Investigation of Evolved Paraoxonase-1 Variants for Prevention of Organophosphorous Pesticide Compound Intoxication

David G. Mata, Peter E. Rezk, Praveena Sabnekar, Douglas M. Cerasoli, and Nageswararao Chilukuri

Physiology and Immunology Branch, Research Division, United States Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland

Received January 29, 2014; accepted April 3, 2014

ABSTRACT

We investigated the ability of the engineered paraoxonase-1 variants G3C9, VII-D11, I-F11, and VII-D2 to afford protection against paraaxon intoxication. Paraaxon is the toxic metabolite of parathion, a common pesticide still in use in many developing countries. An in vitro investigation showed that VII-D11 is the most efficient variant at hydrolyzing paraaxon with a kcat/Km of 2.1 × 106 M⁻¹ min⁻¹ and 1.6 × 10⁵ M⁻¹ min⁻¹ for the enzyme expressed via adenovirus infection of 293A cells and mice, respectively. Compared with the G3C9 parent scaffold, VII-D11 is 15- to 20-fold more efficacious at hydrolyzing paraaxon. Coinciding with these results, mice expressing VII-D11 in their blood survived and showed no symptoms against a cumulative 6.3 × LD50 dose of paraaxon, whereas mice expressing G3C9 experienced tremors and only 50% survival. We then determined whether VII-D11 can offer protection against paraaxon when present at substoichiometric concentrations. Mice containing varying concentrations of VII-D11 in their blood (0.2–4.1 mg/ml) were challenged with doses of paraaxon at fixed stoichiometric ratios that constitute up to a 10-fold molar excess of paraaxon to enzyme (1.4–27 × LD50 doses) and were assessed for tremors and mortality. Mice were afforded complete asymptomatic protection below a paraaxon-to-enzyme ratio of 8:1, whereas higher ratios produced tremors and/or mortality. VII-D11 in mouse blood coeluted with high-density lipoprotein, suggesting an association between the two entities. Collectively, these results demonstrate that VII-D11 is a promising candidate for development as a prophylactic catalytic bioscavenger against organophosphorous pesticide toxicity.

Introduction

Pesticides benefit society by increasing food production, preserving produce, and combating insect infestations. However, because of their inherent toxicities, pesticides also have the potential to be detrimental. The US Environmental Protection Agency (EPA) estimates that 10,000–20,000 physician-diagnosed pesticide poisonings occur each year among the 2 million agricultural workers in the United States. Workers in industries such as pesticide manufacturing, groundskeeping, pet grooming, and building fumigation are also at risk for exposures to pesticides and insecticides. The World Health Organization (WHO) estimates that ~3 million pesticide poisonings occur each year worldwide, with 300,000 people dying each year in the Asian-Pacific region alone as a result of self-poisoning with pesticides (WHO, 1986; WHO, 1990). The toxicity of organophosphorous (OP) pesticides and chemical warfare nerve agents arise from the inhibition of the enzyme acetylcholinesterase (AChE) at neuromuscular junctions and nerve synapses, which results in accumulation of acetylcholine (ACh) at these sites. Excess ACh at synapses causes an overstimulation of cholinergic receptors (Stewart and Anderson, 1968), leading to muscle weakness, increased secretions, respiratory depression, seizures, coma, and ultimately death as a result of respiratory and/or cardiovascular failure. The current treatment regimen for OP pesticide toxicity includes anticholinergic drugs such as atropine sulfate and oximes such as 2-PAM chloride. Although these antidotal regimens are sometimes successful in preventing lethality, they induce side effects and are generally unable to prevent postexposure incapacitation, convulsions, performance deficits, or permanent brain damage (Dirnhuber et al., 1979; Hardman et al., 2001).

An alternative approach to treating OP pesticide poisoning is the use of enzymes to sequester these compounds in circulation before they reach acetylcholinesterase in the nervous system. Plasma-derived human butyrylcholinesterase (BChE), a tetrameric protein with a molecular mass of 85 kDa per subunit, is a viable candidate for human use (Ashani et al., 1991; Raveh et al., 1993, 1997; Allon et al., 1998; Doctor and Dain, 1991; Raveh et al., 1993, 1997; Allon et al., 1998; Doctor and Dain, 1991). The views expressed in this abstract are those of the author(s) and do not reflect the official policy of the Department of Army, Department of Defense, or the US Government.

dx.doi.org/10.1124/jpet.114.213645.
This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: BChE, butyrylcholinesterase; CMP, 3-cyano-4-methyl-2-oxo-2H-chromen-7-yl cyclohexyl methylphosphonate; HDL, high-density lipoproteins; OP, organophosphorous; 2-PAM, pralidoxime [2-[hydroxyimino)methyl]-1-methylpyridin-1-ium]; PON1, paraoxonase-1; vp, viral particles.
Saxena, 2005; Lenz et al., 2005; Geyer et al., 2010). However, BChE is a stoichiometric scavenger in that one equivalent of protein binds and inactivates one equivalent of the OP compound, thus making it less attractive for development as a prophylactic treatment against OP pesticide toxicity. A second and more attractive approach would be to develop a catalytic enzyme that can potentially hydrolyze multiple equivalents of the OP compound without inactivation of the enzyme, thus requiring much less of the enzyme to offer protection. Human paraoxonase-1 (PON1) (EC 3.1.8.1; formerly EC 3.1.1.2), a 354-amino acid calcium-dependent glycoprotein with an estimated molecular mass of 40–45 kDa, is a leading candidate in this category. In circulation, the enzyme exists in association with high-density lipoproteins (HDL) and is believed to play a role in drug metabolism and atherosclerosis by preventing the oxidation of low-density lipoproteins (LDL) (Aviram et al., 1998; Mackness et al., 1998; Sorensen et al., 1999; Luus, 2000; Gaidukov and Tawfik, 2005). Two human polymorphic positions in PON1 exist: Gln/Arg at position 192 and Leu/Met at position 55 (Draganov and La Du, 2004). The Gln/Arg polymorphism has been shown to significantly affect the catalytic efficiency of PON1; the Arg192 isoform hydrolyzes paraoxon faster than the Gln192 isoform, whereas the Gln192 isoform hydrolyzes G agents faster than the Arg192 isoform (Davies et al., 1996). Wild-type PON1 hydrolyzes a range of OP pesticide compounds in vitro, including diazoxon, chlorpyrifos, and paraoxon. Although the enzyme offered in vivo protection against diazoxon and chlorpyrifos, it failed to provide protection against paraoxon (Stevens et al., 2008; DuySEN et al., 2011; HodGINS et al., 2013). The failure of wild-type PON1 to offer protection against paraoxon is attributed to insufficient catalytic efficiency, which results in the OP compound escaping the blood stream before PON1 is able to reduce the level of exposure below a lethal dose (Li et al., 2000).

To enhance the catalytic efficiency of wild-type human PON1 against G-type nerve agents, Goldsmith et al. (2012) performed such methods as DNA shuffling, high-throughput library screening, and mutagenesis to generate a number of PON1 variants with increased catalytic efficiencies and the desired stereochemical preference for the more toxic P (-)-isomer of each agent. In this study, we used an adenosine expression system to express the PON1 variants G3C9, VII-D11, VII-D2, and I-F11 in a mammalian cell culture system to determine their respective catalytic efficiencies against paraoxon. After characterizing each of the mammalian cell-derived PON1 variants in vitro, we chose the most efficacious variant, VII-D11, to assess its in vivo protective efficacy against paraoxon in mice.

**Materials and Methods**

Phenyl acetate and ethyl paraoxon were obtained from Sigma-Aldrich (St. Louis, MO). CMP (3-cyno-4-methyl-2-oxo-2H-chromen-7-yl cyclohexyl methylphosphonate) was a gift from Dr. Thomas Magliery (Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH). Rabbit a-apolI antibody was obtained from Abcam (Cambridge, MA). Cell culture medium, heat-inactivated fetal bovine serum, and supplements were purchased from Life Technologies (Grand Island, NY). PON1 variants expressed in Escherichtia coli were provided as a generous gift from Dr. Thomas Magliery and Srividya Murali (Department of Chemistry and Biochemistry, The Ohio State University) for use as standards.

**Production of Adenovirus and Polyclonal Antibodies against G3C9.** Recombinant adenosivuses expressing PON1 variants G3C9 (Ad-G3C9), VII-D11 (Ad-VII-D11), I-F11 (Ad-I-F11), and VII-D2 (Ad-VII-D2) were generated by Welgen Inc. (Worcester, MA). In brief, the genes of PON1 variants were codon harmonized for mammalian cell expression, cloned into pENT-CMV adenoviral transfer vector, and recombined with an adenosiviral backbone vector by standard cloning protocols. Virus generation and amplification were accomplished in human embryonic kidney epithelial (293TA) cells, and double cesium chloride centrifugation was used to purify the virus. All virus preparations were quality tested for endotoxin levels, and titers were assayed in plaque-forming assays and TCID50 methods. The control virus (Ad-null) used was the same as Ad-PON1 variant viruses except that it lacks the PON1-variant gene. Polyclonal antibodies against G3C9 were produced in two New Zealand white rabbits according to standard antibody production procedures (Washington Biotechnology Inc., Baltimore, MD). Antibodies to G3C9 are expected to cross-react with VII-D11, I-F11, and VII-D2; because these are evolved from G3C9 by targeted mutagenesis, 2 mg of G3C9 purified from E. coli was used as an immunogen. Serum collected after the second booster injection was used for purification of IgG by protein A/G chromatography. Serum was diluted in phosphate-buffered saline (1:10 dilution, pH 7.40) and loaded onto a 5-ml column of protein A/G agar. The bound IgG was eluted with 50 mM glycine-HCl (pH 2.5), and fractions containing IgG were neutralized with 1 M dibasic phosphate buffer (pH 7.5). Reducing SDS-PAGE was used to check the purity of IgGs, and Western blot was used to confirm their reactivity to all PON1 variants used in this study.

**Cell Culture and Adenovirus Infection in Vitro.** 293A cells (2.5 × 10^6) were seeded in 12-well plates. After 24 hours, the cells were infected with 0–50 viral particles/vp/cell for 1–2 hours using 500 μl of infection medium (Dulbecco’s modified Eagle’s medium (DMEM) containing 2% fetal bovine serum (FBS)) at 37°C. After infection with adenovirus, 1.5 ml of growth medium (DMEM containing 10% FBS, 50 μg/ml penicillin and streptomycin, L-glutamine, and 50 μg/ml sodium pyruvate) was added to the cells and incubated at 37°C for 24–96 hours. PON1 activity in the culture medium was quantified using phenyl acetate as the substrate.

**Large-Scale Expression and Partial Purification of 293A Expressed PON1 Variants.** 293A cells (8 × 10^6) were seeded in 150-cm^2 tissue culture dishes. After 24 hours, the cells were infected with Ad-PON1 variant viruses (20 vp/cell) for 1–2 hours using 10 ml of infection medium at 37°C. Growth medium (15 ml) then was added, and the cell cultures were returned to the incubator. After 96 hours, the spent medium was collected and cleared of cell debris by centrifugation at 2500 rpm for 10 minutes at 4°C. Partial purification of recombinant PON1 variants was accomplished by subjecting the medium to anion-exchange chromatography using an AKTA FPLC equipped with a Mono Q 5/50 GL column (GE Healthcare Bio-Sciences, Pittsburgh, PA). Fractions displaying CMP activity were pooled, buffer was exchanged into storage buffer [50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM CaCl_2, 0.1% Tween 20, 1 mM β-mercaptoethanol (BME), 50% glycerol], and fractions were stored at −20°C for future use.

**In Vivo Expression of PON1 Variants.** Adult male mice (n = 4, 25–30 g body weight; Swiss-Webster) were housed at 20–26°C and were provided food and water ad libitum. Saline (60 μl) containing 2 × 10^11 vp of either Ad-G3C9, Ad-VII-D11, Ad-I-F11, Ad-VII-D2, or Ad-null was administrated via injection into the tail vein. Blood (10–15 μl) was drawn on days 4, 5, and 6, while virus injection, collected into tubes containing 1 US Pharmacopeia (USP) unit of heparin, and centrifuged at 14,000 rpm for 10 minutes at 4°C. The plasma was removed and assayed for enzyme activity using phenyl acetate, CMP, and paraoxon as substrates. To determine whether G3C9 is secreted out of the cells, Ad-G3C9-infected cells were lysed on day 3 with 250 μl of mammalian protein extraction reagent (Pierce Chemical Co., Rockford, IL), and PON1 activity in cell culture medium and lysate was determined using phenyl acetate as the substrate.
conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council) and the Animal Welfare Act of 1966 (P.L. 89-544) as amended.

**PON1 Activity Assays and Kinetic Analyses.** Chemical structures of various PON1 substrates used are shown in Supplemental Data. Activity was determined in a 96-well format on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA) series spectrophotometer. To test for arylesterase activity, 10 μl of enzyme at an appropriate concentration (depending on activity) was added to 186 μl of assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM CaCl2). Four microliters of increasing concentrations of phenyl acetate then was added using a multichannel pipette to give final concentrations between 0.06 and 3.3 mM. The rate of formation of phenol was measured from the A405 (ε = 1310 M⁻¹ cm⁻¹) at room temperature for 3 minutes. PON1 activity was measured similarly by adding 4 μl of increasing concentrations of paraoxon to 10 μl of enzyme in 186 μl of assay buffer to give final paraoxon concentrations in the range of 0.05 to 2.6 mM. The formation of p-nitrophenolate was followed at A405 (ε = 17,000 M⁻¹ cm⁻¹) for 3 minutes at room temperature. To test for CMP activity, enzymes were incubated in assay buffer with increasing concentrations of CMP from 0.005 to 0.5 mM in a 96-well plate. The rate of formation of 3-cyano-7-hydroxy-4-methyl-coumarin was monitored at A405 (ε = 37,000 M⁻¹ cm⁻¹) for 5–10 minutes at 25°C. Phenyl acetate, paraoxon, and CMP were prepared as high-concentration stocks in methanol on the day of the assay, and the purity of each substrate was assessed by base hydrolysis. The final methanol concentration in all assays was held constant at 2%. Kinetic parameters were determined by Michaelis-Menten steady-state kinetics using GraphPad Prism (San Diego, CA). Kinetic data for CMP hydrolysis was used as a metric to quantify the amount of adenvirus-mediated PON1 expression, which was confirmed via quantitative Western blot.

**HDL Association of PON1 Variants in Mouse Blood.** Plasma (150 μl) from mice infected with Ad-VII-D11, plasma from mice infected with Ad-null, or purified bacterial VII-D11 incubated for 2 hours at room temperature in mouse plasma was applied onto a Superdex 200 gel filtration column (height 75 cm, width 2 cm) attached to an AKTA FPLC system (Gordon et al., 2010). The samples were processed at a flow rate of 0.5 ml/min in 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM CaCl2. Eluate was collected as one-milliliter fractions at 4°C, and each fraction was assayed for PON1 activity using phenyl acetate (Ad-null plasma) and CMP (plasma containing VII-D11) as substrates.

**LD₉₀ Determination of Paraoxon.** Swiss-Webster male mice weighing 25–30 g were used to assess the protection offered by selected PON1 variants. Since LD₉₀ values depend on the mouse strain, we determined the LD₉₀ of paraoxon subcutaneously by treating Swiss-Webster male mice with various doses of paraoxon (300–750 μg/kg body weight) dissolved in methanol. The mortality response versus the dose administered (expressed in log form) was fit to a nonlinear regression model to calculate the LD₉₀ dose (Supplemental Data).

**Determination of Protection Offered against Multiple Exposures to Paraoxon.** Ad-G3C9 and Ad-VII-D11 were compared for their ability to afford protection against multiple exposures of 0.9 × 1.8 × LD₉₀ doses of paraoxon intoxication. Ad-G3C9, Ad-VII-D11, and Ad-null (2 × 10¹² vp/mouse) were injected into groups of mice via the tail vein. Plasma (20 μl) was collected from each mouse prior to virus injection and on days 4 and 5 prior to toxicant exposure. Plasma concentrations of G3C9 and VII-D11 were determined by CMP hydrolysis. On day 4, mice were challenged subcutaneously with two 0.9 × LD₉₀ doses of paraoxon separated by 2 hours between the two challenges. On day 5, the surviving animals were challenged sequentially with one 0.9 × LD₉₀ dose followed by two 1.8 × LD₉₀ doses separated by 2 hours between each challenge. Mice were observed for cholinergic symptoms (tremors, mortality) for 1 hour after each challenge and survival was scored after 24 hours. All animals were humanely killed 24 hours after the final paraoxon challenge.

**Determination of Stoichiometry of Protection Offered against Exposure to Paraoxon.** Ad-VII-D11 was injected into mice at a dose of 2 × 10¹¹ vp/animal and on days 3, 4, 5, and 6; they were challenged subcutaneously with 1.4–27 × LD₉₀ doses of paraoxon. The dose of paraoxon was determined by the concentration of PON1 in the mouse blood to attain fixed stoichiometric ratios of the agent to enzyme from 2:1 to 10:1. After the agent challenge, mice were observed continuously for 1 hour for cholinergic symptoms as above. Mice were humanely killed 24 hours after the final paraoxon challenge as stipulated in the animal protocol.

**Results**

**In Vitro and in Vivo Adenoviral Expression of PON1 Variants.** The ability of Ad-G3C9, Ad-VII-D11, Ad-VII-D2, and Ad-I-F11 to induce the expression of recombinant PON1 variants was first evaluated in vitro in 293A cells. To quantitate the expression of PON1, we monitored the increase in specific activity against either phenyl acetate or CMP as a function of time. The time course of expression of the PON1 variant G3C9 in 293A cells is shown in Fig. 1A. G3C9 expression was detected in the culture medium on day 3 and was maintained through day 6. A similar pattern of expression was observed for the other variants (data not shown). These data suggest that Ad-G3C9, Ad-VII-D11, Ad-VII-D2, and Ad-I-F11 transduced the expression of biologically active enzymes in vitro in mammalian cells. To quantitate the PON1 distribution in the medium and cell lysate, cells were infected with 10 vp/cell of Ad-G3C9, and PON1 expression was characterized in the medium and cytosolic fractions by aryl esterase activity. In the medium, enzyme activity was 80–85%, whereas only 15–20% was within the cytosol (Fig. 1B). These results suggest that evolved PON1 variants are mostly secreted when expressed in a mammalian expression system.

Next, the apparent molecular weight of virus-transduced PON1 variants was determined by Western blot using a polyclonal rabbit α-G3C9 antibody. G3C9, VII-D11, VII-D2, and I-F11 were identified as doublets/triplets with molecular masses between 37 and 50 kDa (Fig. 1C). In a previous study, adenovirus containing the gene for wild-type human PON1 also transduced the expression of proteins with similar molecular weights in 293A cells and mice (Duyssen et al., 2011). It has been suggested that native huPON1 undergoes post-translational N-glycosylation at positions Asn253 and Asn324 (Kuo and La Du, 1995). To determine whether the modifications of the evolved variants involve N-glycosylation analogous to huPON1, 293A cells were pretreated for 2 hours with 1 or 2 μg of tunicamycin/ml of growth media and were subsequently infected with 20 vp/cell Ad-G3C9 and Ad-VII-D11. The medium was concentrated and tested for the presence of PON1 variants by Western blot. As shown in Fig. 1D, both G3C9 and VII-D11 were detected as a single-band species with the addition of tunicamycin, and migrated faster than the doublet/triplet glycosylated PON1 species displayed in the absence of tunicamycin. These results suggest that evolved PON1 variants undergo N-glycosylation in mammalian cells similar to huPON1.

After confirming that Ad-PON1 is capable of producing PON1 variants in 293A cells, we investigated whether these viruses also induce the expression of PON1 variants in vivo in mice. Each Ad-PON1 virus was injected into mice via the tail vein; blood was collected on days 4, 5, and 6 and assayed for CMP specific activity as a metric to determine protein expression. As shown in Fig. 2A, enzymatic expression of VII-D11 was well above baseline on day 4, reached peak levels on
day 5, and remained well above the baseline on day 6. On day 5, VII-D11 levels in one mouse were as high as 12.2 mg/ml in plasma. A similar expression pattern was observed for Ad-G3C9, Ad-VII-D2, and Ad-I-F11 (data not shown). Western blot analysis of mouse plasma indicated that the adenovirus-expressed PON1 also appears as a doublet/triplet with molecular masses between 37 and 50 kDa (Fig. 2B). Thus, Ad-G3C9, Ad-VII-D11, Ad-VII-D2, and Ad-I-F11 are capable of transducing the expression of functional and full-length PON1 variants in vivo in mice.

Association of VII-D11 with Mouse HDL. Wild-type human PON1, with its ability to bind to HDL, has been shown to prevent oxidation of low-density lipoprotein particles, which are a major contributing factor to atherosclerotic plaque development. Therefore, we used a previously described gel filtration chromatography separation method (Gordon et al., 2010) to investigate whether adenovirus-derived VII-D11 also associates with HDL when expressed in mouse plasma. As shown in Fig. 3A, mouse plasma, mouse plasma spiked with bacterial VII-D11, and mouse plasma containing adenovirus-derived VII-D11 separated into four major peaks: VLDL+LDL (fractions 1, 5, and 9), HDL (fractions 2, 6, and 10), and two lipid free protein peaks (fractions 3, 4, 7, 8, 11, and 12). Analyzing various fractions from each peak for arylesterase (normal mouse plasma) or CMP hydrolysis (plasma containing VII-D11) revealed that most of the enzyme activity was present in the HDL fraction (Fig. 3A, fractions 2, 6, and 10). The fractions were pooled, concentrated, and subjected to Western blot using rabbit α-apo1ipoprotein A1 (ApoA1) and rabbit α-G3C9 antibodies to confirm the presence of HDL and VII-D11 in each peak, respectively. As shown in Fig. 3B, VII-D11 was found in peaks 6 and 10 whether overexpressed in vivo or
added to mouse blood ex vivo, whereas apoA1 was detected in fractions 2, 6, and 10 (Fig. 3C). The endogenous mouse PON1 (peak 2) was not present at a sufficient concentration to be detected using α-G3C9 antibodies. Taken together, these results suggest that VII-D11 in mouse plasma is associated with HDL.

**Partial Purification and Characterization of 293A Cell-Expressed PON1 Variants.** Our goals in this study were to evaluate 1) whether an evolved PON1 variant that has shown enhanced catalytic efficiency against G-type chemical warfare nerve agents would also display enhanced catalytic efficiency against paraoxon and 2) whether that PON1 variant would also display enhanced catalytic efficiency against paraoxon.
variant affords catalytic protection against paraoxon toxicity in vivo.

The culture medium from virus-infected 293A cells could not be used as is for determining the $k_{\text{cat}}/K_m$ values for paraoxon because the specific activity and amount of the PON1 variant in the crude culture medium were too low (Fig. 1; data not shown). The medium samples containing G3C9, VII-D11, I-F11, and VII-D2 were subjected to anion-exchange chromatography for partial purification of PON1 variants. As shown in Fig. 4A, anion-exchange chromatographic purification of VII-D11 expression medium resulted in a 3-fold increase in CMP-specific activity relative to the crude medium. A similar 3- to 4-fold increase in specific activity for G3C9, VII-D2, and I-F11 against CMP were also noted following anion-exchange chromatography (data not shown).

SDS-PAGE of partially purified PON1 variants revealed the presence of a number of additional proteins with molecular masses ranging from 50 to 150 kDa (Fig. 4B, lanes 1–4). These additional proteins are also present in a sample prepared by the same method using Ad-null control virus expression medium (Fig. 4B, lane 5). The 37- to 50-kDa PON1 species are undetected or barely detected in these samples. However, Western blot using α-G3C9 antibodies confirmed the presence of 37- to 50-kDa PON1 species in Ad-G3C9 (Fig. 4C, lane marked G3C9), Ad-VII-D11 (lane marked VII-D11), Ad-I-F11 (lane marked I-F11), and Ad-VII-D2 (lane marked VII-D2) samples but not in the Ad-null sample. None of the additional 50–150-kDa proteins cross-reacted with the α-G3C9 antibodies, suggesting that these proteins are not immunologically related to PON1 variants (Fig. 4C). Furthermore, these contaminating proteins had no effect on the $k_{\text{cat}}/K_m$ of VII-D11 against CMP (data not shown).

**Kinetic Analysis of Engineered PON1 Variants Expressed in E. coli, 293A Cells, and Mouse Blood.** We measured the catalytic efficiency of G3C9, the parent scaffold, and its evolved variants (VII-D11, I-F11, and VII-D2) derived from E. coli, 293A cells (Fig. 4B), and mouse blood (Fig. 2) against phenyl acetate, paraoxon, and CMP. None of the PON1 variants evolved for G-agent hydrolysis (VII-D11, I-F11, and VII-D2) showed appreciable differences in activity toward paraoxon between the bacterial and mammalian-derived sources (Table 1). These studies also revealed that the $k_{\text{cat}}/K_m$ of VII-D11 against paraoxon is 3- to 5-fold higher than that of VII-D2 or I-F11 and is 15- to 20-fold higher than that of mammalian-expressed G3C9; among the variants tested, VII-D11 is the most efficient toward paraoxon hydrolysis. Relative to bacterially expressed G3C9, VII-D11 produced in bacterial or mammalian systems displayed catalytic efficiencies 100- to 200-fold higher against paraoxon (Table 1).

It is surprising that expression of G3C9 via the mammalian expression system in 293A or in mice resulted in a roughly 10-fold increase in catalytic efficiency against CMP, paraoxon, and phenyl acetate versus G3C9 expression in E. coli (Table 1). A closer examination of the Michaelis-Menten parameters reveals that, in the case of CMP, the mammalian-derived protein had a higher $k_{\text{cat}}$ than the bacterial protein (Table 2). To determine whether the stimulation is attributed to the addition of exogenous protein, purified bacterially expressed G3C9 was incubated with either bovine serum albumin (BSA) or medium from 293A cells infected with Ad-null. Ultimately, the increase in $k_{\text{cat}}/K_m$ associated with the expression of G3C9 in mammalian cells could not be recapitulated by the addition of the exogenous proteins to the purified bacterial G3C9. In the case of paraoxon and phenyl acetate, none of the variants was saturable under the conditions of the experiment, and as such the $k_{\text{cat}}$ and $K_m$ could not be explicitly determined. Nonetheless, it is possible that the 10-fold enhancement in the catalytic efficiencies of the mammalian proteins produced by the adenoviral expression system is due to an overall increase in $k_{\text{cat}}$.

**Evolved PON1 Variant VII-D11 Offers Greater Protection than G3C9 Against Paraoxon.** Prior to determining the efficacy of the treatment group, we determined the LD$_{50}$ of untreated groups of animals by administering variable doses of paraoxon. By use of a nonlinear regression model of the mortality-probability dependence on dose administered, we calculated the LD$_{50}$ value to be 0.49 ± 0.07 mg/kg in the untreated group (Supplemental Data). This value was used to quantify the amount of LD$_{50}$ doses injected in each subsequent challenge.

We next evaluated whether adenovirus-mediated expressed VII-D11 would offer a greater level of protection than the parent...
VII-D11 Offers Catalytic Protection against Paraoxon.

As the previous study was performed under conditions in which the enzyme was present in stoichiometric excess relative to paraoxon, we investigated whether VII-D11 offers catalytic protection against super-stoichiometric doses of paraoxon in mice. Beginning on day 4 after Ad-VII-D11 injection, groups of mice (n = 3–12) containing varying blood concentrations of VII-D11 (0.2–4.1 mg/ml) were challenged with single doses of paraoxon at stoichiometric ratios varying from 2:1 to 10:1 with respect to the concentration of protein, corresponding to a range of 1.4–27 LD50 doses (Table 4). At ratios below 8:1, 100% of the mice were protected from paraoxon toxicity. At ratios higher than 8:1, the percentage of mice experiencing tremors or mortality after exposure to paraoxon was significantly higher.

Discussion

Here, we report that an evolved PON1 variant, VII-D11, offers catalytic protection against paraoxon intoxication in mice. This is the first demonstration of any PON1 variant affording asymptomatic protection against paraoxon toxicity. Moreover, the kcat/Km value for VII-D11 against paraoxon is similar irrespective of being expressed in E. coli, in an in vitro mammalian cell culture system, or in vivo in mice. These results suggest that an E. coli expression system for large-scale industrial production of the enzyme is highly feasible. Such a product is urgently needed for protection against OP pesticide poisoning either intentional or accidental as a work hazard. In addition, the enzyme retained the ability to bind to HDL, which may be beneficial in reducing atherosclerotic plaque development and heart disease.

A decade of work using DNA shuffling, library screening, and mutagenesis of PON1 led to the characterization of a number of evolved PON1 variants based on the recombinant variant G3C9 (Gupta et al., 2011; Goldsmith et al., 2012). One such variant, 4E9, increased the survival rate of mice when injected 6 hours before a GF-analog injection (Gupta et al., 2011), which suggests that one or more of the evolved PON1 variants could also offer prophylactic protection against authentic chemical warfare nerve agents. It was unclear, however, whether increases in kcat/Km against G agents would also translate to increases in kcat/Km against OP pesticide compounds such as paraoxon. Thus, we screened PON1 variants that were evolved to efficiently hydrolyze G agents to determine whether paraoxon hydrolysis was also enhanced. G3C9 differs from human PON1 by having 50 amino acid substitutions, whereas the later round variants, VII-D11, I-F11, and VII-D2, differed from G3C9 by having six, seven, and eight amino acid substitutions, respectively. Our results identified that VII-D11 has the highest catalytic efficiency of the variants tested against paraoxon. Although further rounds of selection beyond VII-D11 gave rise to variants such as I-F11 and VII-D2 with increased activity toward the P- isomer of each G agent (Goldsmith et al., 2012), the enhancement in paraoxon hydrolysis obtained through the evolution of VII-D11 from G3C9 was lost. In each round of selection, variants were screened against compounds other than paraoxon. As such, any gain in paraoxon hydrolysis obtained through selection against the G-agent analogs was fortuitous, and the gains were more than likely lost as the enzyme became more specialized at hydrolyzing G agents.

As an additional consideration, we set out to investigate whether there were any significant differences between E. coli

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<td>Catalytic efficiencies against paraoxon, phenyl acetate, and CMP of PON1 variants expressed in E. coli, 293A cells, and mice</td>
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<tr>
<td>I-F11 (293A)</td>
</tr>
<tr>
<td>I-F11 (mouse)</td>
</tr>
</tbody>
</table>

n.d., not detected.

TABLE 2

Michaelis-Menten parameters for G3C9 CMP hydrolysis

<table>
<thead>
<tr>
<th>Variant</th>
<th>kcat</th>
<th>Km</th>
<th>kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>min^-1</td>
<td>mM</td>
<td>M^-1 min^-1</td>
</tr>
<tr>
<td>G3C9 (E. coli)</td>
<td>1.10 ± 0.04</td>
<td>53 ± 7</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>G3C9 (293A)</td>
<td>15.0 ± 0.3</td>
<td>38 ± 3</td>
<td>392 ± 30</td>
</tr>
<tr>
<td>G3C9 (mouse)</td>
<td>9.2 ± 0.5</td>
<td>23 ± 5</td>
<td>400 ± 100</td>
</tr>
<tr>
<td>G3C9 (E. coli) + Ad-null media</td>
<td>1.10 ± 0.05</td>
<td>33 ± 6</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>G3C9 (E. coli) + 0.05% BSA</td>
<td>1.09 ± 0.06</td>
<td>45 ± 9</td>
<td>24 ± 5</td>
</tr>
</tbody>
</table>
TABLE 3

VII-D11 offers superior protection compared with G3C9 against paraoxon

Before challenge on days 4 and 5, blood was drawn and assayed for CMP-hydrolyzing activity, and enzyme concentrations were determined. Multiple challenges on the same day were performed at 2-hour time intervals. The G3C9 concentration ranged from 2.7 to 4.6 mg/ml and 4.8 to 6.7 mg/ml on days 4 and 5, respectively. VII-D11 concentration ranged from 1.6 to 8.8 mg/ml and 4.4 to 12.2 mg/ml on days 4 and 5, respectively. Each protein was present at a stoichiometric excess relative to the dose of paraoxon administered.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 4 (Survival)</th>
<th>Day 5 (Survival)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.9 × LD_{50}</td>
<td>0.9 × LD_{50}</td>
</tr>
<tr>
<td></td>
<td>(Survival)</td>
<td>(Survival)</td>
</tr>
<tr>
<td>Control (n = 3)</td>
<td>3/3 (100%)</td>
<td>0/3 (0%)</td>
</tr>
<tr>
<td>G3C9 (n = 6)</td>
<td>6/6 (100%)</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>VII-D11 (n = 5)</td>
<td>5/5 (100%)</td>
<td>5/5 (100%)</td>
</tr>
</tbody>
</table>

n.a., not applicable.

derived and mammalian-produced PON1 variants. We observed that, with the exception of G3C9, PON1 variants that were produced in E. coli or in mammalian cells hydrolyzed paraoxon with similar catalytic efficiencies within experimental error. We further characterized the in vivo produced VII-D11 to determine whether it associates with endogenous HDL upon synthesis. The present study demonstrates that VII-D11 associates with HDL both when the bacterially produced enzyme was added to mouse blood ex vivo and when it was synthesized in vivo, thus suggesting that the capacity to associate with HDL was not lost during the directed evolution process. It was observed in other studies that HDL association increases enzymatic activity of PON1 against lactones (Draganov et al., 2005; Gaidukov and Tawfik, 2005; Khersonsky and Tawfik, 2005; Gaidukov et al., 2010). In concurrence with previous findings (R. Baldauff and T.J. Magliery, unpublished data), our results suggest that this stimulation in activity may not occur with OP hydrolysis, as the catalytic efficiency of the PON1 variants against paraoxon and CMP did not differ significantly despite an apparent association with HDL. It has also been suggested that the HDL association of PON1 plays a role in increasing the circulatory stability of the enzyme (Moren et al., 2008). Whether that is the case for VII-D11 remains to be investigated.

The adenosine-mediated gene delivery system has been used to induce high-level expression of therapeutic proteins of interest for 4–7 days in various animal model systems. We have successfully used this system to demonstrate the protection offered by BChE and wild-type huPON1 against G- and V-type nerve agents and diazoxon toxicity, respectively (Duysen et al., 2011; Parikh et al., 2011). By use of the same system, we have also found that some candidate catalytic enzymes, such as human liver prolidase, senescence marker protein-30, and KIAA 1360 serine protease, that hydrolyzed V- and G-nerve agents in vitro failed to offer protection in vivo (Rezk et al., 2014; A. Peetambaran, K. Parikh, and N. Chilukuri, unpublished work). By use of the adenosine-mediated gene delivery system, we have demonstrated that VII-D11, with a higher k_{cat}/K_m against paraoxon, offers superior protection compared with G3C9, which has a lower k_{cat}/K_m.

TABLE 4

In vivo stoichiometric assessment of protective efficacy of VII-D11 against paraoxon

Before challenge with paraoxon, blood was drawn and assayed for CMP-hydrolyzing activity and enzyme concentration was determined. Based on the enzyme concentration, mice were exposed to doses of paraoxon at fixed stoichiometric ratios. The mice were observed continuously for 1 hour for signs of paraoxon intoxication (tremors or death), and survival was scored after 24 hours.

<table>
<thead>
<tr>
<th>No. of Mice</th>
<th>Stoichiometry Tested (Paraoxon/VII-D11)</th>
<th>VII-D11 Concentration Range</th>
<th>LD_{50} Dose Range</th>
<th>% Survival/Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2:1</td>
<td>0.7–1.0</td>
<td>1.4–1.9</td>
<td>100% survival</td>
</tr>
<tr>
<td>3</td>
<td>5:1</td>
<td>0.4–0.9</td>
<td>1.6–3.9</td>
<td>100% survival</td>
</tr>
<tr>
<td>10</td>
<td>6:1</td>
<td>0.4–2.0</td>
<td>2.0–10.8</td>
<td>100% survival</td>
</tr>
<tr>
<td>7</td>
<td>7:1</td>
<td>0.5–1.9</td>
<td>3.4–12.2</td>
<td>100% survival</td>
</tr>
<tr>
<td>12</td>
<td>8:1</td>
<td>0.4–4.1</td>
<td>2.9–27.0</td>
<td>92% survival</td>
</tr>
<tr>
<td>9</td>
<td>9:1</td>
<td>0.2–2.2</td>
<td>1.5–18.0</td>
<td>88% survival</td>
</tr>
<tr>
<td>4</td>
<td>10:1</td>
<td>0.4–1.0</td>
<td>3.5–9.0</td>
<td>100% survival</td>
</tr>
</tbody>
</table>

n.a., not applicable.
cholinesterase due to the escape of cumulative, nonlethal amounts of paraoxon from previous doses lowering the LD50 of subsequent doses. Regardless, both factors are a direct result of the low catalytic efficiency of G3C9, as animals containing VII-D11 did not display any perceivable symptoms of toxicity (tremors or mortality) throughout the dose regimen, although it is still possible that nonlethal amounts of agent are able to escape the bloodstream before hydrolysis by VII-D11. Further physiologic studies are needed to quantify the amount of paraoxon, if any, that is able to escape the bloodstream without being hydrolyzed by the enzyme after cumulative doses.

One of the limitations of our adenovirus delivery system was the inability to control the amount of protein that is expressed in a given animal. This limitation makes it difficult to derive traditional statistical protection parameters such as LD50 and protective ratios due to the inability to obtain enough biologic replicates to derive these values with any kind of statistical relevance. To circumvent this issue, we varied the paraoxon dose concentration according to the concentration of VII-D11 in each animal to achieve replicable challenges at fixed stoichiometric ratios. By use of this dosage paradigm, we discovered that each stoichiometric replicate behaved in a reproducible fashion across a broad range of paraoxon dosages. Although there may be limits to this stoichiometric approach in the case of paraoxon doses significantly lower or higher than that screened in our study, the correlation nonetheless was consistent across our experimental conditions. Our data suggest that VII-D11 is able to offer symptom-free protection against a 7-fold stoichiometric excess of paraoxon in vivo. Through interpolation of the data for the mice challenged with the 7-fold stoichiometric excess of paraoxon, a dosage of at least 1.2 mg of VII-D11 should protect a mouse against 5 × LD50 exposure of paraoxon. HuBChE, an established OP stoichiometric bioscavenger (hence capable of affording protection at a 1:1 ratio at best), would require a dose of ~16 mg to bind and sequester an equivalent dose of paraoxon. These results suggest that VII-D11 offers protection against paraoxon at a dose 14-fold lower than the dose of HuBChE predicted to be required to afford protection against the same dose of paraoxon. Furthermore, if a more efficacious prophylactic against paraoxon is desired, VII-D11 can be used as a starting platform to develop one by a targeted mutagenesis approach.

In summary, we described the capacity of an evolved PON1 variant to hydrolyze paraoxon at a rate that is sufficient for it to offer prophylactic protection. Moreover, the enzyme functions in a catalytic manner, thereby significantly lowering the required dose of the enzyme to afford protection. The enzyme also binds to HDL and therefore may be beneficial in reducing plaque formation by hydrolyzing oxidized lipids. Since VII-D11 is expressible as a soluble enzyme in E. coli, large-scale development of the enzyme for human use is highly feasible. Finally, it is plausible that VII-D11 may have retained the ability of wild-type human PON1 to protect against a broad spectrum of OP pesticide compounds, such as diazoxon, chlorpyrifos, malathion, guthion, and dichlorvos. Further experiments could be implemented to investigate and to improve the broad spectrum efficacy of VII-D11 against pesticides.

Acknowledgments

The authors thank Dr. Magliery and Srividya Murali (Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH) for providing E. coli expressed PON1 for use as standards and CMP substrate.

Authorship Contributions

Participated in research design: Mata, Chilukuri.

Conducted experiments: Mata, Rezk, Sabnekar.

Contributed new reagents or analytical tools: Mata, Rezk, Cerasoli, Chilukuri.

Performed data analysis: Mata, Rezk, Cerasoli, Chilukuri.

Wrote or contributed to the writing of the manuscript: Mata, Cerasoli, Chilukuri.

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**Address correspondence to:** Dr. Nageswararao Chilukuri, Physiology and Immunology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010. E-mail: Nageswararao.chilukuri.civ@mail.mil
SUPPLEMENTAL DATA

Investigation of evolved paraoxonase1 variants for prevention of organophosphorus pesticide compound intoxication

David G Mata, Peter E. Rezk, Praveena Sabnekar, Douglas M. Cerasoli, Nageswararo Chilukuri

Physiology & Immunology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010.

Corresponding Author: N Chilukuri, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010, USA
Email: Nageswararo.chilukuri.civ@mail.mil

Present address (Peter E Rezk): Weatherhead School of Management, Case Western Reserve University, Cleveland, Ohio

I. Structures of substrates used

phenyl acetate    paraoxon    CMP

II. Determination of subcutaneous LD$_{50}$ of paraoxon in Swiss Webster mice. A. In order to determine the LD$_{50}$ of untreated Swiss Webster mice against paraoxon, we administered doses ranging from 0.31 – 0.75 mg/kg subcutaneously into groups of mice. B. The LD$_{50}$ was determined using non-linear regression by plotting the response vs. the dose of paraoxon (expressed in log form).

A.

<table>
<thead>
<tr>
<th>Number of mice (n)</th>
<th>Dose (mg/kg)</th>
<th>Log Dose</th>
<th>Observed Mortality</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.31</td>
<td>2.50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.38</td>
<td>2.57</td>
<td>1</td>
<td>25</td>
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<tr>
<td>8</td>
<td>0.50</td>
<td>2.70</td>
<td>3</td>
<td>37.5</td>
</tr>
<tr>
<td>10</td>
<td>0.63</td>
<td>2.80</td>
<td>9</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>0.75</td>
<td>2.88</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>

B.