Investigation of evolved paraoxonase1 variants for prevention of paraoxon intoxication in mice

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Investigation of evolved paraoxonase-1 variants for prevention of organophosphorous pesticide compound intoxication

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Abstract

We investigated the ability of the engineered paraoxonase-1 variants G3C9, VII-D11, I-F11 and VII-D2 to afford protection against paraoxon intoxication. Paraoxon 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 paraoxon with a $k_{\text{cat}}/K_m$ of $2.1 \times 10^6$ M$^{-1}$ min$^{-1}$ and $1.6 \times 10^6$ M$^{-1}$ min$^{-1}$ for the enzyme expressed via adenovirus infection of 293A cells and mice, respectively. Compared to the G3C9 parent scaffold, VII-D11 is 15-20 fold more efficacious at hydrolyzing paraoxon. Coinciding with these results, mice expressing VII-D11 in their blood survived and showed no symptoms against a cumulative $6.3 \times LD_{50}$ dose of paraoxon while mice expressing G3C9 experienced tremors and only 50% survival.

We then determined whether VII-D11 can offer protection against paraoxon when present at sub-stoichiometric concentrations. Mice containing varying concentrations of VII-D11 in their blood (0.2 - 4.1 mg/mL) were challenged with doses of paraoxon at fixed stoichiometric ratios that constitute up to a ten-fold molar excess of paraoxon to enzyme (1.4 - $27 \times LD_{50}$ doses) and were assessed for tremors and mortality. Mice were afforded complete asymptomatic protection below a paraoxon to enzyme ratio of 8:1, whereas higher ratios produced tremors and/or mortality. VII-D11 in mouse blood co-eluted 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 are a benefit to society through increasing food production, preserving produce, and combating insect infestations. However, due to their inherent toxicities, pesticides also have the potential to be detrimental to society. The United States 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 (Aldridge and Davison, 1953). Excess ACh at synapses causes an over-stimulation of cholinergic receptors (Stewart and Anderson, 1968), leading to muscle weakness, increased secretions, respiratory depression, seizures, coma, and ultimately death due to 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 post-exposure 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 AChE in the nervous system.
Plasma-derived human butyrylcholinesterase (BChE), a tetrameric protein with a molecular weight of 85 kDa per subunit, is a viable candidate for human use (Ashani et al., 1991; Raveh et al., 1993; Raveh et al., 1997; Allon et al., 1998; Doctor and 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 weight 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; Sorenson et al., 1999; Lusis, 2000; Gaidukov and Tawfik, 2005). Two human polymorphic positions in PON1 exist: Q/R at position 192 and L/M at position 55 (Draganov and La Du, 2004). The Q/R polymorphism has been shown to significantly affect the catalytic efficiency of PON1; the R192 isoform hydrolyzes paraoxon faster than the Q192 isoform, while the Q192 isoform hydrolyzes G-agents faster than the R192 isoform (Davies et al., 1996). Wild-type PON1 hydrolyzes a range of OP pesticide compounds in vitro, including diazoxon, chlorpyrifos, and paraoxon. While 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 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 and coworkers performed methods such 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 (Goldsmith et al., 2012). In this study, we employed an adenovirus 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-cyano-4-methyl-2-oxo-2H-chromen-7-yl cyclohexyl methylphosphonate) was provided as a gift from Dr. Thomas Magliery (Department of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, OH 43210). Rabbit α-ApoA1 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 E. coli were provided as a generous gift from Dr. Thomas Magliery and Srividya Murali (Department of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, OH 43210) for use as standards.

Production of adenovirus and polyclonal antibodies against G3C9

Recombinant adenoviruses 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). Briefly, the genes of PON1 variants were codon-harmonized for mammalian cell expression, cloned into pENT-CMV adenoviral transfer vector, and recombined with an adenoviral backbone vector by standard cloning protocols. Virus generation and amplification was accomplished in human embryonic kidney epithelial (293A) 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 since 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 beads. 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 × 10^5) were seeded in 12-well plates. After 24 h, the cells were infected with 0-50 viral particles (vp)/cell of viruses for 1-2 h using 500 μL of infection medium (DMEM containing 2% FBS) at 37 °C. Following 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 to 96 h. PON1 activity in the culture medium was quantified using phenyl acetate or CMP 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 M-per cell lysis reagent (Pierce Chemical Co., Rockford, IL), and PON1 activity in cell culture medium and lysate was determined using phenyl acetate as the substrate.

Large-scale expression and partial purification of 293A expressed PON1 variants
293A cells \((8 \times 10^6)\) were seeded in 150 cm\(^2\) tissue culture dishes. After 24 h, the cells were infected with Ad-PON1 variant viruses \((20 \text{ vp/cell})\) for 1-2 h using 10 mL of infection medium at 37 °C. Then, 15 mL of growth medium was added, and the cell cultures were returned to the incubator. After 96 h, the spent medium was collected and cleared of cell debris by centrifugation at 2500 rpm for 10 min at 4 °C. Partial purification of recombinant PON1 variants was accomplished by subjecting the medium to anion exchange chromatography using an Acta FPLC equipped with a Mono Q 5/50 GL column. Fractions displaying CMP activity were pooled, buffer was exchanged into storage buffer \((50 \text{ mM Tris, pH 8.0, 50 mM NaCl, 1 mM } \text{ CaCl}_2, 0.1\% \text{ Tween 20, 1 mM } \beta\text{-mercaptoethanol (BME), 50\% glycerol})\) and fractions were stored at -20 °C for future use.

**In vivo expression of PON1 variants**

Adult male mice \((n=4, 25–30 \text{ g body weight/Swiss Webster})\) were housed at 20 - 26 °C and were provided food and water *ad libitum*. 60 μL of saline containing \(2 \times 10^{11}\) vp of either Ad-G3C9, Ad-VII-D11, Ad-I-F11, Ad-VII-D2 or Ad-null was administered via injection into the tail vein. Blood \((10-15 \mu\text{L})\) was drawn on days 4, 5 and 6 post virus injection, was collected into tubes containing 1 USP unit of heparin, and was centrifuged at 14,000 rpm for 10 min at 4 °C. The plasma was removed and assayed for enzyme activity using phenyl acetate, CMP and paraoxon as substrates. The plasma samples were subjected to Western blot analysis to assess the identity and concentration of each PON1 variant.

**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 upon activity) was added to 186 μL assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM CaCl₂). Then, 4 μL of increasing concentrations of phenyl acetate 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 $A_{270}$ ($\varepsilon = 1,310 \text{ M}^{-1} \text{ cm}^{-1}$) at room temperature for 3 min. PON1 activity was measured similarly by adding 4 μL of increasing concentrations of paraoxon to 10 μL enzyme in 186 μL 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 $A_{405}$ ($\varepsilon = 17,000 \text{ M}^{-1} \text{ cm}^{-1}$) 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 mM to 0.5 mM in a 96-well plate. The rate of formation of 3-cyano-7-hydroxy-4-methyl-coumarin was monitored at $A_{405}$ ($\varepsilon = 37,000 \text{ M}^{-1} \text{ cm}^{-1}$) 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 Prism Graphpad (Irvine, CA). Kinetic data for CMP hydrolysis was used as a metric to quantify the amount of adenovirus-mediated PON1 expression, which was confirmed via quantitative Western blot.

**HDL association of PON1 variants in mouse blood**
150 μL of plasma 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 1 mL 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.

**LD₅₀ determination of paraoxon**

Swiss-Webster male mice weighing 25 to 30 g were used to assess the protection offered by selected PON1 variants. Since LD₅₀ values depend on the mouse strain, we determined the LD₅₀ 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 non-linear regression model to calculate the LD₅₀ 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 × or 1.8 × LD₅₀ doses of paraoxon intoxication. Ad-G3C9, Ad-VII-D11, and Ad-null (2 × 10¹¹ vp/mouse) were injected into groups of mice via the tail vein. 20 μL of plasma 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 × LD₅₀ doses of paraoxon
separated by two hours between the two challenges. On day 5, the surviving animals were challenged sequentially with one $0.9 \times \text{LD}_{50}$ dose followed by two $1.8 \times \text{LD}_{50}$ doses separated by 2 hours between each challenge. Mice were observed for cholinergic symptoms (tremors, mortality) for 1 h after each challenge and survival was scored after 24 h. All animals were euthanized 24 h 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 $2 \times 10^{11}$ vp/animal and on days 3, 4, 5, and 6, they were challenged subcutaneously with 1.4 to $27 \times \text{LD}_{50}$ 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 one hour for cholinergic symptoms as above. Mice were euthanized 24 h 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 Figure 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 %, while only 15 - 20 % was within the cytosol (Figure 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 weights between 37 kDa and 50 kDa (Figure 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 (Duysen et al., 2011). Native huPON1 has been suggested to undergo 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 h with 1 μg or 2 μg of tunicamycin per mL of growth media and were subsequently infected with 20 vp/cell of Ad-G3C9 and Ad-VII-D11. The media was concentrated and tested for the presence of PON1 variants by Western blot. As shown in Figure 1D, both G3C9 and VII-D11 were detected as a single band species with the addition of tunicamycin, which 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 if 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 Figure 2A, enzymatic expression of VII-D11 was well above base line 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 weights between 37 kDa and 50 kDa (Figure 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 employed 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 Figure 3A, mouse plasma, mouse plasma spiked with bacterial VII-D11, and mouse plasma containing adenovirus-derived VII-D11 separated into 4 major peaks, VLDL+LDL (fractions 1, 5 and 9), HDL (fractions 2, 6 and 10) and 2 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 (Figure 3A, fractions 2, 6 and 10). The fractions were pooled, concentrated and subjected to Western blot using rabbit α-Apolipoprotein A1 (ApoA1) and rabbit α-G3C9 antibodies to confirm the presence of HDL and VII-D11 in each peak, respectively. As shown in Figure 3B, VII-D11 was found in peaks 6 and 10 whether overexpressed in vivo or added to mouse blood ex vivo while ApoA1 was detected in fractions 2, 6 and 10 (Figure 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 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 $k_{cat}/K_m$ values for paraoxon since the specific activity and amount of the PON1
variant in the crude culture medium was too low (Figure 1 and Data not shown). The media samples containing G3C9, VII-D11, 1-F11, and VII-D2 were subjected to anion-exchange chromatography for partial purification of PON1 variants. As shown in Figure 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 – 4-fold increase in specific activity for G3C9, VII-D2, and 1-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 weights ranging 50 to 150 kDa (Figure 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 (Figure 4B, lane 5). The 37 kDa 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 kDa to 50 kDa PON1 species in Ad-G3C9 (Figure 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 kDa to 150 kDa proteins cross reacted with the α-G3C9 antibodies, suggesting that these proteins are not immunologically related to PON1 variants (Figure 4C). Additionally, these contaminating proteins had no effect on the $k_{cat}/K_m$ 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, parent scaffold and its evolved variants (VII-D11, I-F11, and VII-D2) derived from *E. coli*, 293A cells (Figure 4B), and mouse blood
None of the PON1 variants evolved for G agent hydrolysis (VII-D11, I-F11, and VII-D2) showed appreciable differences in activity towards paraoxon between the bacterial and mammalian-derived sources (Table 1). These studies also revealed that the $k_{cat}/K_m$ of VII-D11 against paraoxon is 3-5 fold higher than that of VII-D2 or I-F11 and is 15-20 fold higher than that of mammalian expressed G3C9; among the variants tested, VII-D11 is the most efficient towards paraoxon hydrolysis. Relative to bacterially-expressed G3C9, VII-D11 produced in bacterial or mammalian systems displayed catalytic efficiencies 100-200 fold higher against paraoxon (Table 1).

Surprisingly, 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 $k_{cat}$ than the bacterial protein (Table 2). To determine whether or not the stimulation is due to the addition of exogenous protein, purified bacterially expressed G3C9 was incubated with either BSA or medium from 293A cells infected with Ad-null. Ultimately, the increase in $k_{cat}/K_m$ 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 $k_{cat}$ 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 $k_{cat}$.

**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. Using a non-linear regression model of the mortality probability dependence on dose administered, we calculated the LD$_{50}$ value to be $0.49 \pm 0.07$ 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 greater level of protection than the parent scaffold, G3C9, against paraoxon intoxication. Since PON1 variant expression levels were highest between days 4 and 6 following virus injection (Figure 2A), mice were challenged on days 4 and 5 with multiple successive doses of paraoxon, where PON1 variants G3C9 and VII-D11 were present at super-stoichiometric amounts relative to the concentration of paraoxon administered (Table 3). The G3C9 concentration ranged from 2.7 – 4.6 mg/mL and 4.8 – 6.7 mg/mL on days 4 and 5, respectively. VII-D11 concentration ranged from 1.6 - 8.8 mg/mL and 4.4 – 12.2 mg/mL on days 4 and 5, respectively. On day 4, each mouse was challenged with two $0.9 \times$ LD$_{50}$ doses of paraoxon. Control mice (n=3) and the treatment groups (G3C9, n=6, and VII-D11, n=5) were monitored for tremors and death following each dose. Control mice became symptomatic after the first dose and died following the second dose. Mice containing G3C9 and VII-D11 survived both $0.9 \times$ LD$_{50}$ doses and showed no perceptible cholinergic symptoms. The following day, these mice were exposed to three subsequent doses of paraoxon of $0.9 \times$ LD$_{50}$, $1.8 \times$ LD$_{50}$, and $1.8 \times$ LD$_{50}$ as described above. Two mice expressing G3C9 died after the first $1.8 \times$ LD$_{50}$ dose and one more died after the second $1.8 \times$ LD$_{50}$ challenge. The three animals that survived in the G3C9 group showed severe tremors but survived the 24 hour observation period. All of the mice in the VII-D11 group survived 24 hours after the second $1.8 \times$ LD$_{50}$ dose and did not display any cholinergic
symptoms during the duration of the experiments. Thus, 100% survival was observed for the VII-D11 group against a $6.3 \times LD_{50}$ cumulative dose of paraoxon, whereas a 50% survival was observed for the G3C9 group, with all of the animals in this group experiencing severe tremors.

**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 mg/ml to 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 to 27 $LD_{50}$ 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 is 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 $k_{cat}/K_m$ value for VII-D11 against paraoxon is similar irrespective of it 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 employing 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 prior to 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 or not increases in $k_{cat}/K_m$ against G agents would also translate to increases in $k_{cat}/K_m$ against OP pesticide compounds such as paraoxon. Thus, we screened PON1 variants that were evolved to efficiently hydrolyze G agents to determine if paraoxon hydrolysis was also enhanced. G3C9 differs from human PON1 by having 50 amino acid substitutions, while the later round variants VII-D11, I-F11, and VII-D2 differed from G3C9 by having 6, 7, and 8 amino acid substitutions, respectively. Our results identified that VII-D11 has the highest catalytic efficiency of the variants tested against paraoxon. Though further rounds of selection beyond VII-D11 gave rise to
variants such as I-F11 and VII-D2 with increased activity towards 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 or not there were any significant differences between E. coli-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 or not 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 (Magliery et al, unpublished) 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. HDL association of PON1 has also been suggested to play 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 adenovirus-mediated gene delivery system has been employed to induce high-level expression of therapeutic proteins of interest for 4 to 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). Using 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 hydrolyze failed to offer protection against V- and G-type nerve agents (Chilukuri et al., Unpublished data). Using the adenovirus-mediated gene delivery system, we have demonstrated that VII-D11, with a higher \( k_{\text{cat}}/K_m \) against paraoxon, offers superior protection compared to G3C9, which has a lower \( k_{\text{cat}}/K_m \). A similar observation has been made in a previous study by Gupta et al. wherein a later round engineered PON1 variant, 4E9, offered superior protection compared to G3C9 against \( S_{\text{P}}-\text{CMP} \) intoxication (Gupta et al., 2011). In this study, G3C9 was protective against multiple 0.9 × LD50 doses of paraoxon when the enzyme is present in stoichiometric excess (Table 3). When the dose was doubled to 1.8 × LD50 doses, the ability of G3C9 to afford protection began to diminish, with 100% of the G3C9 containing animals displaying either tremors or death after the final cumulative dose. The loss in protection afforded by G3C9 can be attributed to the inability of G3C9 to hydrolyze the increased dose rapidly enough to lower the concentration to nonlethal levels, and/or due to unperceivable residual inhibition of brain and peripheral 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 regime, though it is still possible that nonlethal amounts of agent are able to escape the
bloodstream before hydrolysis by VII-D11. Further physiological 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 deliver 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 LD$_{50}$ and protective ratios due to the inability
to obtain enough biological 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 replicate challenges at fixed stoichiometric
ratios. Using this dosage paradigm, we discovered that each stoichiometric replicate behaved in a
reproducible fashion across a broad range of paraoxon dosages. While 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 suggests that VII-D11 is able to offer symptom free protection against a 7-
fold stoichiometric excess of paraoxon \textit{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 mgs of VII-
D11 should protect a mouse against 5 × LD$_{50}$ exposure of paraoxon. HuBChE, an established OP
stoichiometric bioscavenger (and 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.
Additionally, 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 to reduce 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, malaxon, guthion, and dichlorvos. Further experiments could be implemented to investigate and to improve the broad-spectrum efficacy of VII-D11 against pesticides.
Disclaimers/Acknowledgements

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 U.S. Government. The experimental protocol was approved by the Animal Care and Use Committee of the US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD) and all procedures were 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. The authors are grateful to Dr. Thomas Magliery and Srividya Murali (Department of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, OH 43210) for providing E. coli expressed PON1 for use as standards and CMP substrate.

Authorship Contributions

Participated in research design: Mata and Chilukuri

Conducted Experiments: Mata, Rezk, and Sabnekar

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

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

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

Other: Funding for this work was obtained by Chilukuri
References


Footnotes

This research was supported by the Defense Threat Reduction Agency, Joint Science and Technology Office, Medical S&T Division. Present address (P.E.R): Weatherhead School of Management, Case Western Reserve University, Cleveland, Ohio
**Figure Legends:**

**Figure 1:** Expression and characterization of engineered PON1 variants *in vitro* in 293A cells.

(A) 293A cells were infected with 10 vp/cell of Ad-null control virus or Ad-G3C9, and medium was assayed for arylesterase activity on days 3, 4, 5 and 6 post infection. The results depicted are representative of three independent expressions. (B) 293A cells were infected with Ad-G3C9 at 10 vp/cell, and on day 3, the media and cell lysates were assayed for arylesterase activity. Cells infected with Ad-G3C9 at 25 vp/cell and at 50 vp/cell produced a similar distribution of activity between the medium and cell lysate (not shown here). (C) Western blot of Day 4 culture media from 293A cells infected with Ad-G3C9, Ad-VII-D11, Ad-I-F11, and Ad-VII-D2 using polyclonal antibodies to G3C9. (D) Untreated and 293A cells pretreated with 1 μg/mL and 2 μg/mL tunicamycin were infected with Ad-G3C9 or Ad-VII-D11 at 20 vp/cell, and the media was subjected to Western blot as described in Figure 1C.

**Figure 2:** Expression and characterization of engineered PON1 variants *in vivo* in mice (A)

Expression of VII-D11 was recorded on days 0, 4, 5 and 6 in plasma of mice (n=5) injected with Ad-VII-D11. Expression of VII-D11 was determined using CMP hydrolysis as a metric for expression. (B) Identification of engineered PON1 variants in mice plasma by Western blot. Plasma samples from mice infected with Ad-null, Ad-G3C9, Ad-VII-D11, Ad-I-F11 or Ad-VII-D2 on day 4 were subjected to Western blot as described in Figure 1C.

**Figure 3:** HDL association of VII-D11. A. Separation of plasma proteins by gel filtration chromatography. 100 μL of plasma from a mouse infected with Ad-null, plasma from a mouse infected with Ad-VII-D11, or bacterial VII-D11 incubated in mouse plasma was loaded onto a Superdex 200 gel filtration column. Each peak was assayed for arylesterase (Ad-null plasma) or
CMP (VII-D11-containing plasma) activity, which was plotted onto the chromatograph. **B.** Western blot of each corresponding peak using a rabbit α-G3C9 antibody. **C.** Western blot of each corresponding peak using a rabbit α-ApoA1 antibody.

**Figure 4:** Partial purification of engineered PON1 variants expressed *in vitro* in 293A cells. (**A**) Enrichment of VII-D11 following one-step purification involving anion-exchange chromatography. (**B**) Coomassie blue gel photograph showing the presence of a number of proteins in the partially purified preparations of in vitro expressed engineered PON1 variants. Culture medium infected with Ad-null was similarly partially purified, and its protein pattern was also shown (lane marked Ad-null). (**C**) Western blot image showing the presence of G3C9, VII-D11, I-F11, and VII-D2 in respective partially purified samples, but the partially purified preparation from the control virus sample did not show the presence of G3C9 reactive proteins (lane marked Ad-null).
<table>
<thead>
<tr>
<th>Variant</th>
<th>Phenyl Acetate $k_{cat}/K_m$ ($\times 10^7$ M$^{-1}$ min$^{-1}$)</th>
<th>Paraoxon $k_{cat}/K_m$ ($\times 10^5$ M$^{-1}$ min$^{-1}$)</th>
<th>CMP $k_{cat}/K_m$ ($\times 10^6$ M$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3C9 (E. coli)</td>
<td>0.19 ± 0.01</td>
<td>0.10 ± 0.04</td>
<td>0.021 ± 0.003</td>
</tr>
<tr>
<td>G3C9 (293A)</td>
<td>1.68 ± 0.08</td>
<td>1.03 ± 0.04</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>G3C9 (mouse)</td>
<td>2.4 ± 0.1</td>
<td>1.17 ± 0.06</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>VII-D11 (E. coli)</td>
<td>n.d.</td>
<td>11.3 ± 0.4</td>
<td>3.06 ± 0.08</td>
</tr>
<tr>
<td>VII-D11 (293A)</td>
<td>n.d.</td>
<td>21.4 ± 0.7</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>VII-D11 (mouse)</td>
<td>n.d.</td>
<td>16.1 ± 0.6</td>
<td>1.85 ± 0.09</td>
</tr>
<tr>
<td>VII-D2 (E. coli)</td>
<td>n.d.</td>
<td>2.22 ± 0.02</td>
<td>2.83 ± 0.08</td>
</tr>
<tr>
<td>VII-D2 (293A)</td>
<td>n.d.</td>
<td>5.3 ± 0.2</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>VII-D2 (mouse)</td>
<td>n.d.</td>
<td>1.0 ± 0.6</td>
<td>1.66 ± 0.02</td>
</tr>
<tr>
<td>I-F11 (E. coli)</td>
<td>n.d.</td>
<td>2.04 ± 0.04</td>
<td>2.81 ± 0.06</td>
</tr>
<tr>
<td>I-F11 (293A)</td>
<td>n.d.</td>
<td>5.6 ± 0.1</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>I-F11 (mouse)</td>
<td>n.d.</td>
<td>6.79 ± 0.07</td>
<td>8.9 ± 0.2</td>
</tr>
</tbody>
</table>

**Table 1.** Catalytic efficiencies against paraoxon, phenyl acetate and CMP of PON1 variants expressed in *E. coli*, 293A cells, and mice. n.d. not detected
Table 2. Michaelis-Menten parameters for G3C9 CMP hydrolysis. G3C9 expressed in mammalian cells displayed an increase in $k_{cat}$, which could not be recapitulated by addition of exogenous protein.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<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>
<tr>
<td>Group</td>
<td>Day 4</td>
<td>Day 5</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
<td>---------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Challenge 1 0.9 × LD₅₀ (Survival)</td>
<td>Challenge 2 0.9 × LD₅₀ (Survival)</td>
<td>Challenge 3 0.9 × LD₅₀ (Survival)</td>
</tr>
<tr>
<td>Control (n=3)</td>
<td>3/3 (100%)</td>
<td>0/3 (0%)</td>
<td>N/A</td>
</tr>
<tr>
<td>G3C9 (n=6)</td>
<td>6/6 (100%)</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>
<td>5/5 (100%)</td>
</tr>
</tbody>
</table>

Table 3. VII-D11 offers superior protection compared to G3C9 against paraoxon. Prior to 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 – 4.6 mg/mL and 4.8 – 6.7 mg/mL on days 4 and 5, respectively. VII-D11 concentration ranged from 1.6 - 8.8 mg/mL and 4.4 – 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>Mice (n)</th>
<th>Stoichiometry tested (Paraoxon:VII-D11)</th>
<th>VII-D11 concentration range (mg/ml)</th>
<th>LD$_{50}$ dose range</th>
<th>% Survival/Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2:1</td>
<td>0.7 - 1.0</td>
<td>1.4 - 1.9</td>
<td>100% survival 0% symptomatic</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 0% symptomatic</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 10% symptomatic</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 0% symptomatic</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 8% symptomatic</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 100% symptomatic</td>
</tr>
<tr>
<td>4</td>
<td>10:1</td>
<td>0.4 - 1.0</td>
<td>3.5 - 9.0</td>
<td>25% survival 100% symptomatic</td>
</tr>
</tbody>
</table>

**Table 4.** *In vivo* stoichiometric assessment of protective efficacy of VII-D11 against paraoxon. Prior to challenge with paraoxon, blood was drawn and assayed for CMP hydrolyzing, 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.
Figure 1

(A) Aryl esterase specific activity (μmol min⁻¹ mL⁻¹) over days.

(B) Aryl esterase specific activity in different samples:
- Ad-G3C9 Media
- Ad-G3C9 Cell Lysate
- Ad-null Media

(C) Gel electrophoresis images with bands labeled as:
- M
- G3C9
- VI-D11
- VII-D11
- G3C9 + 2μg tun
- G3C9 + 1μg tun
- VII-D11 + 2μg tun
- VII-D11 + 1μg tun
Figure 4

A

Specific Activity (mM min⁻¹ mg⁻¹)

Crude  |  Partially Purified

B

C

M  G3C9  VII-D11  I-F11  Ad-null

M  G3C9  VII-D11  I-F11  Ad-null
SUPPLEMENTAL DATA

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

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

I. Structures of substrates used

\[
\text{phenyl acetate} \quad \text{paraoxon} \quad \text{CMP}
\]

III. 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).

<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>
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<tr>
<td>4</td>
<td>0.31</td>
<td>2.50</td>
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<td>0</td>
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<tr>
<td>4</td>
<td>0.36</td>
<td>2.57</td>
<td>1</td>
<td>25</td>
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<tr>
<td>8</td>
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<td>2.70</td>
<td>3</td>
<td>37.5</td>
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<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>
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<td>100</td>
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