Supplementary Information

for

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

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Supplementary Results

Supplementary Table 1. Activity of PON1 mutants with both isomers of CMP-coumarin and with Sp-IMP-coumarin

Variants	S_p-CMP coumarin [a] (k_{cat}/K_m) $M^{-1}min^{-1}$	S_p-IMP coumarin [a] (k_{cat}/K_m) $M^{-1}min^{-1}$	R_p-CMP coumarin [a] $\times 10^6$ Apparent (k_{cat}/K_m) $M^{-1}min^{-1}$
Wild-type-like rePON1-G3C9	<200 (1)[b]	n.d.	0.08 ±0.003 (1)
H115W	331 ±39 (>1.6)	1983 ±30 (1)	0.45 (6)
V346A	885 ±76 (>4.4)	3633 ±126 (1.8)	0.2 (2.5)
H115W+V346A	1300 ±500 (>6.5)	10140 ±7 (5)	0.4 (5)

a. For each variant, enzymatic activities (k_{cat}/K_m) were measured with purified proteins and denoted are the average ± standard deviation values obtained from the 3 independent repeats. The values without standard deviations had s.d.≤20% of their values. Values in parentheses denoted the fold increase and decrease as compare to wt like rePON1 for either isomers of CMP and as compared to H115W for Sp-IMP. n.d. denotes non detectable activity.

b. The catalytic efficiency was estimated as described in Ref. [1]

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Supplementary Table 2. Improved variants from 1st and 2nd round libraries starting from rePON1-H115W-V346A.

Variants [a]	Round [b]	Fold improvement with S_p -IMP-coumarin [c]	Non-Synonymous mutations [d]
1G3	1	2x	H115W, P135A, V346A
2A10	1	2.8x	I109T, H115W, S139P, V346A
3G6	1	4.8x	F17S, H115W, V346A
3F11	1	3.7x	H115W, V346A [e]
4B8	1	5.6x	H115W, F347I
5F2	1	3.3x	H115W, F222L, V346A
5F11	1	2.5x	H115W, M289I, V346A
6G1	1	5x	H115W, F222L, V346A
6E3	1	3.1x	H115W, S139P, V167M, V346A
6D10	1	4.3x	H115W, V346A [e]
7F6	1	7.4x	H115W, F222S, D309N, V346A
3A7	2	22x	V97A, H115W, P135A, F222S, M289I
6D10	2	12x	H115W, F222S
6G5	2	16x	L10S, H115W, P135A, F222S

a. **The annotation of the variants:** The first letter relates to the plate number, and the letter-digit to the location of the clone within this plate. For example, variant 1G3= plate # 1, well G3.

b. Round of mutagenesis and screening

c. Shown are all variants that exhibited higher S_p-IMP-coumarin activity in crude cell lysates **relative to the H115W + V346A PON1 mutant**. For each variant, enzymatic activities were measured in crude lysate and denoted are the average values of fold improvement obtained from 3 independent repeats. The values had s.d.≤20% of their value.

d. Non-synonymous mutations observed in each variant. Mutations in active site residues are noted in red

e. These two variants had the same amino acid exchanges. The small differences in activity may relate to differences in the composition and number of synonymous mutations.

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Supplementary Table 3. Improved variants from 3rd round libraries.

Variants [a]	Fold improvement with S_p -IMP-coumarin [b]	Fold improvement with S_p -CMP-coumarin [b]	Non-Synonymous mutations [c]
4D2	0.3x	10x	L69S, H115W, P135A, F222S
8C8	0.4x	13x	L69S, V97A, H115W, P135A, F222S
6C5	2x	3x	V97A, H115W, P135A, F222S, M196V, M289I
1A8	1.3x	1.5x	L4P, V97A, H115W, P135A, F222S, M196V
1E3	0.2x	1.4x	A6T, V97A, H115W, P135A, F222S, D212N, M289I
7G10	0.5x	1.3x	L10S, H115W, F222S, M289I, V346A
8H4	0.7x	0.8x	V97A, H115W, P135A, F222S
8H3	0.8x	0.8x	V97A, H115W, P135A, F222S, I237V, L262F
1G1	1.2x	1.2x	V97A, H115W, F222S, M289I

a. **The annotation of the variants:** The first letter relates to the plate number, and the letter-digit to the location of the clone within this plate. For example, variant 4D2= plate # 4, well D2.

b. Shown are all variants that exhibited higher S_p -IMP-coumarin and S_p -CMP-coumarin activities in crude lysates **relative to the H115W + V346A PON1 mutant**. For each variant, enzymatic activities were measured in crude lysate and denoted are the average values of fold improvement obtained from 3 independent repeats. The values had s.d.≤20% of their value.

c. Non-synonymous mutations observed in each variant. Mutations in active site residues are noted in red.

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Supplementary Table 4. Improved variants from the targeted substitutions library (5th round).

Variants [a]	S_p -CMP-coumarin [b]	Non-Synonymous mutations [c]
2F8	0.3x	T35A, L69S, H115W, F222N
6H2	0.4x	L10S, L69G, H115W, H134K, F222S
6C6	0.3x	L10S, L69S, H115W, P135A, F222S
1D10	0.2x	L69G, H115W, H134T, F222L
3G7	0.3x	L69G, H115W, F222L
6B1	0.3x	H115W, F222V
2G11	2x	L10S, L69G, H115W, P135A, F222S
1H1	4x	L69G, H115W, H134R, F222C
2H4	2x	L10S, L69A, H115W, H134R, F222S
4H7	2x	L69G, H115W, F222S

a. **The annotation of the variants**: The first letter relates to the plate number, and the letter-digit to the location of the clone within this plate. For example, variant 2F8= plate # 2, well F8. **.**

b. Shown are all variants that exhibited higher S_p-CMP-coumarin activities in crude lysates **relative to the 8C8 mutant**. For each variant, enzymatic activities were measured in crude lysate and denoted are the average values of fold improvement obtained from 3 independent repeats. The values had s.d.≤20% of their value.

c. Non-synonymous mutations observed in each variant. Mutations in active site residues are noted in red

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Supplementary Table 5. Improved variants from shuffling of the targeted substitutions library (6th round).

	CMP(Sp)[b]			CMP[b] (R_p)	CMP[b] (racemic)	IMP[b] (S_p)	EMP[b] (S_p)	EMP[b] (S_p)	
Variant[a]	k_{cat} min^{-1}	K_m μM	k_{cat}/K_m $\mu M^{-1}min^{-1}$	k_{cat}/K_m $\mu M^{-1}min^{-1}$					**Non-synonymous mutations[c]**
8C8	15.1 ±0.18	84.5 ±16	0.18 ±0.02 (1)	ND	0.03 (1)	0.022 (1)	0.08 (1)	0.2 (1)	**L69S**, V97A, **H115W**, P135A, **F222S**
2C3	149.5 ±0.87	212.7 ±5.5	0.7 ±0.01 (7.5)	0.088 (1)	0.62 (21)	0.079 (3.6)	0.88 (11)	0.16 (1)	**L69G**, **H115W**, **H134R**, **F222S**, K233E
5H5	126 ±2	102 ±3.8	1.25 ±0.05 (13.4)	0.2088 (2)	2.43 (81)	0.76 (35)	3.8 (48)	0.8 (4)	L10S, F28Y, **L69G**, **H115W**, **H134R**, **F222S**, **T332S**
0C9	185 ±2.5	65 ±3.6	2.85 ±0.1 (31)	0.296 (3)	3.56 (119)	1.04 (47)	6.67 (83)	2.6 (13)	L14M, **L69G**, S111T, **H115W**, **H134R**, **F222S**, **T332S**
2D8	395 ±23	42.5 ±5	9.3 ±0.6 (38)	nd[d]	3.04 (101)	0.85 (39)	6.8 (85)	0.98 (5)	**L69G**, **H115W**, **H134R**, **F222S**, **T332S**
1A4	185 ±1.5	51 ±1.7	3.63 ±0.1 (39)	0.39 (4)	3.11 (104)	0.85 (39)	7.15 (89)	2.2 (11)	A6E, **L69G**, **H115W**, **H134R**, **F222S**, K233E, **T332S**, T326S
3D8	546 ±29	36.7 ±7	12.7 ±4 (125)	nd[d]	4.73 (158)	4.6 (209)	12 (150)	6.2 (31)	**L69G**, **H115W**, **H134R**, M196V, **F222S**, **T332S**

a. The annotation of variants: The first letter relates to the plate number, and the letter-digit to the location of the clone within this plate. For example, variant 3B3 = plate # 3, well B3.

b. For each variant, enzymatic activities (k_{cat}/K_m) were measured with purified proteins and denoted are the average ± standard deviation values obtained from the 3 independent repeats. The individual values exhibited s.d.≤20%. Values in parentheses denoted the fold increase and decrease **as compare to 8C8**, the best variant of the previous round. ND – not determined.

c. Denoted in bold are mutations in active-site residues.

d. Variant exhibited a 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.

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Supplementary Table 6. PON1 variants selected using the AChE inhibition assay.

	Variant name	CMP-coumarin hydrolysis activity [a,c] [fold improvement over 3D8]	CMP-F hydrolysis activity [b,c] [fold improvement over 3D8]
1	V-H10	11.6x	4.3x
2	V-E2	11.0x	6x
3	V-F3	8.8x	6x
4	V-C11	8.3x	6x
5	V-D6	7.2x	3.4x
6	V-A6	6.2x	2.1x
7	VIII-A12	2.3x	3.3x
8	VIII-D1	2.1x	5.5x
9	VI-G8	2.0x	6x
10	VI-H12	1.7x	2x
11	IV-H5	1.6x	6x
12	IV-E9	1.6x	1.6x
13	VI-A3	1.6x	4.5x

a. The variants were tested using AChE (0.5nM) with racemic CMP-coumarin (1μM), and **ranked relative to 3D8**, isolated in Round 6 (**Supplementary Table 5**).
b. The variants were ranked relative to 3D8 using AChE (0.5nM) and in-situ generated racemic CMP-F (1μM)
c. Values are the average values obtained from 3 independent repeats. The values had s.d.≤20% of their values.

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Supplementary Table 7: Activities of selected rePON1 variants on Sp-CMP-coumarin and its fluoridated product CMP-F (cyclosarin). [a,b,c,d]

mutant	(S_p)CMP-coumarin	CMP-fluoridate	fluoridate/ coumarin
8C8	0.18	0.15	0.8
3D8	12.7	3.14	0.2
0C9	2.8	11.1	3.9
2D8	9.3	4.65	0.5
1A4	3.6	11.3	3.1
2C3	0.7	0.47	0.7

a. The figures shown are values of $k_{cat}/K_M x10^6$ ($M^{-1}min^{-1}$). Values are the average values obtained from 2 independent repeats. The values had s.d.≤20% of their values.

b. Data for OP-coumarin are based on release of the chromophore monitored at 400 nm.

c. The k_{cat}/K_m values for the fluoridates were determined by monitoring the rate of loss of anti-AChE potency of the *in situ*-generated compound, assuming K_m>>[P-F]. Calculations are based on a single enzyme concentration selected to fit the dynamic range for determination of the apparent k_{obs} of loss of anti-AChE potency.

d. The coumarin leaving group was replaced by fluoride in racemic CMP-coumarin to yield the racemic fluoridates of CMP (CMP-F). Note that the data for the hydrolysis of CMP-F can be attributed mostly to the toxic (Sp) isomer of CMP-F.

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Supplementary Table 8. List of the oligonucleotides and primers

pET-Nes2-Bc	Forward	5'-GATGGCGCCCAACAGTCC- 3'
pET-Nes1-Fo	Backward	5'- GCGCGTCCCATTCGC-3'
pET-Nes0-Fo	Backward	5'- TGATCTAGTGCGGCCGCCAGCTCACAGTA AAGAGCTTTGTGAAACAC-3'
pET-Nes1-Bc	Forward	5'-GTCCGGCGTAGAGGATCG-3'
L69NNS		5'-GGCTTTCATCAGCTCCGGANNSAAGTATCCTGGAATAATGAGC-3'
H115NNS	Forward	5'-CTTCATTTAACCCTNNSGGGATTAGCACATTC-3'
H134NNS	Forward	5'-CTACTGGTGGTAAACNNSCCAGACTCCTCGTCC-3'
F222NNS	Forward	5'-GTTGATTCCGTTAGCSNNATCAAATCCTTCTGC-3'
V346NNS	Backward	5'-GAGCTTTGTGAAASNNTGTGCCAATCAGCAG-3'
F247NNS	Backward	5'-GTAAAGAGCTTTGTGSNNCACTGTGCCAATCAG-3'
H348NNS	Backward	5'-CAGTAAAGAGCTTTSNNAAACACTGTGCCAATC-3'

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Supplementary Methods

1. **Materials.** All liquid growth media and agar plates were produced by the Bacteriology unit of the Biological Services department of Weizmann Institute of Science (Rehovot, Israel). All plasmids and PCR products were sequenced by the DNA sequencing unit of the Biological Services department of Weizmann Institute of Science (Rehovot, Israel). Primers were synthesized by either IDT® or Metabion®. DNA purifications were done using PCR, miniprep, and gel purification kits (Qiagen). Maxiprep purification kits (Qiagen or Macherey-Nagel). Chemicals and Enzymes: CMP-coumarin (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yl ester cyclohexyl ester), IMP-coumarin (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yl ester isopropyl ester and PinP-coumarin (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yl ester pinacolyl ester) were synthesized as described in [1] (purity≥95% by TLC and NMR), DEPCyC (*O,O*-diethyl phosphoryl 3-cyano-7-O-coumarinyl) was synthesized as described in [2] (purity≥95% by TLC and NMR). Commercial chemicals (analytical or molecular biology grade): IPTG (Inalco), Ammonium Sulfate and Glucose (J.T.Baker), $CaCl_2$ and EDTA (Merck), NaCl and Tris-HCL (Bio-lab), Atropine Sulfate, 2-PAM, ampicillin, DTNB, BSA and Tergitol-NP10 (Sigma). Enzymes: AChE was either recombinant AChE (produced as in Bar, H. et. al. *in preparation)* or *Torpedo californica* AChE (T*c*AChE) purified as in [3], *Not*I, *Nco*I, *Dpn*I (NEB), *Dnase I* (Takara), Ni-NTA (Novagen), Taq polymerase ready mix (Bio-lab), PFU-Ultra (Stratagene), lysozyme (Sigma), Benzonase (Novagen).

2. **Constructing PON1 gene libraries by random mutagenesis**. Site specific mutations such as H115W and V346A, were introduced by primer designing. In general, we used primers pET-Nes2-Bc and pET-Nes1-Fo (**Supplementary Table 8)** for library construction**.** Random mutagenesis using mutator *Taq* polymerase (mutazyme, Genemorph) resulted in ~60% transition and ~40% transversion. The PCR product was treated with *Dpn*I (NEB®, to destroy the template plasmid), purified, and served as a template (10 ng) for another 15 cycles of nested PCR performed with Taq polymerase (Bio-lab).

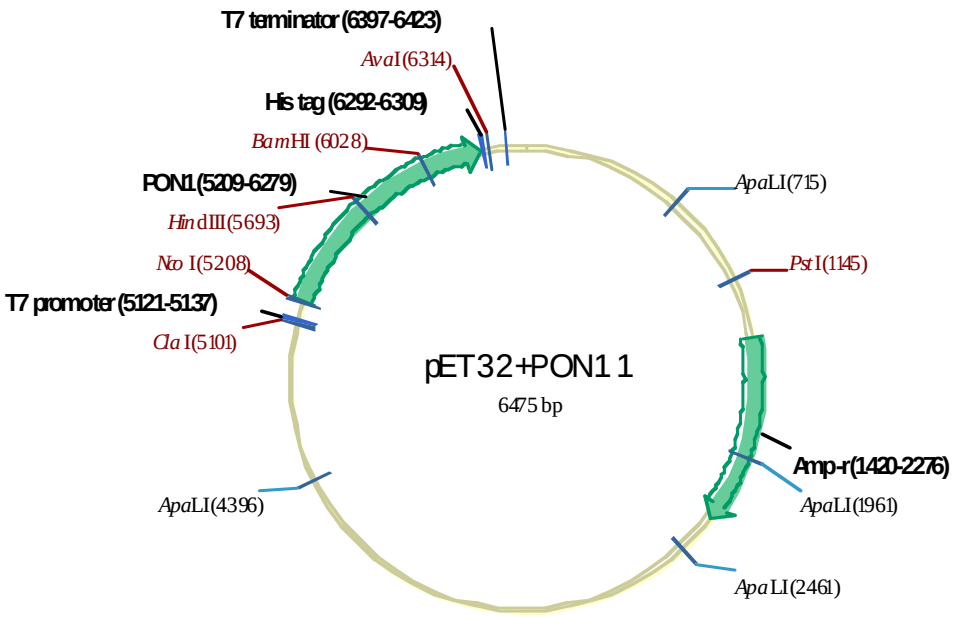
3. **Constructing PON1 gene libraries by gene shuffling**. The improved PON1 variants were separately amplified from their respective plasmids using Taq polymerase (Bio-lab) and primers pET-Nes2-Bc and pET-Nes1-Fo. To facilitate the removal of non-beneficial mutations, the PCR amplified wild-type PON1 gene was added at a 1:3 ratio to a mixture of PCR products from all the improved variants. Approximately 5 µg of purified DNA mixture in 50 µl reactions was digested with 0.01U *DNase*I (Takara) at 37°C for 2, 4, and 6 min. The reactions were terminated with 15µl of 0.5 M EDTA, and heating at 90°C for 10 min, and were run on a 2% agarose gel. Fragments of 50-150 bps size were excised and purified using a gel extraction kit (Qiagen). The PON 1 gene was reassembled

Nature Chemical Biology: doi:10.1038/nchembio.510

using 100ng of purified DNA fragments and thermocycling in a 50µl reaction mixture that contained 2.5 U *Pfu* Ultra (Stratagene). The cycling included: one denaturation step at 96°C for 3 min, then 35 cycles composed of: (i) a denaturation step at 94°C (30 s); (ii) nine successive hybridization steps separated by 3°C each, from 65°C to 41°C, for 1.5 min each (total 13.5 min), and (iii) an elongation step of 1.5 min at 72°C. Finally, a 10 min elongation step at 72°C was performed. The assembly product was amplified by a nested PCR reaction with primers pET-Nes1-Bc and pET-Nes0-Fo. In this step, 1 µl of the assembly reaction was used as a template in a standard 50 µl PCR reaction. The purified PCR product was digested with *NcoI* and *NotI* (NEB), and cloned into the pET32 vector (Novagen) with a C-terminal 6-His tag **(Supplementary Figure 1**).

4. **Constructing PON1 gene libraries by using designed oligonucletides at targeted positions.** The PON 1 gene having H115W mutation was used as a template to construct a library using synthetic oligos by ISOR protocol[4]. Briefly, H115W mutant gene was digested with *DNase*I (Takara). Approximately 5 µg of purified DNA in 50 µl reactions was digested with 0.01 U *DNase*I (Takara) at 37°C for 2, 4, and 6 min. The reactions were terminated with 15µl of 0.5 M EDTA, and heating at 90°C for 10 min, and were run on a 2% agarose gel. Fragments of 50-150 bps size were excised and purified using a gel extraction kit (Qiagen). The PON 1 gene was reassembled using 100ng of purified DNA fragments with oligonucleotides encoded one mutation and 20 flanking nucleotides matching the PON1 gene (**Supplementary Table 8**). Assembly PCR was performed in a 50µl reaction mixture that contained 2.5 U *Pfu* Ultra (Stratagene). The cycling included: one denaturation step at 96°C for 3 min, then 35 cycles composed of: (i) a denaturation step at 94°C (30 s); (ii) nine successive hybridization steps separated by 3°C each, from 65°C to 41°C, for 1.5 min each (total 13.5 min), and (iii) an elongation step of 1.5 min at 72°C. Finally, a 10 min elongation step at 72°C was performed. The assembly product was amplified by a nested PCR reaction with primers pET-Nes1-Bc and pET-Nes0-Fo. In this step, 1 µl of the assembly reaction was used as a template in a standard 50 µl PCR reaction. The purified PCR product was digested with *NcoI* and *NotI* (NEB), and cloned into the pET32 vector with a C-terminal 6-His tag (**Supplementary Figure 1**).

Nature Chemical Biology: doi:10.1038/nchembio.510



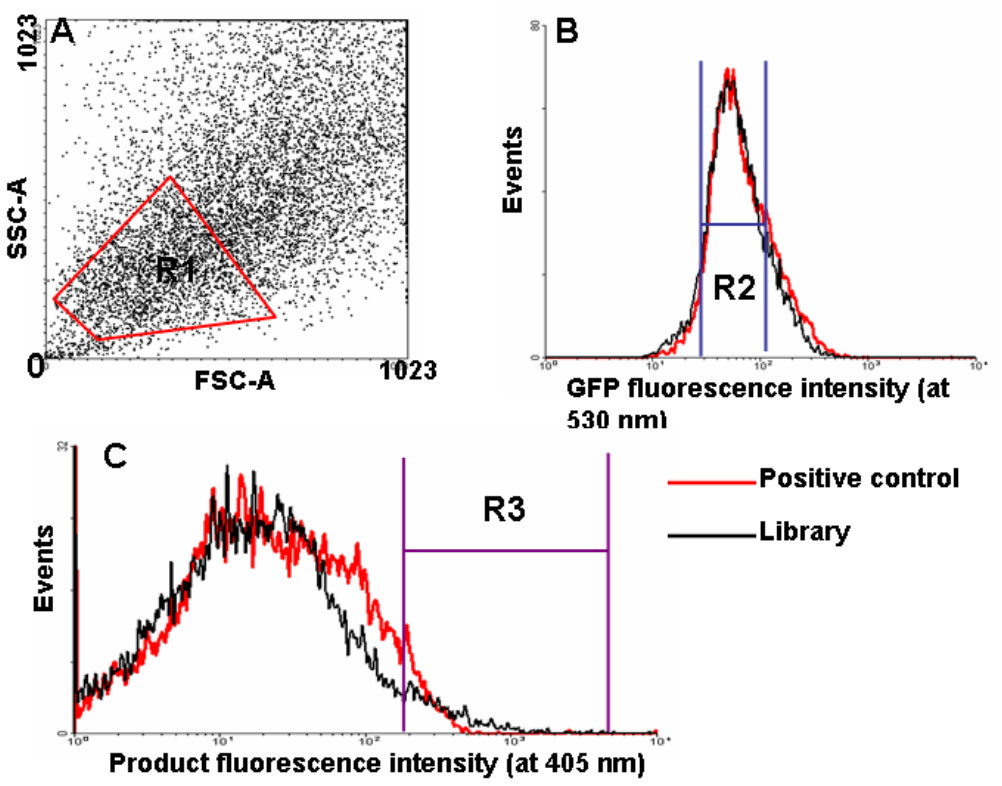
Supplementary Figure 1. **The pET32PON1 plasmid**.

This plasmid was used for the expression of PON1 variants with a C-terminal His-tag and no GFP. The plasmid was derived from pET32b(+) from which the thioredoxin fusion protein and peptide tags were truncated using the *NotI/XhoI* sites. The recombinant PON1 variant G3C9, and library variants, were inserted using the *NcoI/NotI* sites. The NotI restriction site was inserted upstream to the His tag to enable the cloning of various PON1 variants with no alterations to the tag.

Nature Chemical Biology: doi:10.1038/nchembio.510

5. **FACS sorting of emulsion droplets containing bacterial cells.** *E.coli* cells (BL21-DE3) expressing single variants from the substitution libraries were emulsified and sorted by FACS as previously described [5-7]. The cells possessed a genomic copy of the GFPuv gene and expressed PON1 under the T7 promoter. Plasmid DNA was transformed and grown while shaking at 250 RPM in 5ml 2xYT media containing 100μg/ml ampicillin and 1mM $CaCl_2$, for 12 hrs at 30°C, followed by another 24 hrs at 20°C. The cells were centrifuged at 3000g for 10 min at 4°C, resuspended in 2xYT, and kept for 1hr at room temperature. They were then rinsed twice in 0.1M Tris-HCl, 1mM $CaCl_2$, 0.1M NaCl, pH 8.0, resuspended in the same buffer, and passed through a 5 μm filter (Sartorius). Filtered cells were compartmentalized in the first emulsion (water-in-oil), and 100 mM solutions of CMP-MeCyC racemate or DEPCyC was added to the oil phase (0.8 *μ*l, to a final concentration of 50μM). The production of the second emulsion (water-in-oil-in-water) and sorting were performed as described [7]. We sorted >10^6 events, at 2000 events/sec, using FACSAria (Becton-Dickinson) **(Supplementary Figure 2).** Events corresponding to single *E. coli* cells were gated by GFP emission (at 530nm, using blue laser for excitation). Approximately 5000 events were sorted to 96-well plates containing 200 μl of 2xYT media (~1000 events per well). The plates were immediately moved to 37°C, incubated for 1hr while shaking at 250rpm, plated on LB-agar plates containing 100μg/ml ampicillin and 20mM glucose, and grown overnight at 30°C. Recovery of the sorted cells was determined by comparing the number of colonies on the LB plates to the number of events sorted by the FACS, and was found to be 20-40%.

Nature Chemical Biology: doi:10.1038/nchembio.510



Supplementary Figure 2: FACS detection and sorting of PON1-carrying *E. coli* cells in w/o/w emulsion droplets.

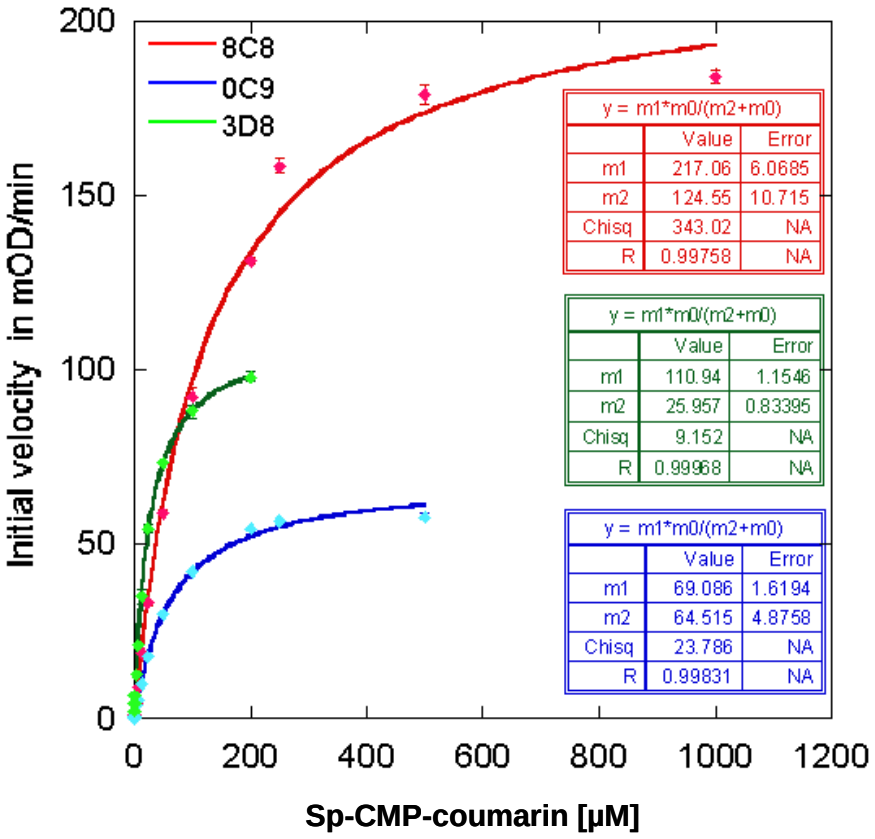
We used E. *coli* BL21 (DE3) cells possessing GFPuv gene in the genome for expression of the PON 1 under the T7 promoter. Cells were emulsified, together with the fluorogenic substrate (DEPCyC). Briefly, filtered cells were compartmentalized in the first emulsion (water-in-oil), and 100 mM solutions of DEPCyC was added to the oil phase (0.8 *μ*l, to a final concentration of 50μM). The production of the second emulsion (water-in-oil-in-water) and sorting were performed as described[7]. We sorted >10^6 events, at 2000 events/sec, using FACSAria (Becton-Dickinson). Events corresponding to single *E. coli* cells were gated by GFP emission (at 530nm, using blue laser for excitation). **(A)** Representative density plot FSC-H (forward scatter) and SSC-H (side scatter) analysis of the double emulsion. **(B)** Histogram of the GFP emission for the R1 population of droplets. Events gated in R2 correspond to droplets that contain GFP expressing cells. **(C)** The R1+R2 gated events were analyzed for the hydrolytic activity. Events gated in R3 represent active variants that were present as 0.5-1 % of total population; these were sorted into liquid growth media.

Nature Chemical Biology: doi:10.1038/nchembio.510

6. **Screens in 96-well plates.** *E.coli* cell colonies were picked randomly and grown as described in the main text. Several repeats of wild-type PON1 were grown as controls. Following growth (OD_{600nm} ≈4), the plates were centrifuged at 3000g for 15mins at 4°C, and pellets were kept at -70°C for few hours. The pellets were resuspended in 200µl of lysis buffer (0.1M Tris-HCl pH 8.0, 1mM $CaCl_2$, 10µg/ml lysozyme (Sigma), 0.2% Triton x-100, and 5 units/ml benzonase (Novagen)), and lysed by shaking at 1300 rpm for 30 min at 37°C. The pellet was removed by centrifugation at 4000 rpm for 20 min at 4°C, and the supernatant was transferred to a new set of plates and stored at 4°C. Apparent enzymatic rates (v_0) for different substrates were measured in a plate reader (Synergy-HT BioTek) using an appropriate volume of clarified lysates (0.1-10 µl depending on the substrate). All rates were determined at the linear range of product release, and background rates (lysates containing no PON1) were subtracted to give the observed initial rate (v_0).

7. **Enzyme purification and kinetics**. Cell colonies expressing active variants were picked and grown O/N at 37°C. Cultures were used to inoculate 50-250 m"l of fresh growth media (2YT), and were then grown at 37°C up to OD_{600nm} ≈0.5. Protein expression was induced by addition of IPTG (1mM) and subsequent growth at 30°C for 3-6h. Cultures were harvested by centrifugation, resuspended, and disrupted by sonication. Ammonium sulfate was added to the lysate to 55% (wt/vol). The precipitate was dissolved, dialyzed and purified on Ni-NTA (Novagen). Fractions were analyzed for paraoxonase activity and purity (by SDS-PAGE), pooled, dialyzed against activity buffer supplemented with 0.02% sodium azide, and stored at 4°C. Protein purity was typically 70-80% by SDS-PAGE gel. Variants 4E9 and rePON1 (G3C9) were further purified by FPLC purification using a mono-Q column (HiPrep 16/10 Q FF, GE healthcare) eluted by activity buffer with 250mM NaCl, concentrated (vivaspin 20 MWCO 20KDa), loaded on a gel filtration column (HiLoad 26/60 Superdex 75, GE healthcare) and dialyzed against activity buffer supplemented with 0.02% sodium azide for long term storage at 4°C. Protein purity was assessed to be >97% by SDS-PAGE gel. For each purified variants, at least three independent repeats were done for kinetic parameters and values were determined by fitting the data directly to the Michaelis–Menten using KaleidaGraph (**Supplementary Figure 3**). The values of the determined kinetic parameters were found to be influenced by the purification method and by buffer components such as detergents. The catalytic activity of evolved variants with R_p-CMP-coumarin was obtained by measuring kinetics of the 2nd phase observed with racemic CMP-coumarin after consumption of the S_p-CMP-coumarin by one of the S_p evolved variants (*e.g.* variant OC9 at 30nM). By measuring initial rates using several substrate concentrations, we could estimate the apparent k_{cat}/K_M for this isomer.

Nature Chemical Biology: doi:10.1038/nchembio.510



Supplementary Figure 3. Determination of kinetic parameters.

Shown is a representative Michaelis-Menten plot for rePON1 variants 8C8, 0C9, and 3D8, evolved towards S_p-CMP-MeCyC hydrolysis. Enzyme concentrations were 0.65 µM for 8C8, and 12.5 nM for 0C9 and 3D8. Substrate concentrations were varied from 0.4 µM up to 1000 µM. Error bars represent s.d. of 3 measurements.

Nature Chemical Biology: doi:10.1038/nchembio.510

8. **Conversion of CMP-coumarin to GF.** CMP-coumarin was converted *in-situ* to GF similarly to what was previously described[1] and product formation was confirmed by chemical characterization as well as three additional biochemical assays (details can be obtained upon request from the corresponding author).

9. **Determination of k_{cat}/K_M values with in-situ generated GF.** The GF stock was diluted 1000-fold in cold distilled water and then further diluted 20-fold into the 0.005 to 0.2 μM PON variant in activity buffer (Tris-HCl 50mM pH=8, $CaCl_2$ 1mM, NaCl 50mM, Tergitol 0.1%.). The nominal racemic GF concentration was set to 40-50 nM. At various time intervals, the reaction mixture is diluted 10- fold into 2.5 nM T*c*AChE in 50 mM phosphate buffer pH 8.0, 25°C. The phosphate buffer that chelates calcium, and the dilution, quenched the PON1 activity with GF. Residual T*c*AChE activity was measured after 10 and after 20 min (to ascertain completion of inhibition) by aliquoting 10 μl into 1 ml Ellman assay solution[8] containing 1 mM acetylthiocholine as substrate. The %-inhibition of T*c*AChE by the same GF solution with out PON was considered as 100% anti-AChE potency attributed to the toxic isomer of GF. This %-inhibition decreased over time of incubation with PON1, and k_{obs} was calculated by fitting the %-loss of anti-T*c*AChE potency versus time to a mono-exponential equation. The concentration of PON1 was set so that degradation of >50% of GF (i.e., gain of 50% AChE activity) occurred within less than 10 min's (although this was impossible with the poorly active variants such as wild-type-like rePON1-G3C9).

10. **Estimation of kinetic parameters for in vivo detoxification.** Enzymes displaying turnover with OP substrates, can provide effective prophylactic protection at low doses, provided that their catalytic efficiency (k_{cat}/K_M) $\geq 1\times10^7$ ($M^{-1}min^{-1}$). We considered a single compartment model of homogenous distribution and the kinetics of detoxification of OP's by PON1 in 3 liter/70 kg human plasma. We calculated the time that will take to lower the plasma concentration estimated to be produced by exposure to $2\times LD_{50}$ G-agent (about 2 μM, see Ref. [9]) by 100 fold to 0.02 μM, an OP level that is in all likelihood symptom-free. Using the integrated form of the Michaelis-Menten equation and assuming $K_M >> [OP]$ we used the following equation to estimate the concentration of PON1 with a given (k_{cat}/K_M) that will reduce 100-fold [OP] t=0 within 1 min, to ascertain sufficient detoxification in less than a single circulation time: $[PON1](k_{cat}/K_M)t=2.3\times\log100$. For t=1 min the above equation yields: $(k_{cat}/K_M)=4.6/[PON1]$. Thus for example, 50 mg of PON1 in 3 liter plasma is expected to produce initial serum levels of 0.42 μM PON1 and the required (k_{cat}/K_M) to reduce by 100-fold the OP concentration within 1 min is theoretically calculated to be about 1.1×10^7 ($M^{-1}min^{-1}$). Since 50 mg/kg is a protein dose 10-fold lower than what would be required from a stoichiometric scavenger such as hBChE we set 1×10^7 ($M^{-1}min^{-1}$) as the goal for the required catalytic activity of evolved PON1 variants. A similar goal for a catalytic OP scavenger has been previously proposed [10].

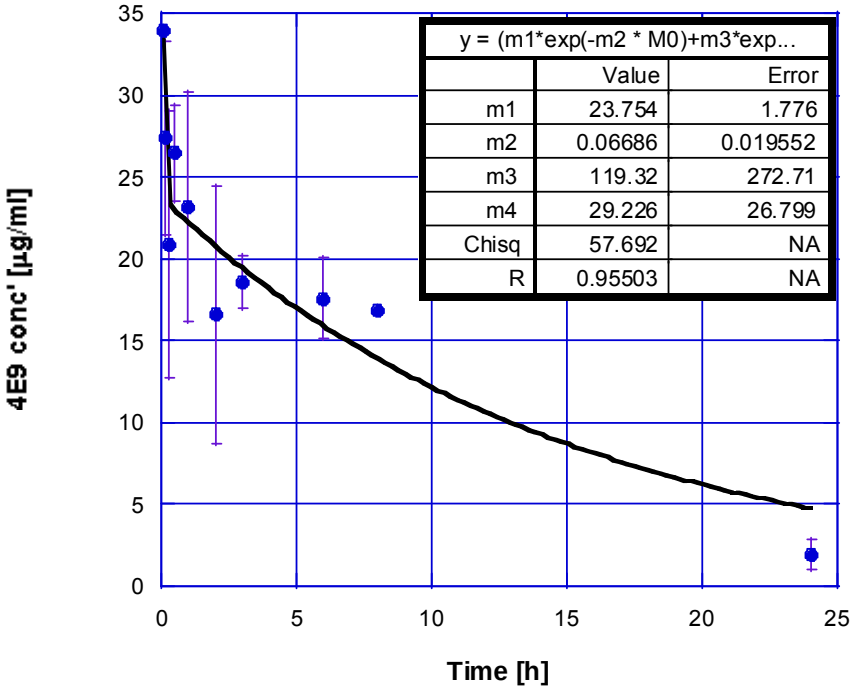
Nature Chemical Biology: doi:10.1038/nchembio.510

11. **Prophylactic activity of 4E9 in a mouse model.** Eight weeks old male mice of strain C57BL/6J, were supplied under germ-free conditions by the Animal Breeding Center of The Weizmann Institute of Science (Rehovot, Israel). The mice were housed in a light- and temperature-controlled room. All animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee (application number 04590909-2). Prior to treatments, blood samples were taken (50-75µl, retero-orbital) into heparin (10µl ,1:10). Mice were then weighted (average weight 24.5(gr) $\pm$ 2.2) and PON1 variant 4E9 or rePON1 (210-260 µg/ml, >97% pure in isotonic activity buffer: Tris 50mM pH=8, CaCl2 1mM, NaCl 100mM, tergitol 0.02%) were injected i.v to the tail vein at different doses(1.1 or 2.2 mg/kg). After 55' or 5h55' blood samples were obtained as described and mice were reweighed. After 1, 6 or 24 hours, intoxication was induced by a single i.v. administration of Sp-CMP-coumarin (26.5 [µg/ml], PBS) at a dose of 290 µg/Kg (1xLD_{100}). All animals were observed closely for clinical signs following CMP-coumarin intoxication during the first 24 hours and were kept for at least 14 days before sacrifice. The toxicity of PON1 variant 4E9 or the isotonic activity buffer were assayed by injecting them to mice without an OP challenge, as described, and monitoring for at least 14 days. All clinical signs noted following Sp-CMP-coumarin intoxication were categorized to mild, moderate or severe reactions. Mild reactions were characterized by straub tail and ataxia. Moderate reactions consisted in addition, decreased motor activity and tremors while animals with severe reactions exhibited in addition ventral position, fasciculation and dyspnea as well. The overall reactions observed following Sp-CMP-coumarin intoxication were scored using semi-quantitative grading of five grades (0-4), taking into consideration the severity of the reactions (0 = No Reactions, 1 = Mild Reactions,2 = Moderate Reactions, 3 = Severe Reactions, 4 = Mortality).

12. **Determination of variant 4E9 plasma concentrations in mice.** A dose of 40 [µg/mouse] of 4E9 was injected (i.v.) into 8 male and 4 female mice (strain C57BL/6J) of similar weights (24$\pm$3 gr). Blood samples (50-75µl, retero-orbital) were taken into heparin (10µl ,1:10) at different times from each mouse following the injection of 4E9. Sampling times for each mouse were spaced by at least 1h and each mouse was sampled only two times in 24h to maintain sufficient blood volume. Blood samples were taken from each mouse prior to introduction of 4E9 in order to determine background CMP-coumarin hydrolytic activity in plasma. The concentration of 4E9 in plasma samples was determined by measuring the rate of CMP-Coumarin hydrolysis *in-vitro* (CMP-coumarin 0.1mM, in Activity buffer (Tris-HCl 50mM pH=8, $CaCl_2$ 1mM, NaCl 50mM, Tergitol 0.1%.) at 405nm in a 96-well plate ELISA reader) and comparing it to a pre-determined calibration curve. The calibration curve was generated by adding increasing amounts of 4E9 into an equal volume of plasma obtained from naïve mice. We employed a noncompartmental analysis that does not require specifying a particular PK

Nature Chemical Biology: doi:10.1038/nchembio.510

model[11] . We used the average concentration of 4E9 in plasma shown in **Supplementary Figure 4** to calculate the mean residence time (MRT) and the terminal elimination phase rate constant (k_{term}) of 4E9 in mice plasma by the a Windows-based Computer Program of noncompartamental analysis[12]. Blood 4E9 levels were normalized to 20 gr mice. The zero time concentration of 2 mg/kg 4E9 injected i.v. in mice was inserted manually at 60 μg/ml plasma (assuming 60 ml WB/kg and 55% plasma fraction.



Supplementary Figure 4. Mice plasma concentrations of 4E9 over time.
A dose of 40 (μg/mouse) of PON1 variant 4E9 was injected to 8 male and 4 female mice. Blood samples were taken at different times and plasma concentrations of 4E9 were determined using CMP-Coumarin hydrolysis (**Supplementary Methods**) and normalized to 20gr mouse weight. Shown are average plasma concentrations for the 12 mice at different times. Error bars represent the s.d. of two independent measurements. The data were fit to a double exponential curve representing the two phases of clearance from the circulation: An initial rapid decline follows the injection of the enzyme i.v. corresponding to its clearance by distribution, and a subsequent slow clearance phase. The differences between male and female mice were not statistically significant (P=0.6, t-test). The mean residence time (MRT) and the terminal elimination phase rate constant (k-term) of 4E9 in mice plasma were found to be: for all 12 mice (8 males + 4 females) MRT=10.1±0.4 h and k-term = 0.1±0.005 h^{-1}. When only male mice were included, very similar values were obtained (MRT=10.7±0.4 h and k-term =0.093±0.005 h^{-1}).

Nature Chemical Biology: doi:10.1038/nchembio.510

Supplementary References

1. Ashani, Y. et al. Stereo-specific synthesis of analogs of nerve agents and their utilization for selection and characterization of paraoxonase (PON1) catalytic scavengers. *Chem Biol Interact* **187**, 362-9 (2010).
2. Aharoni, A. et al. Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proc Natl Acad Sci U S A* **101**, 482-7 (2004).
3. Sussman, J. L. et al. Purification and crystallization of a dimeric form of acetylcholinesterase from Torpedo californica subsequent to solubilization with phosphatidylinositol-specific phospholipase C. *J Mol Biol* **203**, 821-3 (1988).
4. Herman, A. & Tawfik, D. S. Incorporating Synthetic Oligonucleotides via Gene Reassembly (ISOR): a versatile tool for generating targeted libraries. *Protein Eng Des Sel* **20**, 219-26 (2007).
5. Aharoni, A., Amitai, G., Bernath, K., Magdassi, S. & Tawfik, D. S. High-throughput screening of enzyme libraries: thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments. *Chem Biol* **12**, 1281-9 (2005).
6. Amitai, G., Gupta, R. D. & Tawfik, D. S. Latent evolutionary potentials under the neutral mutational drift of an enzyme. *Hfsp J* **1**, 67-78 (2007).
7. Miller, O. J. et al. Directed evolution by in vitro compartmentalization. *Nat Methods* **3**, 561-70 (2006).
8. Ellman, G. L., Courtney, K. D., Andres, V., Jr. & Feather-Stone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**, 88-95 (1961).
9. Ashani, Y. & Pistinner, S. Estimation of the upper limit of human butyrylcholinesterase dose required for protection against organophosphates toxicity: a mathematically based toxicokinetic model. *Toxicol Sci* **77**, 358-67 (2004).
10. Masson, P., Nachon, F. & Rochu, D. [Engineering of catalytic bioscavengers of organophosphorus compounds]. *Bull Acad Natl Med* **191**, 95-111; discussion 112 (2007).
11. Perrier, M. G. a. D. Pharmacokinetics (ed. DeVane, C. L.) (Marcel Dekker, New York, 1982).
12. Laub, P. B. & Gallo, J. M. NCOMP - A windows-based computer program for noncompartmental analysis of pharmacokinetic data. *Journal of Pharmaceutical Sciences* **85**, 393-395 (1996).

Nature Chemical Biology: doi:10.1038/nchembio.510