result in enhanced activity against one or more OP nerve agents may not exhibit additive or synergistic effects when they are simultaneously present on the same molecule.

In comparison to wild-type HuPON1, the H115W mutant has reduced activity against VX and no detectable activity against VR (Figure 3). Since H115W-mediated turnover of VR was not observed, a competitive assay experiment using VX as the reporting substrate was carried out to determine whether either of the two non-hydrolyzed nerve agents, VR or GD, was able to bind to the enzyme. Both VR and GD were found to competitively inhibit VX hydrolysis by the H115W mutant, with K_I values of 0.27 ± 0.09 mM and 0.90 ± 0.38 mM, respectively. These findings suggest that altering a single residue in the active site, i.e., H115, can dictate the specificity of OP substrate binding and hydrolysis for a variety of OP nerve agent substrates. Based on the results of these studies, residue H115 appears to be important for substrate docking in the active site of HuPON1, but is most likely not directly involved in the catalytic hydrolysis of at least OP substrates. Since all of the substrates tested with H115W can bind to the active site (as judged by either substrate hydrolysis or competitive inhibition), it is likely that residue 115 is important for proper orientation of the substrate(s).

These efforts offer valuable insights for the detailed analysis of the mechanism(s) involved in HuPON1 catalysis. The results have identified several active site amino acid residues that can influence both the stereoselectivity and specificity of substrate hydrolysis by HuPON1. Most importantly, the results will inform future genetic engineering efforts to enhance the OP hydrolase activity of HuPON1 against highly toxic OP nerve agents. Based on the relatively limited panel of HuPON1 mutants analyzed to date, if HuPON1 is to be used as an effective bioscavenger against OP nerve agent intoxication, the best approach may be the utilization of a mixture of HuPON1 mutants, each with specificity for a different nerve agent.

4.0 RELATED NATO EFFORTS

The status of bioscavenger research summarized here has, due to space constraints, omitted a host of related efforts carried out in the laboratories of several of the NATO countries. To ensure recognition of the scope of the bioscavenger effort in the NATO community over the past 10 years, a highly condensed summary of those efforts is included in this section.

A significant program on the structure and chemistry of PON has been carried out at the French laboratories at CRSSA (Josse et al. 1999, 2001, 2002; Masson et al. 1998; Rochu et al. 2007 and references therein). In addition, this group has reported on the crystallization of BuChE (Nicolet, et al. 2003) as well as examined the interaction of OPs with this enzyme (Nachon, et al. 2005 and references therein).

Innovative physiological-based pharmacokinetic (PB/PK) studies on the nerve agent soman carried out at the TNO laboratories in The Netherlands (Benschop et al. 1995; Benschop and deJong, 1991; Langenberg, et al. 1997; Spruit et al. 2000) have now been amplified by complimentary efforts in the US to include the effects of administration of a bioscavenger on the toxicity of a nerve agent (Sweeney and Maxwell, 2003; Sweeney et al.

2006). These combined efforts will allow for realistic estimates of human doses of bioscavengers when such drugs are submitted for approval by regulatory agencies such as the US Food and Drug Administration (USFDA). Additional efforts in the Czech Republic have focused on examining the breadth of efficacy afforded by bioscavengers (Sevelova et al. 2004).

5.0 SUMMARY

The threat of OP nerve agent usage not only against military personnel in the field but also against the public at large is quite real (Ember 1991, Aas 2003). Terrorist groups have already used nerve agents against a civilian population, and due to their low cost and relative ease of synthesis, they are likely to be used again in the future (Masuda et al. 1995). Compounding the concern is the fact that many commonly used pesticides and chemical manufacturing by-products act as anticholinesterases, posing an occupational low-dose exposure hazard to workers in a variety of professions. The use of anticholinesterase pesticides against civilians in a terrorist context also exists as a possibility. Current therapeutic regimes for acute nerve agent exposure are generally effective at preventing fatalities if administered in an appropriate time frame. While the use of nerve agents on the battlefield may be somewhat predictable, their use in a terrorist situation will be, in all probability, an unanticipatable event. The potential to afford long-term protection to first responders at risk for exposure to toxic or incapacitating concentrations of OP inhibitors is a notable advantage of biological scavengers.

In addition, the use of bioscavengers has several psychological benefits that are likely to result in a higher degree of user acceptability than exists for conventional therapy. No post-exposure auto-injectors are necessary, and protection is afforded with little chance of short- or long-term side effects. Of particular significance is the fact that current candidate bioscavenger proteins are, exclusively, enzymes of human origin. From a scientific standpoint, these proteins are good candidates because they are less likely to be recognized by cells of the immune system and will enjoy prolonged residence times in circulation. From a user point of view, individuals are in essence being protected against nerve agents using a substance that their bodies already produce, rather than being injected with drugs and enzyme inhibitors that alone can produce potent side effects; such a distinction may enhance the comfort and compliance of end users.

Based on the data summarized above, the decision was made by the US Army in October 2004 to transition plasma-derived HuBuChE to advanced development. HuBuChE currently has been granted investigational new drug (IND) status by the USFDA. It is being produced under good manufacturing practice (GMP) by Baxter BioScience, using outdated human blood as the source material. This product is currently undergoing Phase Ia human safety trials. More recently (February 2006), r-HuBuChE was also transitioned to advanced development, although the details for GMP production and human clinical safety trials for this material have not yet been established. HuBuChE has dual use potential for both military personnel and civilian first responders, and a decision about how to proceed to development of one of these products to full licensure is under consideration.

The next phase in these efforts is to design a recombinant version of a naturally occurring human enzyme that is capable of being developed as a catalytic biological scavenger to protect against nerve agent poisoning. Since HuPON1 is a naturally occurring plasma enzyme produced in the liver, an alternative approach might be to enhance endogenous enzyme biosynthesis by inducing increased activity of the HuPON1 gene promoter. Recent results on increased expression levels of HuPON1 by HuH7 hepatoma cells upon action of fibrates are promising in this regard (Gouédard et al. unpublished).

While the catalytic enzyme human PON 1 has not yet been tested in mammalian systems, it is indicative of the types of therapies that may soon be available for use in animals and eventually in humans. While r-HuBuChE and mutants of HuPON1 are based on human proteins, it is recognized that the immunogenicity and serum half-life of the scavenger(s) must be determined in humans, and that efforts may be required to minimize the former and maximize the latter. Additionally, appropriate dosages of scavenger(s) must be determined that will, based on animal models, protect against concentrations of nerve agents likely to be encountered under a wide range of scenarios. While the research efforts of numerous NATO laboratories to date have resulted in the successful transition to advanced development of stoichiometric scavengers, the use of either naturally occurring or genetically engineered enzymes with catalytic activity holds the greatest theoretical promise for the development of a broad specificity, high efficacy prophylactic scavenger. Current efforts in several countries are now focused on designing and expressing such enzymes, and characterizing their *in vivo* anti-nerve agent efficacy in animal models acceptable to the USFDA or other regulatory agencies in NATO countries.

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Table 1: Plasma-Derived Human Butyrylcholinesterase (HuBuChE) Protection against Nerve Agent Poisoning in Guinea Pigs and Monkeys

Protection	Species	Nerve Agent	Dose LD 50
Hu BuChE	Guinea Pig (N=10)	VX	5 +
		GD	5 +
Hu BuChE	Cynomolgus Monkey (N=6)	GD	5+

Table 2: Recombinant Goat Milk Human Butyrylcholinesterase (HuBuChE) Protection against Nerve Agent Poisoning in Guinea Pigs

Pre-treatment	OP	Therapy*	Dose LD 50	Survival	Toxic Signs
PEG-rBuChE	VX	None	5.5	12 / 12	Minimal to none
PEG-rBuChE	GD	None	5.5	12 / 12	Minimal to none
PEG-rBuChE	None	None	None	8 / 8	Minimal to none
None	None	None	None	12 / 12	Minimal to none
None	VX	ATR/2-PAM/ Diazepam	1.5	10 / 10	Mild to moderate (6 / 10), Severe (4 / 10)
None	GD	ATR/2-PAM/ Diazepam	1.5	2 / 4	Severe (4 / 4)

* Atropine (ATR), 2-pralidoxime Cl (2-PAM)

Table 3: Site-Directed Mutations of Human PON1 and Effect on Enzymatic Activity

		Phenyl Acetate		Paraoxon	
Predicted Role	reHuPON1	K_m*(mM)	%K_m to wt (a)	K_m*(mM)	%K_m to wt (a)
N-Terminus Signaling Peptide	G11A	0.74	121	0.18	113
	G11C	0.74	121	0.19	119
	G11S	0.64	105	0.18	113
Catalytic Calcium Binding	N168E	nd	-	nd	-
	N224A	nd	-	nd	-
	D269E	nd	-	nd	-
Structural Calcium Binding	D54N	nd	-	nd	-
Substrate Binding Site	L69F	nd	-	nd	-
	H115W	nd	-	0.42	262
	N133S	0.59	97	0.17	106
	H115W/N133S	nd	-	0.15	83
	H134W	nd	-	nd	-
	H134Y	nd	-	nd	-
	F222D	nd	-	nd	-
	F222Y	0.89	146	nd	-
	N224A	nd	-	nd	-
	C284D	nd	-	nd	-
Catalytic Site	H285D	nd	-	nd	-
	H285Y	nd	-	nd	-
Surface Residue	E313A	0.51	84	0.14	88
	E314A	0.67	106	0.19	119
	V304A	nd	-	nd	-

(a) Relative to recombinant wild-type K_m values for Phenyl Acetate (K_m = 0.61 mM) and Paraoxon (K_m = 0.16 mM) as substrates, respectively

nd = not detectable at 3.3 mM and 2.6 mM substrate concentrations for Phenyl Acetate and Paraoxon, respectively

*K_m values are averages of at least three experiments with supernatants from independent transfections

Table 4: Affinity and Turnover of Each of the Four Soman Stereoisomers by HuPON1

GD Isomer	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($mM^{-1}min^{-1}$)
C+P+	0.27 ± 0.08	1030 ± 94	3814
C-P+	0.58 ± 0.23	593 ± 54	1022
C+P-	0.71 ± 0.49	553 ±163	779
C-P-	0.91 ± 0.34	501 ± 45	550

Table 5: Kinetic Parameters for the Enzymatic Hydrolysis of VX by Recombinant HuPON1 Enzymes

Samples	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})
Wild-type	2.74 ± 1.51	107.91 ± 31.13	44.99 ± 12.51
H115W	2.58 ± 0.56	53.91 ± 7.80	21.37 ± 8.06
S193G	2.14 ± 0.45	152.26 ± 65.97	69.41 ± 16.28
S193A	3.33 ± 0.24	393.25 ± 0.76	118.29 ± 16.28
R214Q	2.08 ± 0.56	284.48 ± 47.03	138.98 ± 15.06
S193A/R214Q	3.32 ± 1.79	125.30 ± 30.19	41.89 ± 11.05

Results shown are the average and standard deviation of at least three independent ($n \geq 3$) transfections.

Table 6: Kinetics of VR Hydrolysis Mediated by Recombinant HuPON1 Enzymes

Samples	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})
Wild-type	0.57 ± 0.08	14.37 ± 1.03	25.49 ± 5.23
H115W	ND	ND	ND
S193G	0.31 ± 0.02	11.99 ± 3.72	39.71 ± 15.62
S193A	0.39 ± 0.12	40.98 ± 12.05	107.61 ± 17.80
R214Q	0.48 ± 0.14	35.93 ± 6.32	75.93 ± 9.18
S193A/R214Q	0.43 ± 0.05	9.47 ± 0.63	22.34 ± 2.19

Results (averages and standard deviations) shown were determined from at least three independent ($n \geq 3$) transfections. ND: Catalysis was not detectable at VR concentrations ≤ 1.80 mM.

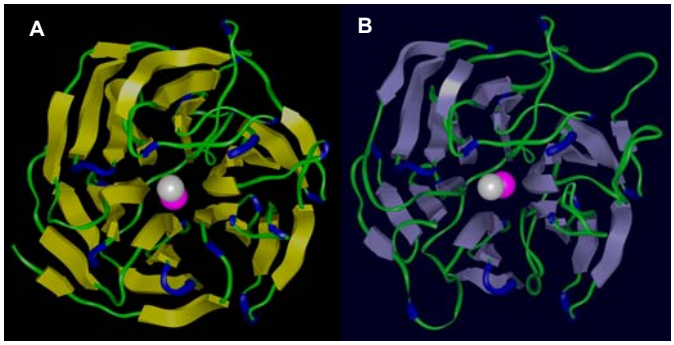

Figure 1: Crystal structure of DFPase used as a template to model a proposed secondary structure of HuPON1 (Yeung et al. 2004; Josse et al. 2002). (A) View of a ribbon diagram of the DFPase structure as determined from x-ray crystallography experiments (Scharff et al. 2001). (B) Ribbon diagram representation of the proposed secondary structure of HuPON1 (PDB: 1XHR) viewed along the axis with the catalytic calcium (white) above the structural calcium (pink). The model displays a slightly distorted six-bladed β-propeller shown in the same relative orientation as (A).

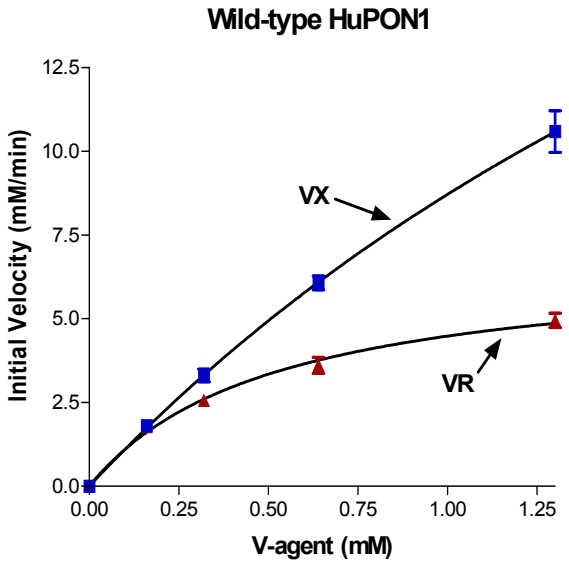

Figure 2: Kinetics of V-agents hydrolysis. Michaelis-Menten plots for the hydrolysis of VX (■) and VR (▲) by wild-type (HuPON1). K_M for VX = 2.74mM vs. K_M for VR = 0.57 mM whereas k_{cat} of VX = 108 min^{-1} vs. k_{cat} of VR = 14.4 min^{-1}.

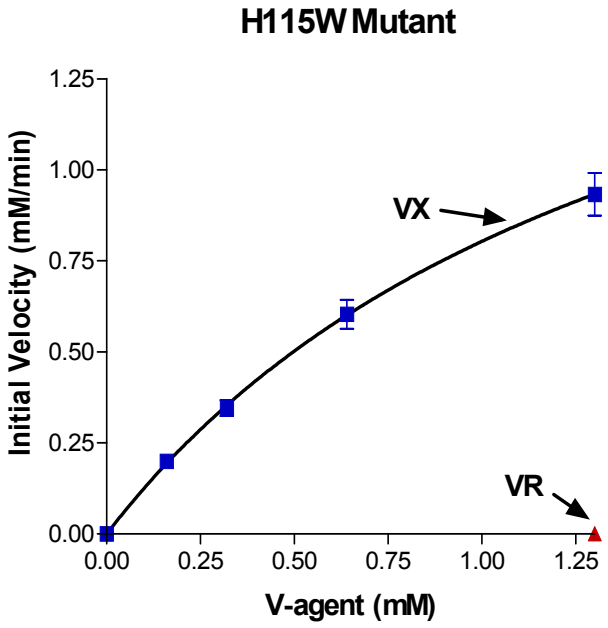

Figure 3: Michaelis-Menten plots for the hydrolysis of VX (■) and VR (▲) by mutant H115W recombinant HuPON1.