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# *In vitro* detoxification of cyclosarin in human blood pre-incubated *ex vivo* with recombinant serum paraoxonases

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ABSTRACT

An *ex vivo* protocol was developed to assay the antidotal capacity of rePON1 variants to protect endogenous acetylcholinesterase and butyrylcholinesterase in human whole blood against OP nerve agents. This protocol permitted us to address the relationship between blood rePON1 concentrations, their kinetic parameters, and the level of protection conferred by rePON1 on the cholinesterases in human blood, following a challenge with cyclosarin (GF). The experimental data thus obtained were in good agreement with the predicted percent residual activities of blood cholinesterases calculated on the basis of the rate constants for inhibition of human acetylcholinesterase and butyrylcholinesterase by GF, the concentration of the particular rePON1 variant, and its $k_{cat}/K_m$ value for GF. This protocol thus provides a rapid and reliable *ex vivo* screening tool for identification of rePON1 bioscavenger candidates suitable for protection of humans against organophosphorus-based toxicants. The results also permitted the refinement of a mathematical model for estimating the efficacious dose of rePON1s variants required for prophylaxis in humans.



## 1. Introduction

A directed evolution strategy was recently described for generating large libraries of mammalian PON1 variants (recombinant mammalian paraoxonase 1, rePON1) that could be over-expressed in *Escherichia coli* and screened for their ability to hydrolyze chemical warfare nerve agents (CWNAs) (Gupta et al., 2011). Structural analysis was used to direct the mutagenesis of relevant active-site positions so as to generate highly effective bioscavengers capable of detoxifying organophosphate-(OP)-based G-type CWNAs. The combined strategy of enhanced evolution and protein engineering, together with a specific interception screening protocol (Gupta et al., 2011), permitted identification of mutants with $k_{cat}/K_m$ values for hydrolysis of an *in situ*-generated cyclosarin (GF) that approached levels required for them to be considered as efficacious catalytic bioscavenger drugs ($k_{cat}/K_m \geq 10^7\ M^{-1}\ min^{-1}$). Briefly, variation at several active-site residues, including 115, 69 and 222, seems to be the key to obtaining improved variants for hydrolysis of G-agents in general, and GF, in particular.

To ascertain that the evolved PON1 variants maintain their capacity to perform the expected detoxification in the circulation, one effort of this project has been geared to exploring the effects of association of rePON1 with human blood constituents, such as HDL, on PON1's catalytic activity and circulatory life-time, followed by subsequent protein engineering to improve these two parameters *in vivo*. For example, we have shown that the PON1–HDL complex may exhibit a potential for *in vivo* treatment of mice against OP intoxication (Gaidukov et al., 2009). In addition, the association of rePON1 variants with HDL seems to significantly prolong the serum life time in mice (Goldsmith et al., unpublished). It thus seemed to us that it would be important to examine the effect of human blood on the stability and proficiency of rePON1 variants. Recently, Valiyaveettil et al. (2011) demonstrated the *in vivo* protection of blood acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), as well as of brain AChE, against slow microinstillation exposure to sarin and soman in guinea pigs pretreated with human and rabbit PON1. Survival of animals could be correlated with the degree of protection of the cholinesterases (ChEs).

We have now developed an *ex vivo* protocol to screen the potential antidotal capacity of rePON1 variants via their ability to protect the endogenous AChE and BChE in human whole blood obtained

*Abbreviations:* AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChE, cholinesterase; rePON1, recombinant mammalian paraoxonase 1; CWNA, chemical warfare nerve agent; GF, cyclosarin; ATC, acetylthiocholine iodide; WB, whole blood.

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from a local blood bank. This protocol enabled us to correlate the concentrations of the rePON1s in whole blood, and their kinetic parameters ($k_{cat}/K_m$) determined in buffer solution, with the level of protection conferred on ChEs in human blood, following a challenge with GF. The latter was generated *in situ* at a non-hazardous concentration (Gupta et al., 2011). Validation of this protocol provides a rapid and reliable *ex vivo* screening tool for identification of rePON1 bioscavenger candidates suitable for administration to humans for the purpose of providing protection against OP intoxication.

## 2. Materials and methods

### 2.1. Chemicals

5,5′-dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide (ATC) and butyrylthiocholine iodide (BTC) were obtained from Sigma (Rehovot, Israel). Tergitol was purchased form Aldrich Chemicals (Rehovot, Israel). *O*-cyclohexyl methylphosphonofluoridate (cyclosarin, GF) was generated *in situ*, from its coumarin surrogate, in dilute aqueous solution, as previously described (Ashani et al., 2010; Gupta et al., 2011). For further information please contact the corresponding author.

*Caution:* Although both the aqueous concentration and total volume of the *in situ*-generated GF solution were below threat toxic levels of GF, all handling of stock and diluted solutions followed safety precautions. Specifically, manipulations were performed in a chemical hood, and gloves were worn. In addition, decontamination of glassware and leftover solutions was performed overnight in 1 N NaOH.

### 2.2. rePON1 variants

Library construction, selection, sequencing, expression and purification procedures that resulted in the directed evolution of rePON1 variant 4E9 were reported by Gupta et al. (2011). All stock solutions of the rePON1 variants were in 0.1% tergitol/1 mM $CaCl_2$/50 mM Tris, pH 8.0.

### 2.3. Human blood source

Outdated whole blood samples, obtained from a local blood bank, were collected above an anticoagulant citrate–phosphate–dextrose solution (United States Pharmacopeia) ('citrate buffer'), to prevent clotting. Since rePON1 requires the presence of free $Ca^{2+}$ to maintain its catalytic activity, and citrate reduces the $Ca^{2+}$ concentration, blood samples were spiked with 2 mM $CaCl_2$, and the pH was adjusted from 6.5 to 7.4 with 1 M Tris base. The added $CaCl_2$ did not produce coagulation for at least 2 weeks at 4 °C, and the tested rePON1s performed their catalytic activity in blood only 30–40% faster than in the absence of the added $CaCl_2$.

To measure the individual rate constants for inhibition of the erythrocyte AChE and the plasma BChE, whole blood samples were centrifuged for 5 min at 1000 × *g*, followed by removal of the upper layer of clear plasma containing the BChE. The erythrocyte pellet was washed 3 times with 2 vol. of phosphate-buffered saline. Both fractions were kept at 4° until used.

### 2.4. Assays of AChE and BChE

Baseline and residual activities were measured by the Ellman procedure (Ellman et al., 1961) using 1 mM ATC and BTC as substrates for AChE and BChE, respectively, and 0.6 mM DTNB (Ellman's reagent), in 50 mM sodium phosphate, pH 8.0, at 25°. The combined cholinesterase (ChE) activity of human whole blood was assayed with 1 mM ATC. Release of the Ellman chromophore was monitored at 412 nm for BChE, and at 436 nm for the erythrocyte AChE, so as to minimize interference due to absorption by hemoglobin (Worek et al., 1999). In all cases, the substrate was added to the Ellman assay mixture after 4 min of pre-incubation of the tested sample with DTNB, so as to allow reaction of non-specific thiols with the Ellman reagent to go to completion.

### 2.5. Determination of rate constants for inhibition of human blood ChEs by GF

#### 2.5.1. Plasma BChE

The separated plasma was diluted 250-fold into cold 50 mM sodium phosphate, pH 7.4, to produce a ~0.2 nM active site concentration of BChE. Following equilibration at 25° for 5 min, 1 ml of the solution was spiked with 2.0–4.0 nM GF, and residual enzyme activity was assayed at appropriate time intervals, using BTC as substrate, as described above.

#### 2.5.2. RBC AChE

Washed packed erythrocytes were diluted 100-fold in 50 mM sodium phosphate, pH 7.4, containing 0.1% tergitol and 0.05% BSA, to yield a clear solution with a ~0.1 nM active site concentration of AChE. The diluted AChE solution was spiked with 2.0–4.0 nM GF, and residual enzyme activity was assayed at appropriate time intervals, using ATC as substrate, as described above.

### 2.6. Determination of the catalytic proficiency of the rePON1s

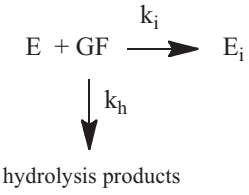
The catalytic proficiency, $k_{cat}/K_m$, of rePON1s reacting with GF was performed as described by Gupta et al. (2011). Briefly, a 0.1–0.01 μM solution of the rePON1 variant in Tris activity buffer (0.1% Tergitol/1 mM $CaCl_2$/50 mM Tris, pH 8.0) was spiked with GF freshly generated *in situ* to produce a final concentration of 0.05–0.2 μM GF. At appropriate time intervals, aliquots were mixed with ~4.5 nM purified *Torpedo californica* AChE (*Tc*AChE; Sussman et al., 1988) in 0.05% BSA/50 mM phosphate, pH 8.0, so as to produce a 5–10% excess of *Tc*AChE over stoichiometry. The process of inhibition was complete within 10–15 min at RT. It should be noted that phosphate quenches the activity of rePON1. For each data point the % residual GF in the Tris reactivity buffer was determined from the level of inhibition of *Tc*AChE. Hundred percent GF was taken as the level of inhibition of *Tc*AChE in the absence of the rePON1.

### 2.7. Monitoring of the protection of ChEs in whole blood by rePON1 variants

A 0.5 ml aliquot of whole blood was spiked with a rePON1 variant to produce a final enzyme concentration of 0.2–4 μM. After incubation at 25° for 5 min, GF, freshly generated *in situ*, was added to a final concentration of 0.08–0.4 μM. The test tube was vortexed gently, and after 10 min the sample was diluted 50-fold into distilled water to hemolyze the erythrocytes. Total ChE activity was assayed using ATC as described above. A control blood sample, containing the same amount of rePON1, was assayed in the absence of GF, and was taken as 100% baseline activity.

## 3. Theory and calculations

To test the relationship between the proficiency of the rePON1 ($k_{cat}/K_m$), its molar concentration in blood, the rate of inhibition of ChEs by GF ($k_i$), and the level of ChE protection, we calculated the expected % residual activity of blood ChEs, and compared it to the observed values. Calculations were based on the following kinetic scheme:




where $k_i$ is the second-order rate constant for the inhibition of blood ChEs (denoted as E), and $k_h$ is the rate of detoxification of GF by rePON1. Assuming that $[GF]_0$ (80–400 nM) is well below the $K_m$ value, $k_h$ is approximated by $(k_{cat}/K_m)[rePON1]_0$. As time increases, a limiting value is approached, with the following mathematical solution (Ashani et al., 1972):

$$\ln\left(\frac{E_\infty}{E_0}\right) = -[GF]_0\left(\frac{k_i}{k_h}\right) \quad (1)$$

where $E_\infty$ and $E_0$ are residual ChE activity at $t = \infty$ and at $t = 0$, respectively, and $[GF]_0$ is the initial concentration of GF. Substituting $k_h$ by $(k_{cat}/K_m)[rePON1]_0$ in Eq. (1) gives:

$$\ln\left(\frac{E_\infty}{E_0}\right) = -[GF]_0\left(\frac{k_i}{\{(k_{cat}/K_m)[rePON1]_0\}}\right) \quad (2)$$

Thus, Eq. (2) describes the % ChE residual activity at $t = \infty$ $(E_\infty/E_0)$ as a function of the concentration and catalytic efficiency of rePON1.

## 4. Results

### 4.1. Precision

Whole blood samples diluted into the Ellman reaction mixture, and assayed with 1 mM ATC, showed a coefficient of variation (CV, $n = 3$), of 2.6%, and 18% for the highest (0.100 OD/min) and lowest (0.005 OD/min) activity slopes, respectively. Regardless of the activity levels, the accuracy of the individual activity slopes in the

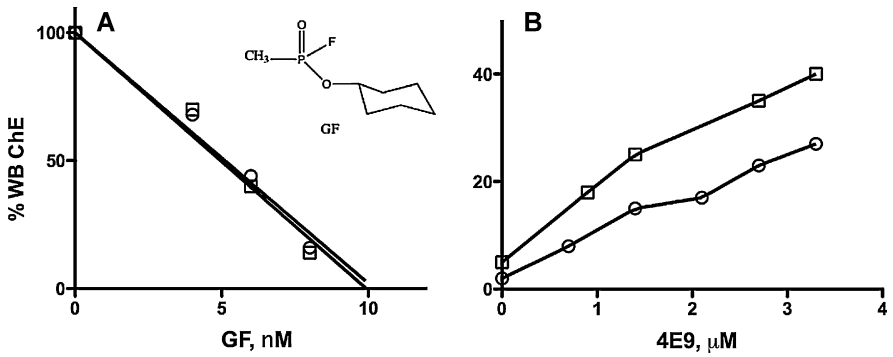



**Fig. 1.** Reactions of GF with human whole blood collected with citrate buffer. Panel A: titration of 1:10 dilution of whole blood in 50 mM sodium phosphate, pH 8.0, with GF. The concentration of GF was determined by titration of a known amount of *Tc*AChE. Shown are titration curves determined after 10 min (□ – □) and 30 min (○ – ○) of incubation of GF with the enzyme. $r^2 > 0.97$. Panel B: effect of spiking whole blood with 2 mM $CaCl_2$ (□ – □) on the rate of hydrolysis of 100 nM GF by rePON1 variant 4E9. Activity is presented as the percentage of residual blood ChE activity. ○ – ○, whole blood without addition of $CaCl_2$. Data points are average from duplicates with CV < 16%. WB ChE activities determined 10 min after spiking with GF.

Ellman assay (i.e., OD/min) were at a SD lower than 7% of the mean and with $r^2 > 0.9930$. $r^2$ was >0.97 for the titration curve of blood ChEs by GF (Fig. 1A), with 95% confidence intervals of the *X*-intercept of the titration curve of 8–14.

### 4.2. Titration of whole blood ChEs

The active-site concentration of ChEs in human whole blood, collected above citrate buffer, was obtained by titration of the total ChE activity content of a sample diluted 1:10 dilution into 50 mM sodium phosphate, pH 8.0, using *in situ* generated GF as the titrant, and ATC as the substrate (Fig. 1A). A period of 10 min was sufficient to achieve complete inhibition at each GF concentration used, and from the linear titration curve it was concluded that 100% inhibition of blood ChE activity was achieved with ~100 nM racemic GF. The fresh human whole blood used had been diluted 12% by the added citrate buffer. Assuming that only the highly toxic GF isomer ($S_P$), which accounts for half of the racemic mixture, mediates inhibition over the first 10 min, the calculated active-site concentration, when normalized to whole blood prior to dilution with the citrate buffer, is ~55 nM. This value is consistent with previously reported active-site concentrations of AChE and BChE in human whole blood (Ashani and Pistinner, 2004).

### 4.3. Effect of externally added $CaCl_2$

As already mentioned, the human whole blood obtained from the local blood bank was collected over citrate buffer (see Section 2) by mixing approximately 450 ml blood with 63 ml of the anticoagulant citrate buffer. Since rePON1 activity is $Ca^{2+}$-dependent (Pla et al., 2007), and citrate chelates calcium ions, whole blood was spiked with 2 mM $CaCl_2$ to ensure an adequate concentration of free calcium. Fig. 1B shows the activity of a rePON1 variant, 4E9 (Gupta et al., 2011), in blood, in the presence and absence of added $CaCl_2$. As seen, the addition of 2 mM $CaCl_2$ increased the proficiency of the rePON1 against GF by ~35%. Thus, all PON assays were carried out in blood supplemented with 2 mM $CaCl_2$, and adjusted to pH 7.4.

### 4.4. Selection of blood matrix for ex vivo protection experiments

To simplify the ChE protection protocol, and thus allow rapid screening, we determined the bimolecular rate constants ($k_i$) for the inhibition of human RBC AChE and human plasma BChE by GF in 50 mM sodium phosphate, pH 7.4, at 25°. The first-order rate constants ($k_{obs}$) were obtained by fitting the residual ChE activity data points, monitored over 75% of the inhibition reaction, to a mono-exponential decay function. $k_i$ was subsequently calculated by dividing $k_{obs}$ by $[GF]_0$ (not shown). The $k_i$ values for RBC AChE and plasma BChE were found to be $(2.65 \pm 0.11) \times 10^8\ M^{-1}\ min^{-1}$ ($n = 4$), and $(2.02 \pm 0.26) \times 10^8\ M^{-1}\ min^{-1}$ ($n = 3$), respectively.

We estimated that AChE and BChE contribute 85 and 15%, respectively, to the total ChE activity assayed with 1 mM ATC. This estimate was based on assays using 1 mM ATC and 1 mM BTC, and employing a BTC/ATC activity ratio for human plasma BChE of 1.95, while that for RBC AChE is <0.02 (not shown). The weighted average $k_i$ value for whole blood ChE inhibition by racemic GF was then taken as $2.5 \times 10^8\ M^{-1}\ min^{-1}$. Thus, whole blood ChE assayed with 1 mM ATC was selected as the enzyme matrix to evaluate the protection capabilities of rePON1 against GF inhibition.

### 4.5. Correlation between $k_{cat}/K_m$ of rePON1s and protection of blood ChEs

Variants 4E9 ($k_{cat}/K_m = 1.7 \times 10^7\ M^{-1}\ min^{-1}$) (Gupta et al., 2011) and VIID2 ($k_{cat}/K_m = 7.0 \times 10^7\ M^{-1}\ min^{-1}$) (Goldsmith et al., in preparation) were both shown to significantly enhance the hydrolysis of the toxic isomer of GF relative to the wt rePON1. Thus, the two were selected as representative rePON1s for validating quantitative aspects of the proposed protocol. When 4E9 was added at a final concentration of 3.6 μM to whole blood, residual ChE activity was found to be 48%, 30%, and 13%, following spiking with 80, 150, and 200 nM GF, respectively, compared to a value of 5% for unprotected blood (Fig. 2A). As expected, the more potent VIID2 (Fig. 2B) conferred greater protection at lower concentrations. In the presence of 1 μM VIID2, residual ChE activity was found to be 31% at 200 nM GF. Residual ChE activity (12%) was also observed after spiking blood with 400 nM GF.

Calculations of the expected efficacy *vs*. the observed efficacy of rePON1 variants in protecting the human blood ChEs was carried out using Eq. (2), and the values obtained are summarized in Table 1. Due to uncertainty with respect to the distribution of both GF and rePON1 in whole blood fractions, calculations were normalized per whole blood volume. For the entire range of rePON1 variants used, the observed values for % survival of blood ChE activity at $t = \infty$, at all GF concentrations employed, are in reasonable agreement with the calculated values. It should be noted that the predicted values were slightly but consistently greater than the observed protection of blood ChEs.

## 5. Discussion

Prophylaxis against CWNA and OP-based pesticides by catalytic scavengers, such as human PON1 and rePON1s, has been demonstrated in mice (Duysen et al., 2011; Gaidukov et al., 2009; Gupta

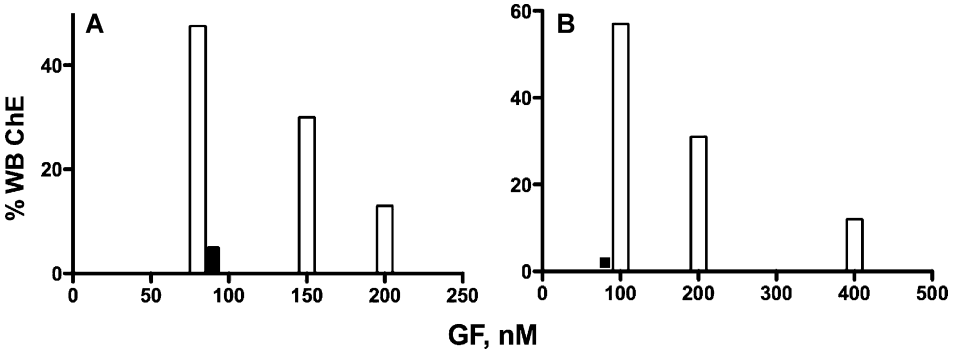



**Fig. 2.** The proficiency of 3.6 μM 4E9 (Panel A) and 1 μM VIID2 (Panel B) in human whole blood spiked with increasing concentrations of GF. Protection of AChE + BChE is expressed in terms of residual activity of whole blood ChEs, assayed with ATC at $t = \infty$. Black bars denote % ChE activity without rePON1 pre-treatment. Data points are average from duplicates with CV < 16%.

et al., 2011; Li et al., 1995) and guinea pigs (Valiyaveettil et al., 2011). Since human PON1 has been shown to associate with high density lipoprotein (HDL) in human blood (Moren et al., 2008; Sorenson et al., 1999), it seemed important to ascertain that evolved rePON1variants, selected on the basis of their capacity to catalyze the hydrolysis of CWNA in aqueous buffer solutions, maintain their capacity to perform the expected detoxification in blood. The *ex vivo* protocol developed here offers a simple, rapid, and sensitive procedure to evaluate the proficiency of rePON1 in human whole blood. Repetitive determinations of total ChE activity, in the presence and absence of rePON1, gave high reproducibility, with CV values ranging between 2.6% and 18% for the highest and lowest recorded activities, respectively, as determined by the Ellman assay, using ATC as substrate.

The equation utilized for calculating the degree of protection by rePON1variants of whole blood ChEs against inhibition by GF requires knowledge of the inhibition rate constants of blood AChE and BChE by GF ($k_i$), and the $k_{cat}/K_m$ value of the rePON1 variant for hydrolysis of GF (Eq. (2)). Human RBC AChE and plasma BChE were inhibited by GF at $(2.65 \pm 0.11) \times 10^8\ M^{-1}\ min^{-1}$ and $(2.02 \pm 0.26) \times 10^8\ M^{-1}\ min^{-1}$, respectively. Worek et al. (1998), who determined $k_i$ using the initial velocity method and authentic GF reacting with human RBC AChE ($7.4 \times 10^8\ M^{-1}\ min^{-1}$) and human plasma BChE ($3.8 \times 10^8\ M^{-1}\ min^{-1}$), performed the assay at 37° (pH 7.4). The $k_i$ values reported here, that were determined at 25° (pH 8.0) with *in situ* generated GF, seem to be in reasonable agreement with those reported for the authentic GF at 37 °C. Taking into account the similar $k_i$ values of the two ChEs, and the relatively small contribution of plasma BChE to whole blood activity when reacting with 1 mM ATC, the 2 ChEs were assumed to behave kinetically as a single enzyme when inhibited with GF, with $k_i$ normalized at $2.5 \times 10^8\ M^{-1}\ min^{-1}$.

Predictions of the % survival of ChE activity using this value of $k_i$, together with the appropriate $k_{cat}/K_m$ value of the various rePON1variants, correlated reasonably well with the experimentally observed values (Table 1). However, the calculated values were slightly but consistently greater than the experimental values for residual ChE activity at $t = \infty$. A possible explanation for this discrepancy may reside in the $k_{cat}/K_m$ values. The pH-rate profiles for rePON1 variants showed that $k_{cat}/K_m$ values decrease 20–30% when the enzyme is transferred from pH 8.0 to pH 7.4 (Khersonsky and Tawfik, 2006). Re-calculation of the expected % survival of ChE activity using $k_{cat}/K_m$ values 70% of those determined provided predicted values that matched the experimental data (Table 1), and thus gives a measure of the sensitivity of the calculated theoretical value to the uncertainty with respect to the $k_{cat}/K_m$ values. Less likely explanations, that cannot, however, be ruled out, are that the effective initial concentrations of rePON1 and GF in whole blood may differ from the values obtained *in vitro*, and/or that the concentrations differ due to the distribution properties of both the enzyme and the OP in human whole blood.

The advantages of catalytic scavengers for pre-treatment against CWNA intoxication are two-fold: (1) protection is likely to be achieved by protein doses several fold lower than the hundreds of milligram doses envisaged for the best stoichiometric antidote currently available, *viz.*, human plasma BChE (Ashani and Pistinner, 2004); (2) a clearly defined biochemical kinetic parameter, $k_{cat}/K_m$, governs the rate of detoxification of the OP challenge. Since any toxicokinetic model developed to predict *in vivo* protection by pre-treatment with rePON1 will require knowledge of its $k_{cat}/K_m$ value in whole blood, rather than in buffer solution, use of Eq. (2) and the proposed *ex vivo* protocol implemented in human blood can provide this kinetic constant reliably under a variety of experimental conditions. Thus, different blood concentrations of both the CWNA and of the rePON1 tested, as well as different incubation times of the blood with the rePON1, prior to spiking with the CWNA, can be employed to generate a wide range of exposure–protection scenarios. The *ex vivo* protocol developed could also be applied to testing the effect of prolonged incubation of the rePON1s variants with human whole blood prior to spiking with CWNA, and thus to examining the effect of blood constituents on PON1's stability and activity.

**Table 1**
Calculated and observed protection of human blood ChEs.

| rePON1(μM)[a] | GF (nM)[a] | % ChE activity | |
|---|---|---|---|
| | | Calculated[b] | Observed[c] |
| 4E9 (3.6) | 80 | 72 (62)[d] | 48 |
| 4E9 (3.6) | 150 | 54 (42) | 30 |
| VIID2 (0.45) | 80 | 53 (40) | 42 |
| VIID2 (0.45) | 120 | 39 (27) | 27 |
| VIID2 (0.45) | 160 | 28 (16) | 16 |
| VIID2 (1.0) | 100 | 70 (60) | 57 |
| VIID2 (1.0) | 200 | 49 (36) | 31 |
| VIID2 (1.0) | 400 | 24 (13) | 12 |

[a] Concentrations were calculated per whole blood volume.
[b] Calculated according to Eq. (2), using $k_i = 2.5 \times 10^8\ M^{-1}\ min^{-1}$ and $k_{cat}/K_m$ values of $1.7 \times 10^7$ and $7.0 \times 10^7\ M^{-1}\ min^{-1}$ for 4E9 and VIID2, respectively, as determined at pH 8.0.
[c] Figures are average of duplicates with CV < 16%.
[d] Figures in parentheses were recalculated assuming a decrease of 30% in $k_{cat}/K_m$ at pH 7.4, relative to $k_{cat}/K_m$ at pH 8.0.

## 6. Conclusions

An *ex vivo* protocol permitted us to address the relationship between blood rePON1s concentrations, together with their kinetic parameters, and the level of protection conferred on the endogenous ChEs in human blood, following an *in vitro* challenge with

GF. This protocol can be utilized to determine rePON1 proficiencies in human whole blood, and thus be used to estimate: (1) the catalytic efficiency required for a rePON1 variant to confer protection on humans against a given OP agent; (2) the *in vitro* stability and activity of a rePON1 variant when interacting with blood constituents.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

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