

# Directed evolution of hydrolases for prevention of G-type nerve agent intoxication

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**Organophosphate nerve agents are extremely lethal compounds. Rapid *in vivo* organophosphate clearance requires bioscavenging enzymes with catalytic efficiencies of  $>10^7$  ( $M^{-1} \text{ min}^{-1}$ ). Although serum paraoxonase (PON1) is a leading candidate for such a treatment, it hydrolyzes the toxic  $S_p$  isomers of G-agents with very slow rates. We improved PON1's catalytic efficiency by combining random and targeted mutagenesis with high-throughput screening using fluorogenic analogs in emulsion compartments. We thereby enhanced PON1's activity toward the coumarin analog of  $S_p$ -cyclosarin by  $\sim 10^5$ -fold. We also developed a direct screen for protection of acetylcholinesterase from inactivation by nerve agents and used it to isolate variants that degrade the toxic isomer of the coumarin analog and cyclosarin itself with  $k_{\text{cat}}/K_M \sim 10^7 M^{-1} \text{ min}^{-1}$ . We then demonstrated the *in vivo* prophylactic activity of an evolved variant. These evolved variants and the newly developed screens provide the basis for engineering PON1 for prophylaxis against other G-type agents.**

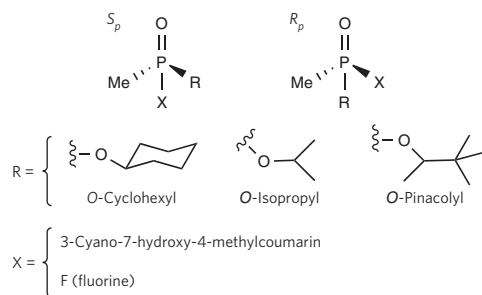
Inactivators of acetylcholinesterase (AChE), including organophosphate-based pesticides and nerve agents, threaten both military and civilian populations. A timely pharmacological treatment with atropine and oxime AChE reactivators can save lives but in many cases does not prevent cholinergic crisis and the resulting onset of secondary toxic manifestations induced by organophosphate intoxication. Side effects associated with drugs used as protective treatment before organophosphate exposure such as pyridostigmine have also prompted the search for effective prophylactics and antidotes<sup>1,2</sup>. Rather than minimizing the damages caused by the organophosphate, the goal of prophylactic drugs is intercepting the organophosphates before they even reach their target organs. A stoichiometric bioscavenger based on human butyrylcholinesterase has been recently developed. However, owing to the daunting mass ratio of organophosphate to protein, hundreds of milligrams of protein are required to confer protection against exposure above a single LD<sub>50</sub> (lethal dose, 50%) dose<sup>3</sup>. Catalytic scavengers, namely enzymes showing multiple turnovers, may allow rapid and efficient protection against high organophosphate doses using low protein amounts<sup>4</sup>. However, with few exceptions<sup>5</sup>, xenobiotics such as organophosphates are promiscuous substrates for natural enzymes and are degraded with low catalytic efficiencies. Improved organophosphate-hydrolyzing enzyme variants have been engineered (for example, refs. 6–8), and the ability of such enzymes to provide prophylactic protection from organophosphate intoxication has been demonstrated (refs. 9–11). However, for prophylactic protection from  $\geq 1 \times \text{LD}_{50}$  doses of G-type agents at reasonable protein amounts, we anticipated that far higher catalytic efficiencies would be required.

The G-agents cyclosarin (known as GF; also referred to as CMP-F) and soman (known as GD; also referred to as Pin-F) are prime targets for scavenger-based prophylaxis because of the low efficacy of pharmacological drugs used to counteract their toxicity<sup>12</sup>. They are applied as racemates, but it is their  $S_p$  isomers

that are the tangible threat (Scheme 1). Unfortunately, enzymes tested thus far primarily hydrolyze the less toxic  $R_p$  isomer<sup>13,14</sup>. Here, we report the engineering of enzyme variants with highly improved catalytic efficiencies toward the toxic  $S_p$  isomer of cyclosarin. We chose the mammalian serum paraoxonase PON1 as our starting point. PON1 is a lactonase that has only promiscuous organophosphate hydrolase activities. It hydrolyzes G-agents at low catalytic efficiencies and reacts primarily with their  $R_p$  isomer<sup>2,15</sup>. We used a recombinant variant dubbed rePON1-G3C9 (referred to as rePON1 hereafter), which, unlike human PON1, is expressed functional and soluble in *Escherichia coli*. Its amino acid sequence is 94% and 85% identical to rabbit and human PON1s, respectively<sup>16</sup>. The enzymatic specificity of rePON1 is essentially identical to human PON1, yet its stability is much improved, and its *in vivo* applicability and efficacy have been confirmed<sup>17</sup>.

We applied both random and targeted mutagenesis to rePON1 and screened mutants for organophosphate hydrolysis using both high-throughput fluorescence-activated cell sorting (FACS) screening and low-throughput plate screening. We also developed a screen for the protection of acetylcholinesterase from inactivation by nerve agents, which enabled us to isolate variants that specifically degrade the toxic isomers of G-agents. For safety and facile detection, we initially used fluorogenic coumarin analogs, including CMP-coumarin that inhibits AChE with a  $k_i$  that is  $\sim 30$ -fold lower than cyclosarin<sup>15,18</sup>. We thereby enhanced PON1's activity toward the toxic  $S_p$  isomer of a coumarin analog of cyclosarin by  $\sim 10^5$ -fold. To increase the specificity of variants toward cyclosarin itself, we developed a nonhazardous procedure for the *in situ* generation of cyclosarin and used it during advanced screening rounds. We were thus able to isolate variants that hydrolyze the  $S_p$ -coumarin analog of cyclosarin and cyclosarin itself with  $k_{\text{cat}}/K_M \sim 10^7 M^{-1} \text{ min}^{-1}$ . We then demonstrated the *in vivo* prophylactic activity of an evolved variant by showing, in mice, that it can provide considerable protection from a lethal dose of  $S_p$ -CMP-coumarin. Our evolved variants

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**Scheme 1 | The organophosphates used in this study.** Shown are the two enantiomers of G-agents: cyclosarin, R = O-cyclohexyl, X = F (**1**); sarin, R = O-isopropyl, X = F (**2**); and soman, R = O-pinacolyl, X = F (**3**). For consistency, these agents are dubbed CMP-F, IMP-F and Pin-F, respectively, and their fluorogenic analogs (X = 3-cyano-7-hydroxy-4-methylcoumarin) are dubbed CMP-coumarin (**4**), IMP-coumarin (**5**) and Pin-coumarin (**6**), respectively.

and the newly developed screens provide the basis for engineering PON1 prophylactics against other G-type nerve agents.

## RESULTS

Our model indicated that prophylactic protection from  $\geq 1 \times \text{LD}_{50}$  doses of G-type agents at reasonable protein amounts requires catalytic scavengers whose efficiencies in  $k_{\text{cat}}/K_M$  terms are  $\geq 10^7 \text{ M}^{-1} \text{ min}^{-1}$  (see **Supplementary Methods**). Our engineering efforts aimed at achieving these high rates, and the protection afforded by the engineered variants validated the assumed model.

### Directed evolution of PON1 for $S_p$ -CMP hydrolysis

We have previously isolated several variants of rePON1 with an enhanced activity toward a racemic mixture of CMP-coumarin by screening ‘neutral drift’ libraries of rePON1 (for example, 1G3, 2G9)<sup>19</sup>. The most active variant was found to be 3B3 with  $\sim 250$ -fold-higher catalytic efficiency ( $k_{\text{cat}}/K_M 2 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ ) compared to the wild-type-like rePON1 ( $k_{\text{cat}}/K_M 8 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$ ; **Table 1**;

**Supplementary Results**). However, the hydrolysis by rePON1-3B3 was also restricted to the  $R_p$  isomer (**Fig. 1a,b**). Nonetheless, we used its high catalytic efficiency and  $R_p$ -stereoselectivity to isolate the  $S_p$  isomers of CMP-coumarin and IMP-coumarin from the corresponding racemates, as previously described<sup>18</sup>, and applied these  $S_p$  isomers in subsequent screens. We also identified two rePON1 mutants, H115W and V346A, with low activity toward  $S_p$ -CMP-coumarin<sup>15</sup>. However, their activity with  $S_p$ -CMP-coumarin was too low for detection under library screening conditions. We therefore used for the first screening rounds IMP-coumarin, a less bulky G-agent analog whose  $S_p$  isomer is more reactive with PON1. As the starting point for random mutagenesis, we incorporated the two identified mutations into rePON1 to give rePON1-H115W-V346A (**Supplementary Table 1**).

Screening of the resulting library in 96-well plates with  $S_p$ -IMP-coumarin yielded several improved variants that typically carried one mutation in addition to H115W and V346A (**Supplementary Table 2**). A second round of mutagenesis and screening with  $S_p$ -IMP-coumarin led to the isolation of variants in which V346A was removed and the H115W and F222S mutations dominated (**Supplementary Table 2**). As the evolving variants became more reactive with the  $S_p$  isomer, the third-generation library could be screened with both  $S_p$ -IMP- and  $S_p$ -CMP-coumarin. Indeed, this round resulted in several variants with improved activities toward  $S_p$ -CMP-coumarin (for example, 3A7, 8C8; **Table 1**; **Supplementary Table 3**). However, the fourth round of mutagenesis and screening yielded no further improvements. We therefore designed a structure-based targeted library and subjected it to high-throughput screening ( $>10^6$  variants per run) by FACS sorting<sup>20,21</sup>.

### Highly proficient variants selected by emulsion sorting

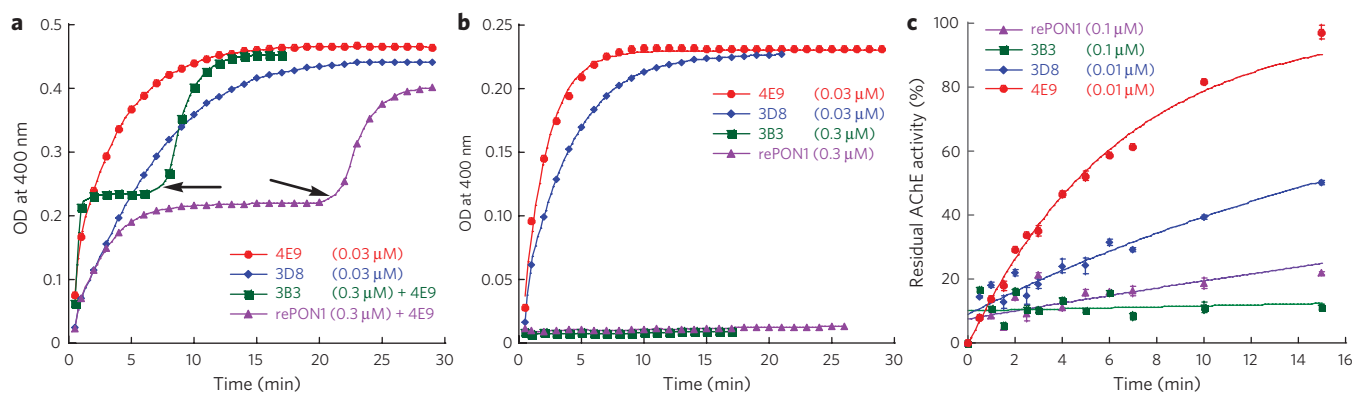
The targeted substitutions library was based on PON1’s active-site structure (PDB: 1V04): residues 69 and 134, which are in direct contact with position 115, were randomized. We also randomized the more remote positions 346–348, because their side chains were found to be rearranged in a crystal structure of variant re-PON1-H115W (data not shown). However, in accordance with the fact that none of the selected variants carried a mutation at positions 346–348,

**Table 1 | Representative PON1 variants along the directed evolution process**

Variant <sup>a</sup>	Mutations <sup>b</sup>	$S_p$ -CMP-coumarin <sup>c</sup>			$R_p$ -CMP-coumarin	$S_p$ -CMP-F
		$k_{\text{cat}}$ (min <sup>-1</sup> )	$K_M$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_M$ ( $\mu\text{M}^{-1} \text{ min}^{-1}$ )	Apparent $k_{\text{cat}}/K_M^c$ ( $\mu\text{M}^{-1} \text{ min}^{-1}$ )	Apparent $k_{\text{cat}}/K_M^c$ ( $\mu\text{M}^{-1} \text{ min}^{-1}$ )
rePON1-G3C9	(Wild-type-like)	ND	ND	<0.0002 (1) <sup>d</sup>	0.08 ± 0.003 (1)	0.13 ± 0.03 (1)
3B3	N41D, S110P, <b>L240S</b> , H243R, F264L, N324D, <b>T332A</b>	ND	ND	<0.0002 (1) <sup>d</sup>	20 ± 2 (250)	-0.0001
H115W-V346A	<b>H115W</b> , <b>V346A</b>	ND	ND	0.0013 ± 0.0005 (>6)	0.4 ± 0.01 (5)	0.018 ± 0.002 (0.14)
3A7	V97A, <b>H115W</b> , P135A, <b>F222S</b> , M289I	ND	ND	0.003 ± 0.0005 (>15)	0.16 ± 0.008 (2)	0.011 ± 0.004 (0.08)
8C8	<b>L69S</b> , V97A, <b>H115W</b> , P135A, <b>F222S</b>	15.1 ± 2	84.5 ± 16	0.18 ± 0.02 (>900)	– <sup>e</sup>	0.15 ± 0.05 (1.1)
2D8	<b>L69G</b> , <b>H115W</b> , <b>H134R</b> , <b>F222S</b> , <b>T332S</b>	395 ± 23	42.5 ± 5	9.3 ± 0.6 (>46000)	– <sup>e</sup>	4.65 ± 0.1 (36)
3D8	<b>L69G</b> , <b>H115W</b> , <b>H134R</b> , M196V <b>F222S</b> , <b>T332S</b>	546 ± 29	36.7 ± 7	12.7 ± 2 (>63,000)	– <sup>e</sup>	3.14 ± 0.5 (24)
4E9	<b>L69G</b> , S111T, <b>H115W</b> , <b>H134R</b> , <b>F222S</b> , <b>T332S</b>	540 ± 37	32.8 ± 10	17.3 ± 4 (>86,000)	– <sup>e</sup>	17.5 ± 3 (135)

<sup>a</sup>Annotation of variants: The first digit relates to the plate number and the following letter-digit to its location within this plate. For example, variant 3A7 = plate #3, well A7. <sup>b</sup>Denoted in bold are mutations in active-site residues. <sup>c</sup>Enzymatic parameters were measured with purified proteins and comprise the average obtained from three independent repeats. Data presented are mean ± s.d. Values in parentheses describe the fold change of improved variants compare to the starting point, rePON1-G3C9. The kinetic parameters for  $S_p$ -CMP-coumarin were spectrophotometrically measured with optically pure samples<sup>18</sup>. Parameters for  $R_p$ -CMP-coumarin were determined with the racemate as described in **Supplementary Methods** and for CMP-F with the *in situ*-prepared substrate and an AChE inhibition assay (see **Methods** and **Supplementary Methods** for details). ND, not detectable. <sup>d</sup>The catalytic efficiency was estimated as described in ref. 18. <sup>e</sup>Variants showed single-phase kinetics of product release when reacted with racemic CMP-coumarin, suggesting that the rates of hydrolysis for  $R_p$ - and  $S_p$ -CMP-coumarin are similar.





**Figure 1 | Hydrolysis of CMP-coumarin and CMP-F by rePONI variants.** Enzyme concentrations were varied depending on the variant's activity and are noted in the figure. **(a)** Hydrolysis of racemic CMP-coumarin (12 μM) in the presence of variants 4E9, 3D8, 3B3 (plus addition of 0.03 μM 4E9 after 6 min, indicated by the black arrow) and wild-type-like rePONI (plus addition of 0.03 μM 4E9 after 20 min). **(b)** Hydrolysis of *S<sub>p</sub>*-CMP-coumarin (6 μM) in the presence of variants 4E9, 3D8, 3B3 and rePONI. **(c)** Residual AChE activity was assayed after the incubation of *in situ*-generated CMP-F (40 nM) and 4E9, 3D8, 3B3 and rePONI at the concentrations noted in the figure (data were fitted to a first-order rate equation to derive the apparent rate constant for hydrolysis of CMP-F; error bars represent the s.d. of two or more independent measurements).

we later discovered that these movements were because of differences in crystallization pHs and not because of the tryptophan substitution at 115. We also randomized residues 115 and 222 that were found to be mutated in all active variants of the third round (Supplementary Table 3). We applied an oligo-spiking strategy that incorporated the randomizing oligos onto re-PON1-H115W in a combinatorial manner<sup>22</sup> so that each library variant carried on average four mutated positions. Because of the intense level of mutagenesis and the targeting of the active site, the libraries comprised mostly inactive variants. To purge the inactive clones, we used a high-throughput FACS screen using a fluorogenic phosphotriester dubbed DEPCyC. The hydrolytic activity of variants with DEPCyC was found to correlate well with their activity with *S<sub>p</sub>*-CMP-coumarin<sup>15</sup>. *E. coli* cells transformed with the plasmid library were compartmentalized in water-in-oil emulsion droplets. The fluorogenic substrate was added, and the primary emulsion droplets were converted to double-emulsion droplets that were sorted by FACS. Cells in isolated droplets were plated, picked and assayed in 96-well plates for *S<sub>p</sub>*-CMP-coumarin activity.

The most active variants isolated from this fifth round (Supplementary Table 4) carried mutations L69G/A and H134R, in addition to H115W and F222S from the previous rounds. These variants were shuffled, together with random mutagenesis at low rates (~1.7 amino acid exchanges per gene), and the resulting sixth-round library was sorted by FACS and then screened in 96-well plates. The most improved variants carried five key mutations: L69G, H115W, H134R, F222S and T332S (Supplementary Table 5). The best variant, 3D8, had a  $k_{\text{cat}}/K_M$  value of  $1.3 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$  with *S<sub>p</sub>*-CMP-coumarin (Table 1 and Fig. 1a). Further, 3D8 and other variants from the sixth round (for example, 2D8) showed similar rates with both the *S<sub>p</sub>* and *R<sub>p</sub>* isomers, as indicated by the complete hydrolysis of the racemic CMP-coumarin with monophasic kinetics (Fig. 1a).

### Acetylcholinesterase protection assay

During the selection for variants with higher rates, we decreased the concentration of the CMP-coumarin substrate to enable the isolation of variants with improved  $K_M$  as well as  $k_{\text{cat}}$ . However, to be able to screen at concentrations that correspond to the very low toxic concentrations of G-type nerve agents *in vivo* (~1 μM)<sup>23</sup> and to screen for variants that efficiently degrade cyclosarin itself, we developed a screen that was based on monitoring the rescue of AChE, the organophosphate's physiological target. AChE was added to crude bacterial lysates expressing the library PON1 variants. The organophosphate was added, and the residual activity of AChE was

subsequently measured using a chromogenic assay to indicate the level of organophosphate degradation by the tested variant. The FACS-sorted sixth-round library was rescreened in 96-well plates using the AChE assay and 1 μM of racemic CMP-coumarin. We screened 730 randomly picked colonies and isolated 13 variants improved by 2–12-fold relative to 3D8 (Supplementary Table 6).

Although at this stage we identified variants that had sufficiently high catalytic proficiency, these were selected and tested with coumarin surrogates. However, the fluoride leaving group of the actual threat agents substantially differs from coumarin (Scheme 1)—fluoride is a more reactive and much smaller leaving group. Because the toxicity of nerve agents prevents their use in ordinary labs, we developed a nonhazardous screening protocol based on cyclosarin (CMP-F) generated *in situ*, in dilute aqueous solutions, by replacing the coumarin leaving group of CMP-coumarin with fluoride. This exchange was spectroscopically monitored by following the release of the coumarin. The inhibition of *Torpedo californica* AChE (TcAChE) by *in situ*-generated CMP-F proceeded 40-fold faster than with *S<sub>p</sub>*-CMP-coumarin, with the expected  $k_i$  of  $1.3 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$  (Supplementary Methods). Using the *in situ*-generated CMP-F, we measured the  $k_{\text{cat}}/K_M$  values of the evolved variants under pseudo-first-order conditions (CMP-F ≤ 50 nM, well under a likely  $K_M$ ). Encouragingly, the activities with the coumarin and fluoride *S<sub>p</sub>* isomers were comparable, and at least two variants (0C9, 1A4) had  $k_{\text{cat}}/K_M$  values ≥  $10^7 \text{ M}^{-1} \text{ min}^{-1}$  with CMP-F (Supplementary Table 7). The AChE protection assays therefore confirmed the ability of the evolved variants to protect AChE from cyclosarin *in vitro* and validated the coumarin analogs as faithful surrogates of the actual G-agents. The assay also indicated that although the starting point, rePONI-G3C9, is much more active with CMP-F than CMP-coumarin (Table 1), as is human PON1 (ref. 24), its  $k_{\text{cat}}/K_M$  (~ $10^5 \text{ M}^{-1} \text{ min}^{-1}$ ) is >100-fold too low for *in vivo* detoxification using reasonable amounts of enzyme.

The evolved variants were sufficiently active to enable library screens using the *in situ*-generated agent. This approach is highly attractive, as the assay of AChE protection against the actual threat agent mimics the *in vivo* protection challenge whereby the catalytic scavenger must be sufficiently active to intercept the threat agent before the latter reacts with AChE. We therefore rescreened the 13 most improved variants from the last round using CMP-F at the expected plasma concentration for  $1 \times \text{LD}_{50}$  exposure (1 μM). Nine variants had improved activities relative to 3D8 (Supplementary Table 6). Of these, three variants (4E9, 5F3 and 6A3) had the highest specific activity upon examination

**Table 2 | Prophylactic protection in mice**

Time before organophosphate challenge (hours) <sup>a</sup>	Treatment group <sup>b</sup>	Enzyme dose <sup>c</sup> (mg kg <sup>-1</sup> )	Percent survival recorded at:			
			12 h	24 h	96 h	14 d
-	Untreated (12)	-	0%	0%	0%	0%
1	Buffer (3) <sup>c</sup>	-	0%	0%	0%	0%
5 min	Atropine plus 2-PAM (9) <sup>d</sup>	-	66%	22%	22%	0%
1	rePON1 (18)	2.2	0%	0%	0%	0%
1	4E9 (11)	1.1	45%	45%	45%	45%
1	4E9 (16)	2.2	75%	75%	63%	63%
6.3	4E9 (14)	2.2	50%	50%	50%	50%
24	4E9 (5)	2.2	0%	0%	0%	0%

<sup>a</sup>S<sub>p</sub>-CMP-coumarin was injected intravenously at 290 μg kg<sup>-1</sup> (1× LD<sub>100</sub>) to male mice weighing on average 24.5 ± 2.2 g. <sup>b</sup>Treatment given before organophosphate challenge. All figures in parentheses relate to the number of mice in each group. <sup>c</sup>Buffer content: Tris 50 mM pH 8, CaCl<sub>2</sub> 1 mM, NaCl 100 mM, Tergitol 0.02% (w/v). <sup>d</sup>Atropine plus 2-PAM: Atropine sulfate (20 mg kg<sup>-1</sup>) plus 2-PAM (25 mg kg<sup>-1</sup>) in PBS. <sup>e</sup>Purified rePON1-G3C9 or variant 4E9 (see Methods) was injected intravenously at the indicated dose before organophosphate challenge.

of the amount of soluble expressed protein. After sequencing and protein purification, we identified 4E9 as the most active variant with both S<sub>p</sub>-CMP-coumarin and S<sub>p</sub>-CMP-F ( $k_{\text{cat}}/K_M = 1.73 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ , and  $1.75 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ , respectively; **Table 1** and **Fig. 1c**). We then examined 4E9's ability to hydrolyze two other G-agents, sarin (also known as GB) and soman, using the AChE protection assay and the *in situ*-generated agents. We found that it was able to hydrolyze both of these agents, soman in particular, at relatively high rates (apparent  $k_{\text{cat}}/K_M$  value for IMP-F is  $\leq 3 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$  and for Pin-F,  $7.4 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ , and  $0.58 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ , for the two toxic isomers respectively).

### Prophylactic protection assays

To validate our model that hydrolysis of the toxic isomer by a variant with  $k_{\text{cat}}/K_M$  values of  $>10^7 \text{ M}^{-1} \text{ min}^{-1}$  can protect against lethal organophosphate exposure at a low protein dose, we tested rePON1-4E9 as a prophylactic in a mouse model. Because of safety issues, we applied the CMP-coumarin surrogate, but we upgraded the challenge by using the toxic isomer only (S<sub>p</sub>-CMP-coumarin) and by administering it directly by intravenous injection. The results indicated a survival rate of 45% for mice pretreated with 1.1 mg kg<sup>-1</sup> 4E9 one hour before the organophosphate exposure (**Table 2**). Increasing the 4E9 dose to 2.2 mg kg<sup>-1</sup> increased the percent of surviving animals to 75% (**Table 2**). As expected, a similar dose of the wild-type-like rePON1-G3C9 ( $k_{\text{cat}}/K_M$  for S<sub>p</sub>-CMP-coumarin  $\leq 2 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$ ), which served as a starting point for the directed evolution of 4E9, conferred no protection *in vivo* (**Table 2**), supporting the predicted correlation between catalytic efficiency and *in vivo* protection levels (**Supplementary Methods**). Notably, treatment of mice with atropine and 2-PAM, even 5 min before challenge, gave very poor protection against the cyclosarin coumarin surrogate, as is the case with cyclosarin itself<sup>25</sup>: the 24-h survival was only 22%. Further, most mice that were pretreated with 4E9 and survived the challenge in the first 2 h showed only mild intoxication symptoms 2–12 h after the challenge and completely recovered later on. However, all mice treated with atropine and 2-PAM that had survived the first 2 h after challenge showed severe intoxication symptoms and subsequently died.

Analysis of 4E9 plasma concentrations over time (**Supplementary Fig. 4**) indicated that the mean residence time of circulating 4E9 is ~10 h. Thus, after 6 h, the initial 4E9 blood concentrations dropped by about 50%. This drop is also seen in the reduction in survival of mice challenged after 6.3 h (50%) relative to mice challenged only

1h after 4E9 administration (75%; **Table 2**). The time limit of prophylactic protection granted by a dose of 2.2 mg kg<sup>-1</sup> of 4E9 against a lethal dose of S<sub>p</sub>-CMP-coumarin was found to be within 24 h (**Table 2**). However, 4E9 was still present at detectable concentrations in mouse blood after 24 h (**Supplementary Fig. 4**), suggesting that it may still provide protection against sublethal doses of CMP.

### DISCUSSION

Combining random mutagenesis and combinatorial exploration of mutations in key active-site positions<sup>26</sup> with high-throughput screens based on sorting by FACS of double-emulsion droplets<sup>20</sup> yielded dramatic improvements in the catalytic efficiency of rePON1 for both isomers of the fluorogenic cyclosarin surrogate CMP-coumarin. Driven by the need to engineer variants that detoxify the actual threat agents, we also developed a direct screen based on a competition between AChE, the nerve agent's physiological target, and the assayed detoxifying PON1 variant. Thus, we were able to increase the catalytic efficiency of rePON1 with cyclosarin to the level required for effective protection at reasonable protein doses ( $k_{\text{cat}}/K_M \geq 10^7 \text{ M}^{-1} \text{ min}^{-1}$ ). Our detoxification approach was also validated by demonstrating prophylactic protection in an animal model. The advantages of using a catalytic scavenger are manifested in the differences in survival and intoxication symptoms between mice pretreated with the evolved variant 4E9 and mice pretreated with the conventional atropine-oxime treatment—atropine plus 2-PAM aims to minimize the damages of the organophosphate, whereas rePON-4E9 neutralizes the agent before it even reaches its target. There is also a clear correlation between the improvement in the catalytic efficiency of the evolved PON1 variant 4E9 relative to wild-type-like rePON1 and the ability of 4E9 to act as an effective prophylactic *in vivo*.

The newly isolated rePON1 variants and the methodologies described here also provide the basis for further engineering of PON1 and other enzymes toward other G-type nerve agents, such as sarin and soman. Our evolved variants hydrolyze these agents, soman in particular, at high rates that enable detection of AChE protection activity in crude cell lysates, thus enabling library screens and additional improvement using directed evolution. The adaptation of the evolved rePON1 variants for clinical applications may also demand a reduction in immunogenicity, for example by pegylation. Alternatively, the identified mutations could be introduced into human PON1, although its low stability is an obstacle<sup>17</sup>. Overall, it appears that enzyme engineering and directed evolution in particular, have become sufficiently powerful to tackle challenging tasks in the biomedical area ranging from nerve agent detoxification to drug synthesis<sup>27</sup> and imaging<sup>28</sup>.

### METHODS

**Library construction.** Recombinant PON1 variant G3C9 (GeneBank: AY499193), cloned in the described pET vector<sup>16</sup>, was used as a template to construct the double mutant H115W-V346A. For random mutagenesis, 10 ng of plasmid template was amplified with a mutator *Taq* polymerase (Genemorph II, Stratagene) in 25 μl of reaction mixture, for 15 cycles. Sequencing indicated an average of  $1.7 \pm 0.65$  amino acid exchanges per gene. For generating libraries of shuffled genes, selected PON1 variants were separately amplified from their respective plasmids and digested by *DNaseI* (Takara). The targeted library was constructed using synthetic oligonucleotides and the ISOR protocol<sup>22</sup>. The assembly PCR reactions were further amplified by nested PCR, digested with *NcoI* and *NotI* (New England BioLabs) and recloned into pET32 (Novagen) with a C-terminal 6-His tag.

**Double emulsions and fluorescence-activated cell sorting.** The target libraries were sorted by compartmentalization of single *E. coli* cells, each expressing an individual library variant, in double-emulsion droplets and sorting these droplets by FACS, essentially as described<sup>20</sup>. Briefly, *E. coli* BL21(DE3) cells carrying a genomic *GFPuv* gene were transformed with the pET plasmids expressing the PON1 library variants. The cells were grown, isolated and compartmentalized in the first emulsion (water-in-oil). The substrate (DEPCyC) was added to the oil phase, and production of the second emulsion (water-in-oil-in-water) was prepared and sorted by FACS. We sorted  $>10^6$  events, at 2,000 events per sec, using FACSAria

(Becton-Dickinson). Events corresponding to single *E. coli* cells were gated by GFP emission, and those showing coumarin fluorescence that corresponded to DEPCyC hydrolysis were isolated. Approximately 5,000 events were sorted to 96-well plates containing growth medium. The plates were incubated at 37 °C for 1 h, plated on LB-agar plates containing ampicillin and glucose and grown overnight at 30 °C.

**Screening in 96-well plates.** Randomly picked colonies (from either FACS-sorted libraries or directly transformed libraries with no prior selection) were individually grown in 96-well plates, with shaking, using 0.5 ml 2× YT medium containing 100 µg ml<sup>-1</sup> ampicillin (Sigma) and 1 mM CaCl<sub>2</sub> (Merck), for 8 h at 30 °C; another 16 h at 20 °C followed. In each plate, several colonies of a reference variant (rePON1-G3C9, or the best variant from the previous round) were grown as controls. Following growth to optical density at 600 nm (OD<sub>600</sub>) ≈ 4, the cells were centrifuged, collected and lysed by incubation in buffered detergent and lysozyme (Sigma). The clarified supernatant was used to measure the initial rates ( $v_0$ ) for different substrates. We used 25 µM of IMP-coumarin and CMP-coumarin<sup>18</sup>, and coumarin release was measured at 405 nm. To screen for hydrolysis of the *S<sub>p</sub>* isomer, we used ~10 nM of purified variant rePON1-3B3 that hydrolyzed the *R<sub>p</sub>* isomer from the 25 µM of racemic CMP-coumarin, and the resulting reaction mixture was added to crude lysates.

**Enzyme purification and kinetics.** Following the screen in 96-well plates, variants showing the highest rates with the target substrate were grown in 50-ml cultures. Cells were harvested by centrifugation, resuspended and disrupted by sonication. Ammonium sulfate-precipitated proteins were resuspended, dialyzed against activity buffer (Tris-HCl 50 mM, pH = 8, CaCl<sub>2</sub> 1 mM, NaCl 50 mM, Tergitol 0.1% (w/v)) and purified by Ni-NTA chromatography. A range of enzyme concentrations (0.01–4 µM) and substrate concentrations were applied (from 0.3× *K<sub>M</sub>* up to 2–3× *K<sub>M</sub>*, subject to substrate solubility). The substrates were prepared as described<sup>18</sup> and were added from stock solutions in methanol (the organic solvent concentration in the final reaction mixture was kept at <0.1%). Product formation was monitored in a plate reader using 96-well plates with 200-µl reaction volumes. The kinetic parameters were determined by fitting the data directly to the classical Michaelis-Menten model and comprise the average of ≥3 independent measurements.

**AChE protection assays.** The assays were performed by preincubation of the PON1 variant and the organophosphate (as exemplified in the below protocol for CMP-coumarin) or by direct competition of the PON1 variant and AChE as exemplified in the second protocol with CMP-F. Briefly, randomly picked colonies of library variants were grown and lysed as above. Clarified cell lysates were diluted 1:4 in activity buffer, and 50 µl diluted lysate were mixed with 10 µl of 6 µM CMP-coumarin. The reactions were incubated (15 min), and an equal volume of 0.25 nM AChE (in PBS, 0.1% (w/v) BSA) was added. After 15 min of incubation, samples (20 µl) were mixed with Ellman's reagent and the AChE substrate (180 µl, 0.85 mM DTNB, 0.55 mM acetylthiocholine, in PBS), and initial rates were measured at 412 nm. Residual AChE activity was determined by comparing initial rates to AChE rates without organophosphate. The screen with CMP-F was performed with the following modifications: undiluted cell lysates were mixed with an equal volume of AChE solution (0.5 nM), and freshly made CMP-F (Supplementary Methods) was added to 1 µM final concentration. Reactions were incubated for 15 min, and residual AChE activity was determined as above. The catalytic specificity ( $k_{cat}/K_M$ ) of purified variants was measured by mixing the *in situ*-prepared organophosphate-fluoridates (40 nM) with purified PON1 variants (0.1–0.01 µM) in activity buffer. Samples of this reaction mix were taken at different times, diluted (1:10) with the AChE solution (4 nM AChE, 0.1% (w/v) BSA, 1 mM EDTA, in PBS), incubated for 15 min, and residual AChE activity was determined as above. The apparent  $k_{cat}/K_M$  values were derived from the slope of the resulting single exponential curve as exemplified in Figure 1c.

**Prophylactic activity of 4E9 in a mouse model.** Re-PON1 variant 4E9, or G3C9, purified as described in Supplementary Methods (0.21–0.26 mg ml<sup>-1</sup>, >97% pure, in isotonic activity buffer: 50 mM Tris pH 8.0, 1 mM CaCl<sub>2</sub>, 100 mM NaCl, 0.02% (w/v) Tergitol) were injected intravenously into 8-week-old male C57BL/6J mice at different doses (1.1 or 2.2 mg kg<sup>-1</sup>). After 1, 6 or 24 h, *S<sub>p</sub>*-CMP-Coumarin (26.5 µg ml<sup>-1</sup>, in PBS) was administered by a single intravenous injection of a dose of 290 µg kg<sup>-1</sup> (1× LD<sub>50</sub>). Animals were observed closely for clinical signs of CMP-coumarin intoxication during the first 24 h and up to 14 days before they were killed. Control mice were injected intravenously with either isotonic activity buffer (200 µl) or atropine sulfate (20 mg kg<sup>-1</sup>) and 2-PAM (25 mg kg<sup>-1</sup>) in PBS, 5 min before organophosphate exposure. All animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science (Rehovot, Israel).

Other methods including the determination of 4E9 plasma concentrations as well as detailed information on all methods, can be found in the Supplementary Methods.

Received 17 May 2010; accepted 22 November 2010; published online 9 January 2011

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## Acknowledgments

Financial support by US National Institutes of Health (W81XWH-07-2-0020) and the Defense Threat Reduction Agency (HDTRA 1-07-C-0024) is gratefully acknowledged. We thank D. Tal and the Israel Structural Proteomics Centre for assistance with protein production and T. Magliery, D. Lenz and D. Cerasoli for a fruitful collaboration.

## Author Contributions

R.D.G. constructed and screened variant libraries by FACS and plate screening with coumarin substrates, characterized evolved variants, analyzed data and wrote the paper. M.G. constructed and screened variant libraries using coumarin and *in situ*-generated substrates, characterized selected variants, performed *in vivo* experiments, analyzed data and wrote the paper. Y.A. designed and synthesized coumarin and fluoride substrates, established the AChE assay, characterized selected variants, designed and performed *in vivo* experiments and contributed to writing and editing the paper. Y.S. screened variant libraries. G.M. constructed and screened the first-generation variant libraries.

H.B. generated recombinant AChE. M.B.-D. assisted the design of targeted libraries. H.L. synthesized the coumarin and fluoride substrates. R.M. performed *in vivo* experiments. I.S. contributed to writing and editing the paper. J.L.S. performed structural analysis and contributed to writing the paper. D.S.T. designed the experiments, analyzed data and wrote the paper.

## Competing financial interests

The authors declare no competing financial interests.

## Additional information

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