Title: **Targeting of Organophosphorus Compound Bioscavengers to the Surface of Red Blood Cells**

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Abstract

To develop a prophylactic for organophosphorus (OP) poisoning utilizing catalytic bioscavengers, the circulatory stability of the enzymes needs to be increased. One strategy for increasing the bioavailability of OP bioscavengers is to target them to the surface of red blood cells (RBCs). Given the circulatory lifespan of 120 days for human RBCs, this strategy has the potential for creating a persistent pool of bioscavenger. Here we report the development of fusion proteins with a single chain variable fragment (scFv) of Ter119, a molecule that associates with glycophorin A on the surface of RBCs, and the VIID11 variant of paraoxonase 1 (scFv-PON1). We show that scFv-PON1 variants expressed by *Trichoplusia ni* larvae are catalytically active and that one variant in particular can successfully bind to the surface of murine RBCs both *in vitro* and *in vivo*. This study represents a proof of concept for targeting catalytic bioscavengers to the surface of RBCs and is an early step in developing catalytic bioscavengers that can remain in circulation for an extended period of time.

Introduction

Organophosphorus compounds (OP) are highly poisonous chemicals that prevent normal function of the nervous system through the irreversible binding and inactivation of acetylcholinesterase (AChE) [1, 2]. Inhibition of AChE leads to a buildup of acetylcholine in the central and peripheral nervous system, resulting in a cholinergic syndrome consisting of respiratory and oral secretions, diarrhea and vomiting, sweating, altered mental status, autonomic instability, general weakness, and progression to paralysis and respiratory arrest [2, 3]. In the United States the standard of care for OP poisoning is treatment with atropine, which reduces binding of acetylcholine to muscarinic receptors, and pyridinium-2-aldoxime (2-PAM Cl), which can reactivate inhibited AChE [2-5]. If administered shortly after intoxication, these medical countermeasures are successful in preventing death, but do not alleviate performance deficits in the warfighter caused by the symptoms of OP poisoning [4, 6]. This, in addition to the

side-effects from the therapeutics themselves, can leave units temporarily depleted and vulnerable to future attack. The issue of how to treat OP exposures with a minimum of physiological and cognitive consequences has led to the concept and development of OP bioscavengers [4, 7-12]. Desirable characteristics for prophylactic bioscavengers include easy administration (i.e., intramuscular rather than intravenous injection), broad specificity, lack of physiological, cognitive, behavioral, or immunological effects, and sustained stability in the circulation. One strategy for designing a catalytic bioscavenger is based on paraoxonase 1 (PON1), which has been the subject of a concerted, directed evolution effort to improve its activity vs. G-agents [13]. PON1 variants have been identified with sufficient activity to be candidates for further development, and new technologies are needed to improve the *in vivo* retention of this protein for effective prophylactic use.

A strategy for prolonging the bioavailability of OP bioscavengers is to sequester them onto red blood cells (RBCs), improving their retention and stability. Approximately 30×10^{12} RBCs at any given time are circulating in the human body [14], providing great potential for surface labeling, distribution, and retention in the vascular compartment. Additionally, human RBCs have an average circulatory lifetime of 120 days [15]. Use of a RBC-specific membrane protein such as glycophorin A (GPA), the major cell surface sialoglycoprotein which accounts for 1.6% of the total RBC membrane protein, equaling about 10^6 copies/cell [16-18], as a target with which to associate an enzyme could theoretically yield an *in vivo* C_{max} of 40 μ M and create a bioscavenger depot of fully accessible and reactive enzyme. This technique has been previously utilized successfully in rodent models to prolong the *in vivo* retention of blood coagulation therapeutic proteins such as decay accelerator factor (DAF) and tissue plasminogen activation factor (tPA). The *in vivo* results were compelling as RBC fusion dramatically enhanced recombinant protein retention times. DAF exhibited a loss of 10-12% per day [19], while over 80% of tPA activity was retained after 2 days [20]. The decay rates of these fusion proteins parallel the circulatory half-life of mouse RBCs [21].

Here we describe work to develop an OP bioscavenger with a GPA-associating single chain variable fragment (scFv) based on the Ter119 antibody to increase the bioavailability of PON1 by targeting the bioscavenger to RBCs. The Ter119 antibody recognizes the antigen Ter119, a 52 kDa molecule on the surface of murine RBCs that is known to highly associate with GPA [22]. We observed no significant impact on PON1 activity from fusion with the scFv, and we observed the successful binding of a scFv-PON1 variant to murine RBCs both *in vivo* and *in vitro*. Proteins delivered in this manner could theoretically improve the retention times to

as long as those of the native RBC (approximately 120 days). With such treatment it would be possible to retain effective concentrations of the proteins over a time course relevant to warfighters and first responders and to reduce or eliminate the need for multiple dosing to maintain protective levels of enzyme in the circulation.

Materials and Methods

Fusion Proteins: Fusion proteins of a scFv for Ter119 [19] and the VIID11 [23, 24] variant of PON1 were purchased from Chesapeake PERL Inc. (Savage, MD). The enzymes were expressed by Chesapeake PERL using their *Trichoplusia ni* larval expression system [25]. Variants were constructed according to the strategy in Figure 1. A GSSSGSSS linker was used between scFv and PON1 sequences, and a C terminal His6x tag was included in the fusion protein to assist with isolation and purification [26].

SDS-PAGE of fusion proteins: Protein samples were prepared for SDS-PAGE with 2X Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and 2-Mercaptoethanol (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Gels were run using Mini-Protean TGX gels (Bio-Rad, Hercules, CA) in Tris/Glycine/SDS Buffer (Bio-Rad, Hercules, CA) and Mark 12 unstained standard (Life Technologies, Carlsbad, CA). Gels were run at 100-130V and then stained with eStain2.0 and protein staining pads (GenScript, Piscataway, NJ) according to the manufacturer's protocol. Gels were then imaged on an Odyssey CLx (LiCor Biosciences, Lincoln, NE) using Image Studio software.

In vitro PON1 activity: *In vitro* enzyme activity was assessed using a spectrometric paraoxonase activity assay [27]. The assay was run in a 96-well plate format with a final reaction volume of 200 μ l per well. PON1 fusion proteins were added at a volume of 10 μ l to the plates, along with 180 μ l of assay buffer consisting of 50 mM HEPES pH 7.2 and 10 mM CaCl_2 (Sigma-Aldrich, St. Louis, MO). Increasing concentrations of paraoxon (Chem Service Inc., West Chester, PA) were added at volumes of 10 μ l to each well. Immediately following the addition of paraoxon the plate was read using a SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA) in kinetic mode at a wavelength of 412 nm. Following a 30 sec plate shake, the plate was read in 20 sec intervals for 10 min. The velocity of the reaction in milli-absorbance units per minute (mAU/min) was calculated by the instrument's software. Using the Beer-Lambert Law and the extinction coefficient of $1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, the reaction rate was converted

from mAU/min to M/min and plotted vs. paraoxon concentration. $V_{\max}$ and k_m were calculated from the plot using the Michaelis-Menton model with GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA), and k_{cat} and the catalytic efficiency (k_{cat}/k_m) were calculated from those values.

In vitro RBC targeting: Approximately 25 μl of mouse whole blood was obtained from male C57BL/6 mice aged 2-4 months, washed with PBS, 0.2% BSA, and centrifuged for 5 min at 200 x g at 4°C. The RBC pellet was resuspended in 100 μl PBS pH 7.4, 0.2% BSA and incubated with increasing concentrations of Ter119 scFv-PON1 fusion proteins (30, 45, and 75 $\mu\text{g/ml}$) for 45 min on ice. Cells were centrifuged for 5 min at 200 x g at 4°C and resuspended in 100 μl PBS pH 7.4, 0.2% BSA. 6xHis Epitope Tag Antibody, DyLight 680 conjugate (Thermo Scientific, Rockford, IL) was added to the cells at 1:100 and incubated for 30 min on ice. Cells were washed with PBS pH 7.4, 0.2% BSA, centrifuged for 5 min at 200 x g at 4°C, and resuspended in 300 μl PBS pH 7.4, 0.2% BSA. RBCs were analyzed by flow cytometry using a guava easyCyte™ flow cytometer (EMD Millipore, Billerica, MA), gating on live cells (as determined by forward and side scattering).

In vivo experiments: C57BL/6 background mice, age 2-6 months, were dosed with 5 mg/kg of fusion enzyme via intraperitoneal (IP) injection. Approximately 25 μl of blood was collected at 0, 2, 24, and 50 h via tail snip in a heparin blood collection tube. RBCs were separated from plasma via centrifugation at 10,000 x g for 10 mins at 4°C. Plasma was removed and frozen at -80°C for further analysis. Cells were then analyzed by flow cytometry as stated above.

Plasma PON1 activity assay: Plasma PON1 activity of the mice was determined using similar methods to the *in vitro* assay described above. Frozen plasma was thawed, and 10 μl of plasma and 0.035 M paraoxon were used in the same assay conditions as the *in vitro* assay. The PON1 activity for each mouse at each time point was normalized to its baseline (activity in plasma samples collected at time point 0).

Data analysis: Statistical significance was determined for the *in vitro* data by using a Student's T-test with appropriate Bonferroni corrections and a significance threshold of 0.017. A two-factor ANOVA with repeated measures was used to compare the *in vivo* data's three treatment groups and three observation times. A significant treatment group by time interaction ($p <$

0.0001) was observed, indicating that the differences among treatment groups varied with time. Therefore, a Bonferroni post-test was used to compare pairs of treatments at each time point.

Results

Ter119 scFv-PON1 catalytic efficiency

We obtained Ter119 scFv-PON1 (VIID11) fusion protein variants from Chesapeake PERL, with an added 6xHis residue to aid in isolation and purification [26], which produced them using a *Trichoplusia ni* larval expression system [25] (Figure 1.). Samples were characterized by SDS-PAGE gel to confirm molecular weights correlated with each construct. As expected, the scFv-PON1 proteins (Figure 1) (AB771: PON1-scFv-His, AB772: scFv-PON1-His) and unconjugated control (PON1-His) ran at the predicted molecular weights (scFv-PON1 – 64 KDa, PON1 – 38 KDa) (Figure 2.). To confirm that the PON1 fusion proteins still retained enzymatic activity following the addition of the Ter119 scFv sequence we performed paraoxonase activity assays [27]. While all of the PON1 variants expressed by *Trichoplusia ni* larvae had lower catalytic efficiencies than previously reported [23] (10-25%), the addition of the Ter119 scFv did not appear to have any significant deleterious effect on activity (Figure 3.).

Ter119 scFv-PON1 in vitro binding

To test whether the scFv-PON1 fusion proteins could bind RBCs, we incubated murine RBCs with increasing concentrations of AB743, AB771, and AB772 *in vitro*. Using a fluorescence antibody against the 6xHis residue, we determined the percentage of RBCs with bound PON1 via flow cytometry. Only the AB771 fusion protein was able to significantly bind to the RBCs at 45 µg/ml and 75 µg/ml (Figure 4.). At the highest concentration tested, AB771 was on the surface of 66.56% of murine RBCs, whereas at the lowest concentration it was on the surface of over 35% of the RBCs. Both AB743 and AB772 were unable to bind murine RBCs *in vitro*.

Ter119 scFv-PON1 in vivo binding

Following the *in vitro* results we tested AB771 in a small scale *in vivo* mouse study. C57BL/6 background mice were dosed at 5 mg/kg with AB743, AB771, or PBS alone. During the experimental procedure none of the mice showed any outward appearance of illness or distress. Blood was obtained via tail snip at times 0, 2, 24, and 50 h. Using a fluorescence antibody against 6xHis, we determined the percentage of RBCs labeled with PON1 by flow cytometry. A significant treatment group by time interaction ($p < 0.0001$) was observed,

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4 indicating that the differences among treatment groups varied with time. AB771 was observed
5 on the surface of a significant percentage of murine RBCs at 2 and 24 h after dosing (Figure 5.,
6 $p < 0.0001$, two-factor ANOVA with Bonferroni post-test), and although not significant a
7 detectable amount was seen in some mice at 50 h. At 2 h after dosing, the percentage of RBC
8 with AB771 on the surface was 13.36%, and over the next 48 h the percentage decreased to
9 2.35%. The flow cytometry results were confirmed by measuring plasma PON1 activity (Table
10 1.). As expected, there was a spike in plasma PON1 activity in mice dosed with the
11 unconjugated control (AB743) at 2 h after dosing. Mice administered AB771 showed no
12 significant increased level of plasma PON1 activity when compared to control mice, and the
13 levels were significantly lower than in AB743-treated mice at 2 and 24 h ($p < 0.0001$, two-factor
14 ANOVA with Bonferroni post-test). The lack of substantial increase in plasma PON1 activity in
15 mice treated with AB771, but seen in mice dosed with unconjugated PON1, supports the flow
16 cytometry data in suggesting that AB771 can successfully bind to murine RBCs *in vivo*.
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18 To summarize, all of the variants tested displayed no deleterious impact on enzymatic
19 activity from the addition of the scFv sequence. AB771 showed an ability to bind RBCs both *in*
20 *vitro* and *in vivo* as demonstrated by flow cytometry data and the lack of increased plasma
21 PON1 activity, whereas AB772 showed no such ability. As expected, the variant AB743, which
22 lacked the Ter119 scFv sequence and therefore was unable to bind RBCs, was found to
23 increase PON1 plasma activity but not to associate with RBCs when administered to mice.
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26 Discussion

27 Desirable characteristics for any prophylactic bioscavenger include easy administration,
28 broad specificity, lack of physiological, psychological, or immunological effects, and persistence
29 in the circulation. For catalytic bioscavengers to reach their full potential as prophylactics
30 against OP poisoning, increased circulatory stability is a key requirement. One strategy to
31 increase the bioavailability of catalytic bioscavengers is to sequester them on the surface of
32 circulating RBCs, which have a long circulatory lifespan in humans. [15]. Glycophorin A is a
33 promising candidate for targeting prophylactics to RBCs, because of its high surface level
34 expression [16, 17], and this strategy has been previously employed in rodent models with anti-
35 blood coagulation therapies [19, 20] by utilizing a scFv for Ter119 [22].
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37 In this study we showed that a fusion protein of Ter119 scFv and the bioscavenger
38 PON1 (variant VIID11) could successfully be targeted to murine RBCs. When fused to PON1,
39 the scFv sequence showed no significant deleterious effect on PON1 enzyme activity. One
40 fusion protein (AB771) was able to bind a high percentage of RBCs in *in vitro* experiments.
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4 Interestingly, we did not observe AB772 associated with the surface of RBCs in *in vitro*
5 experiments. Although we do not have any results to help explain why AB771 associates with
6 RBCs and AB772 does not, it is most likely due either to inaccessibility of the Ter119 binding
7 site in the AB772 fusion protein or to disruptions in the structure of the scFv binding site when it
8 is located at the N-terminus of this variant. We do not believe that the folding of AB772 has
9 blocked the 6xHis tag from being recognized by our fluorescent probe, since the tag was utilized
10 in the isolation and purification of the enzyme.
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16 In a small scale *in vivo* mouse study, we were able to detect AB771 (PON1-scFv-His) on
17 the surface of RBCs at least 24 h after dosing (5 mg/kg), although the percentage of labeled
18 RBCs was much lower than in the *in vitro* study. The normal lifespan of a mouse RBC is around
19 40 days compared to 120 in humans [15, 21, 28], which, if we assume the results are scalable
20 to humans, suggests the possibility of this strategy lasting at least 3 days in circulation. When
21 comparing plasma PON1 activity of mice dosed with either AB771 or the unconjugated control
22 (AB743), we observed a significant initial increase in activity in AB743 mice which decreased
23 over time. The initial increase of paraoxonase activity was absent in AB771-treated mice. The
24 results were interesting given the lower percentage of RBCs with AB771 bound *in vivo*, when
25 compared to the *in vitro* results, as an increase in plasma PON1 activity greater than the 2-fold
26 increase observed could be expected. This leads to the possibility that the flow cytometry data
27 could be underrepresenting the true amount of enzyme binding to RBCs, since it cannot
28 account for the amount of enzyme on each positively stained cell. It is possible that when
29 introduced via IP injection, the fusion protein binds a small subset of RBCs with a high
30 efficiency, resulting in a lower percentage of cells decorated with a higher concentration of
31 bioscavenger enzyme. Alternatively, it is also possible that the fusion proteins are being
32 sequestered in the intraperitoneal space or once bound to RBCs are increasing the RBC
33 turnover rate, leading to their more rapid clearance; these issues will be the topic of future
34 investigation.
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48 Previous investigators have showed near uniform labeling of RBCs by a Ter119 scFv-
49 DAF fusion protein *in vivo* [19], while our fusion protein was only able to label around 13% of the
50 RBCs at the earliest time point. Since the RBC count for a normal mouse is $7-11 \times 10^6/\mu\text{l}$ [29]
51 labeling 13% still raises the possibility of about $1.8-2.9 \times 10^9$ cells carrying the bioscavenger in
52 these mice; whether this amount of binding represents a dose of enzyme that will be protective
53 against OP intoxication still needs to be addressed. It is also important to note that in the prior
54 experiments, the scFv-DAF was administered via intravenous injection, while the PON1-scFv
55 was administered via IP injection, which could account for some of the differences. Given the
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4 substantial decrease in PON1-scFv targeting the RBCs *in vivo*, when compared to *in vitro*,
5 adopting a strategy similar to Zaitzev *et al.* [20] where RBCs are collected, loaded with the
6 Ter119 scFv fusion protein, and then reintroduced via IV injection may ultimately be necessary
7 to afford protection against OP agents with AB771.
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11 Further work is still necessary to determine a prophylactically relevant binding affinity of
12 PON1-scFv for murine RBCs and, if required, to develop strategies to improve this binding
13 affinity. This study represents a proof of concept that a catalytically active OP bioscavenger can
14 be successfully targeted to the surface of RBCs and remain in circulation for an extended period
15 of time. The strategy we employed can be used to develop a prophylactic for long-term
16 protection against OP nerve agent and pesticide poisoning.
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51 The views expressed in this article are those of the authors and do not reflect the official policy
52 of the Department of Army, Department of Defense, or the US Government.
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References

1. Sidell, F. and J. Borak, *Chemical warfare agents: II. Nerve agents*. Annals of Emergency Medicine, 1992. **21**(7): p. 865-871.
2. Leikin, J., et al., *A review of nerve agent exposure for the critical care physician*. Critical Care Medicine, 2002. **30**(10): p. 2346-2354.
3. CDC. *Toxic Syndrome Description: Nerve Agent and Organophosphate Pesticide Poisoning* 2013 Feb 14 2013.
4. Otto, T.C., et al., *Identification and characterization of novel catalytic bioscavengers of organophosphorus nerve agents*. Chemico-Biological Interactions, 2013. **203**(1): p. 186-190.
5. Eddleston, M., et al., *Management of acute organophosphorus pesticide poisoning*. Lancet, 2008. **371**(9612): p. 597-607.
6. Maynard, R. and F. Beswick, *Organophosphorus compounds as chemical warfare agents*, in *Clinical and Experimental Toxicology of Organophosphates and Carbamates*, B. Ballantyne and T.C. Marrs, Editors. 1992, Butterworth: Oxford. p. 373-385.
7. Valiyaveetil, M., et al., *Protective efficacy of catalytic bioscavenger, paraoxonase 1 against sarin and soman exposure in guinea pigs*. Biochemical Pharmacology, 2011. **81**(6): p. 800-809.
8. Lenz, D., et al., *Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: A mini review*. Toxicology, 2007. **233**(1-3): p. 31-39.
9. Ashani, Y., et al., *Stereo-specific synthesis of analogs of nerve agents and their utilization of selection and characterization of paraoxonase (PON1) catalytic scavengers*. Chemico-Biological Interactions, 2010. **187**(1-3): p. 362-369.
10. diTargiani, R.C., et al., *In search of catalytic bioscavenger for the prophylaxis of nerve agent toxicity*. Chemico-Biological Interactions, 2010. **187**(1-3): p. 349-354.
11. Myers, T., et al., *Characterization of human serum butyrylcholinesterase in rhesus monkeys: behavioral and physiological effects*. Neurotoxicology and Teratology, 2012. **34**(3): p. 323-330.
12. Genovese, R. and B. Doctor, *Behavioral and pharmacological assessment of butyrylcholinesterase in rats*. Pharmacology, Biochemistry, and Behavior, 1995. **51**(4): p. 647-654.
13. Gupta, R., et al., *Directed evolution of hydrolases for prevention of G-type nerve agent intoxication*. Nature Chemical Biology, 2011. **7**: p. 120-125.
14. Ballas, S., *Erythrocyte concentration and volume are inversely related*. Clinica Chimica Acta, 1987. **164**(2): p. 243-244.
15. Shemin, D. and D. Rittenberg, *The life span of the human red blood cell*. Journal of Biological Chemistry, 1946. **166**: p. 627-636.
16. de Oliveira, S. and C. Saldanha, *An overview about erythrocyte membrane*. Clinical Hemorheology and Microcirculation, 2010. **44**: p. 63-74.
17. Poole, J., *Red cell antigens on band3 and glycophorin A*. Blood Reviews, 2000. **14**(1): p. 31-43.
18. Gahmber, C., et al., *Biosynthesis of the major human red cell sialoglycoprotein, glycophorin A: A review*. Revue Française de Transfusion et Immuno-hématologie, 1981. **24**(1): p. 53-73.
19. Spitzer, D., et al., *ScFv-mediated in vivo targeting of DAF to erythrocytes inhibits lysis by complement*. Molecular Immunology, 2003. **40**: p. 911-919.
20. Zaitzev, S., et al., *Targeting of a mutant plasminogen activator to circulating red blood cells for prophylactic fibrinolysis*. The Journal of Pharmacology and Experimental Therapeutics, 2010. **332**(3): p. 1022-1031.
21. Magnani, M., et al., *Effect of age on some properties of mice erythrocytes*. Mechanisms of ageing and development, 1988. **42**(1): p. 37-47.

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22. Kina, T., et al., *The monoclonal antibody Ter-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage*. British Journal of Haematology, 2000. **109**(2): p. 280-287.
 23. Mata, D.G., et al., *Investigation of evolved paraoxonase-1 variants for prevention of organophosphorous pesticide compound intoxication*. Journal of Pharmacology and Experimental Therapeutics, 2014. **349**(3): p. 549-558.
 24. Goldsmith, M., et al., *Evolved stereoselective hydrolases for broad-spectrum G-type nerve agent detoxification*. Chemistry and Biology, 2012. **19**(4): p. 456-466.
 25. Kovaleva, E., et al., *Recombinant protein production in insect larvae: host choice, tissue distribution, and heterologous gene instability*. Biotechnology Letters, 2009. **31**(3): p. 381-386.
 26. Schmitt, J., H. Hess, and H.G. Stunnenberg, *Affinity purification of histidine-tagged proteins*. Molecular Biology Reports, 1993. **18**(3): p. 223-230.
 27. Yeung, D., D. Lenz, and D.M. Cerasoli, *Analysis of active-site amino-acid residues of human serum paraoxonase using competitive substrates*. The FEBS Journal, 2005. **272**(9): p. 2225-2230.

AB771 **AB772** **AB743**

Figure 1. Schematic of Fusion Protein Construction. The design strategy for scFv-PON1 fusion proteins. The sequence for the VIID11 variant of PON1 was linked to the Ter119 scFv sequence by a GSSSSGSSSS sequence. A C terminal His6x residue was added to aid in isolation and purification.

Figure 2. SDS-PAGE of fusion proteins. An SDS-PAGE was run on fusion proteins obtained from Chesapeake PERL to confirm their identities. All proteins ran at the sizes expected, indicating that AB771 and AB772 are the fusion proteins of scFv-PON and that AB743 is the unconjugated PON control.

Figure 3. *In vitro* Paraoxonase activity. Activity of the three fusion proteins was tested against paraoxon. The assay was run in a 96-well plate format with a final reaction volume of 200 μl per well. PON1 fusion proteins were added at a volume of 10 μl to the plates (final concentration of 0.13 μM), along with 180 μl of assay buffer consisting of 50 mM HEPES pH 7.2 and 10 mM CaCl_2 . Increasing concentrations of paraoxon were added at volumes of 10 μl to each well, and plates were read in kinetic mode at a wavelength of 412 nm, and read at 20 sec intervals for 10 min. The reaction rate was converted from mAU/min to M/min and plotted vs. paraoxon concentration. V_{max} and k_m were calculated from the plot using the Michaelis-Menton model, and k_{cat} and the catalytic efficiency (k_{cat}/k_m) were calculated from those values. $n = 3$, Error Bars = Standard Deviation.

Figure 4. *In vitro* binding of scFv-PON variants to RBCs. RBCs were incubated with scFv-PON variants and then with a fluorescent antibody for 6xHis. Cells were analyzed by flow cytometry to determine the percentage of cells labeled with fusion protein. There was no detectable binding of AB743 (unconjugated PON1) or AB772 (scFv-PON1) at any of the concentrations tested. AB771 (PON1-scFv) had significant binding at 45 µg/ml and 75 µg/ml when compared to the unconjugated PON variant. The results indicate that AB771 can successfully bind to the surface of RBCs. * $p < 0.017$, Student's T-test vs. AB743 with Bonferroni correction. $n = 3$, Error Bars = Standard Deviation.

Figure 5. *In vivo* binding of AB771 to RBCs. C57BL/6 background mice were dosed with 5 mg/kg of PON1-scFV (AB771), unconjugated PON1 (AB743), or PBS only. Blood samples were obtained at 2, 24, and 50 h, and the percentage of RBCs labeled with enzyme was determined by flow cytometry using a fluorescent antibody for His6x. The values for individual mice are represented by open circles (AB771), gray circles (AB743), and closed squares (PBS). Average values for each group are represented by a line. Background fluorescence was measured on $0.15\% \pm 0.06\%$ of red blood cells. A two-factor ANOVA with repeated measures was used to compare the three treatment groups and three observation times. A significant treatment group by time interaction ($p < 0.0001$) was observed, indicating that the differences among treatment groups varied with time. Therefore, a Bonferroni post-test was used to compare pairs of treatments at each time point. * $p < 0.0001$ vs. PBS; ^ $p < 0.0001$ vs. AB743; PBS and AB743 $n = 5$, AB771 $n = 9$

Table 1. Normalized plasma PON1 activity

	PBS	AB771	AB743
2 h	1.08 ± 0.19	2.48 ± 0.80 [^]	22.91 ± 5.33 [*]
24 h	1.26 ± 0.34	1.96 ± 0.61 [^]	7.12 ± 1.50 [*]
50 h	1.03 ± 0.26	1.50 ± 0.46	2.24 ± 0.51

PON1 activity was normalized to baseline at time 0 for each mouse (1.00), ± standard deviation. A two-factor ANOVA with repeated measures was used to compare the three treatment groups and three observation times. A significant treatment group by time interaction ($p < 0.0001$) was observed, indicating that the differences among treatment groups varied with time. Therefore, a Bonferroni post-test was used to compare pairs of treatments at each time point. ^{*} $p < 0.0001$ vs. PBS, [^] $p < 0.0001$ vs. AB743, $n = 4-9$

Table 2. Normalized plasma PON1 activity

	PBS	AB771	AB743
2 hrs	1.08 ± 0.19	2.61 ± 0.74*^	22.91 ± 5.33*
24hr	1.26 ± 0.34	2.01 ± 0.63*^	7.12 ± 1.50*
50 hrs	1.03 ± 0.26	1.62 ± 0.32*	2.24 ± 0.51*

PON1 activity was normalized to baseline at time 0 for each mouse (1.00), ± Standard Deviation, * $p < 0.0167$ Student's T Test with Bonferroni correction vs. PBS, ^ $p < 0.001$ Student's T Test with Bonferroni correction vs. AB743, $n = 5-9$