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14. ABSTRACT

Project Summary: The long-term objective of this effort is to develop a generic gene shuffling-based technology to rapidly screen libraries of 10^{10} proteins/peptides encoded by DNA libraries, for identifying biomolecules that can intercept both existing and emerging organophosphate-based chemical warfare nerve agents (CWNA). In the first year of the renewal contract we focused on: (a) Design and generation of libraries VXG6 and VXG7 by screening with VX, and (b) HDL association and stabilization of PONs. Libraries VXG6 and VXG7 provided variants that hydrolyzed VX at k_{cat}/K_m approaching $3x10^5$ $M^{-1}min^{-1}$. A "thio effect" was defined and raised as a potential barrier that slowed the improvement of PON1 variants towards V-agents. The most active variants found (167-mix1-F5 and 268-2-E8), were sent to OSU for large-scale production intended for future *in-vivo* experiments at ICD. Targeting key positions in helixes H1 and H2 revealed a H-bonding network responsible for structural changes that stimulate PON1 activity that occur upon binding to HDL. Positions 185 and 198 were found to be key residues associated with increased stability and activity stimulation.
Relevance: this technology is envisaged to provide rapid discovery of pre- and post-treatment therapeutic drugs against existing and emerging CWNA threats and may shorten the time from emergence of a threat to identification of potential counter-measures to a few days or weeks.

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Introduction

The long-term objective of this effort is to develop a generic gene shuffling-based technology to rapidly screen DNA libraries encoding mammalian paraoxonase 1 (PON1), and thus to identify variants that can intercept both existing and emerging organophosphate-based chemical warfare nerve agents (CWNA). Enzymes identified in these screens should be capable of catalytically neutralizing the target agent under physiological conditions, thereby providing a basis for development of a new generation of therapeutic agents against CWNA, in either pre- or post-treatment modality. The major milestone is to integrate established components of enhanced molecular evolution techniques so as to provide a means of screening libraries with both greater sensitivity and higher throughput.

G- and V-type type nerve agents: tabun (GA), sarin (GB), soman (GD), cyclosarin (GF) and VX**,** are highly toxic organophosphates (OPs) that penetrate the body both by inhalation and skin absorption. In the last 6 years we have used a strategy that utilizes enhanced evolution, protein engineering and 3D structural analysis, together with a specific interception screening protocol, to generate large libraries of mammalian PON1 variants that could be expressed in *E. coli* and screened for their ability to hydrolyse OP nerve agents. Structural analysis was then used to direct the mutagenesis of relevant active-site positions so as to obtain highly effective bioscavengers. The combined approach permitted the isolation of mutants with k_{cat}/K_M values for the detoxification of *in-situ*-generated G agents, such as GD and GF, that approached levels required for them to be considered as efficacious catalytic bioscavenger drugs (>10^7 $M^{-1}min^{-1}$). *In vivo* experiments conducted at ICD showed that several variants generated by our group (*e.g.*, 4E9, 2D8, 0C9, and VII-D-11) conferred full protection in pretreated guinea pigs at doses as low as 1 mg/kg protein against 2xLD_{50} doses (sc) of GA, GB, GD, and GF. To date, the best variants hydrolyze GD and GF at 3.0-5.0x10^7 $M^{-1}min^{-1}$ and GA and GB at ~7-10-fold lower rates. Key variability positions at which changing the residue produces improved hydrolysis of G agents are 69, 115, 134, 136, 222, 291 and 332.

Progress for the V agents has been slower than for the G agents, due both to the lack of a suitable surrogate molecule that would mimic the methylphosphonyl(*N*,*N*-dialkylamino ethylthiolate) moiety of V-type agents, and to the fact that under current screening conditions the starting point, wild-type (wt) rePON1, does not hydrolyze the V-type surrogate, amiton, [$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$], at a measurable rate, certainly not at a level detectable in

crude bacterial lysates. However, variants in recent libraries (VXR4 and VXR5), screened directly with VX, displayed k_{cat}/K_M values of ~$1x10^5$ $M^{-1}min^{-1}$, and could be used as lead mutants for advanced directed evolution of antidotes against VX and several VX analogs, such as VR.

Finally, we have preliminary data concerning the influence of the association of wt rePON1 with HDL on both the biological lifetime and the catalytic activity of wt rePON1. *In vivo*, we have seen that removing the HDL-anchoring helix results in very rapid clearance of the enzyme. It is thus imperative to monitor the effect of HDL association on the circulatory stability and proficiency of mutant rePON1s, so as to ascertain that the evolved variants maintain their capacity to bind to HDL, and to perform detoxification *in vivo* as anticipated on the basis of their catalytic proficiency *in vitro*.

In view of the above, our recent efforts under the mission of the center have been directed at improving k_{cat}/K_m values for rePON1 variants towards VX, and understanding the molecular parameters that govern the association of PON1 with HDL.

Body

I. Specific Aims

The overall specific aims of the project research at the Weizmann Institute are:

1. Development of high-throughput assays for OP hydrolase variants exhibiting high specificity factors and turnover.

2. Provision of proof-of-concept for the proposed core technology employing directed evolution of new recombinant PON and AChE variants.

3. Isolation of interceptors for G- and V-type nerve agents, and expression in soluble form.

4. Design, generation and selection of libraries for V- and G-type agents.

5. Large-scale production of selected enzyme candidates, and their kinetic, structural and pharmacological evaluation

6. Establishment of "off-the-shelf" libraries for rapid identification of antidotes against emerging future threats

Specific topics explored during the reported period are:

1. Generation of libraries VXR6 and VXR7 and their screening with VX

2. Validation of the hypothesis concerning the structural motifs in PON1 that are involved in association with HDL, and identification of specific residues.

II. Year 06 milestones (of the renewal NIH project):

The Center milestones for year 06 that reflect on our effort at WIS are:

Milestone #1. Identify modifications or formulations of proteins that increase the circulatory stability of two candidate anti-OP catalytic enzymes in guinea pigs.

Milestone #2. Identify one or more catalytic enzymes that, when administered together with a single dose of conventional therapeutic drugs (atropine and 2-PAM) at the onset of signs of OP poisoning, affords enhanced protection relative to treatment with a single dose of conventional drugs alone. Protection must be afforded against parathion and at least one G or V nerve agent, with each compound administered at a minimum of twice the established median lethal dose (2 x LD_{50}).

The tasks to be performed at the Weizmann Institute to achieve these two major goals of the Center are outlined below:

#1: Design, generate and screen libraries VXG6 and VXR7

In our 06 year progress report (18/6/2012) we described the rationale underlying the construction and generation of 5 rounds of libraries directed towards hydrolysis of VX. Briefly, the results obtained demonstrate the level of specific selection with VX, as well as the sensitivity of the proficiency of the PON1 variants to modifications in the *O*-alkyl and *N*,*N*-dialkyl substituents of the V-type nerve agents. The encouraging results with the 1-4-B9 of library VXG4 prompted the construction of a newly designed VXG5 library, which provided an even more potent rePON1 compared to 1-4-B9, namely, 5-2-G7, that hydrolyzes VX at ~2.5-3.0 x10^5 $M^{-1}min^{-1}$.

Sub-libraries of variants of the 6th generation, VXG6, were constructed based on the 9 most catalytically efficient variants evolved in round VXG5 of directed evolution for V-agent hydrolysis. In addition, these were shuffled with oligonucleotides encoding for particular site-specific mutations. Specifically, we targeted residues 72, 189, 192, 193, 196 and 292, which our docking model of VX with PON1 implicated in substrate binding, and especially in leaving-group binding. In addition, we targeted residues 69, 71, 73, 74, 115, 134, 222, 291 and 346. We also decided to generate a mutant library based only on the putative leaving-group-binding residues. We screened 1200 clones from this library (VXG6-3), and initially identified 50 improved clones. Following verification assays, we identified 8 clones that had improved up to

3-fold in VX hydrolysis as compared to the best variants in the previous round. However, this phenomenon was restricted to assays of the bacterial cell lysates, and upon purification we found no improvement relative to the previous round, VXG5. MD simulations suggested that the side-chains of novel mutations incorporated in this round (P72E, S193Y) tend to point outside the active site into the solvent. We thus consider it likely that these mutations enhance solubility by increasing the protein's surface charge, thus increasing the apparent activity of these variants in cell lysates, but not that of the purified variants. The best scores of round 6 are summarized in **Table 1**. **Table 2** summarizes the catalytic activity of the best variants in the VXG4, VXG5, and VXG6 libraries. It seems that the size of the *O*-alkyl moiety is one of the factors that affects the rate of OP hydrolysis.

Since there was no improvement in rounds VXG5, and VXG6, we embarked on the design of the 7th round library, VXG7. Before describing the design of the 7th library it is important to mention briefly the "thio effect" that poses a kinetic barrier to the hydrolysis of V-type nerve agents by PON1s, as well as by bacterial PTEs. It is likely that this effect originates from the combined effects of the large size of the S atom (relative to oxygen), reduction in the electropositivity of the P atom, poor proton acceptor capacity of the sulfur atom, and the ability to assume numerous P-S-alkyl bond angles that may destabilize the projection of the leaving group in a favorable position for productive encounter with the attacking water molecule. In order to identify trends that might decrease the "thio effect", we developed a protocol for assessing the "thio-effect" in V-type agents, based on comparison of the enzymatic hydrolysis of V-agent analogs bearing either a thio- or an oxo-based leaving group with a similar pK_a (**Fig. 1**). Thus, we assayed the best variants from libraries VXG5 and VXG6 using the thiophenyl and coumarin analogues of VX, which have an identical pK_a of 6.5. The results (**Tables 3, 4**) show, on average, a reduction in the ratio of O/S enzymatic activity in round 6 relative to round 5. This indicates a trend that may slightly overcome the "thio effect".

In an attempt to further reduce the "thio-effect" in PON1 variants and to improve their ability to hydrolyze V-agents, in particular VX, we generated a new library of site-specific targeted mutants (VXG7).

The 7th round PON1-libraries of variants, for V-type hydrolysis, were generated by targeting mutagenesis to residues that flank both sides of essential active-site residues (E53,

W115, N168, M222, N224, D269, S332), which are involved in catalysis and with which the catalytic Ca^{+2} interacts. The underlying rationale was that changes in the closest neighbors of residues essential for catalysis may help to overcome steric, geometric and electronic constraints that reduce catalytic efficiency in the current variants. To improve screening coverage, we restricted substitutions to only two residues at a time and substituted each residue with amino acids that could be found at that same position in one of the PON family members (**Table 5, Figure 2)**. Thus, we generated and screened 8 separate variant libraries that were obtained by shuffling the eight best clones from round 6 and the best two from round 5 together with diversifying oligonucleotides. We screened 200 random colonies from each library (1600 total). Initially, we identified 105 improved variants. Following verification, 24 variants were found to be improved by ~2-6 fold over controls. We sequenced the 12 best variants, purified and characterized the 7 most improved variants (**Table 6**). Of the 13 positions targeted for mutagenesis, only 3 appeared mutated in improved variants (**Table 6**), however, two mutations (D169N and I117M) were found in the most improved variants 167-mix1-F5 and 114-2-C1 and one mutation (S52A) appeared in two different clones (**Table 6**). While the rates of hydrolysis of the two VX isomers were similar or identical in most clones, they were different by about 7 fold in favor of the fast isomer in variant 268-2-E8. Overall, the improvements of selected clones in catalytic efficiencies towards VX were modest (i.e. 1.5-2 fold with respect to variant 2-G7). However, most variants from this round displayed a reduced "thio-effect" relative to variants from previous rounds (**Table 7, Figure 3**). This indicates that the selection pressure aimed at protecting AChE from inhibition by VX in our assay, may eventually direct the enzyme to efficiently hydrolyze the P-S bond of V-agents. Finally, the most active variants found (167-mix1-F5 and 268-2-E8), were sent (late March 2013) to OSU for large-scale production intended for future *in-vivo* experiments at ICD.

#2. Studies on the association of rePON1 with endogenous HDL

In order to increase the stability of PON1s in the circulation we examined the influence of its known capacity to interact with HDL, which is accompanied by an in increase in the catalytic activity of the PON1. Our suggested model is shown below in **Figure 4,** and we aimed to validate it.

The structural data on rePON1 in both its apo form and in complex with the lactone analog, 2-hydroxyquinoline, assigned a major role for helixes H1 and H2 in the binding to HDL. Furthermore, it revealed an H-bond interaction network that starts at the connecting loop of helix H2 and ends in the active site, including interaction with the flexible active-site loop. These interactions may explain the stimulation of PON1 activity observed upon its association with HDL. In order to test the importance of the individual residues we developed a cell-free system that would permit the synthesis of rePON1 and its various mutants in the presence of HDL **(Fig. 5).** The preliminary conclusions from this study are shown below in **Fig. 5**, and highlight the initial findings of involvement of Y185 and F186 in the H2 connecting loop. This approach results in tighter and more catalytically effective association of rePON1 with HDL. Another advantage of this approach is that we need only the mutant DNA (no protein purification is needed), so that it can be used for analysis of many mutants in parallel. We also noted that residue Y71 of the flexible loop, which shows high mobility upon binding of 2HQ, is likely to be a key residue in stimulation of the lipo-lactonase activity of rePON1. Two mutants were examined, Y71F and Y71G, for both of which the mutation produced a ~5-fold decrease in lactonase activity relative to WT rePON1. However, the HDL-induced stimulation of activity decreased significantly, supporting our hypothesis regarding the role of Y71 in stimulation. Currently, we are testing additional mutants for changes in the level of stimulation of activity by HDL.

A set of mutations examined the interaction of helix H2's connecting loop residues (**Fig. 6A**). We prepared the following mutants: Y185A, F186A and Y185A/F186A. Both activity stimulation and the inactivation assays indicated that residues Y185 and F186 have major role in the activity stimulation, especially F186 that shows a significant decrease in the activity stimulation with TBBL (**Fig. 7 and Table 8**). On the other hand, F185 shows comparable activity stimulation and improved stabilization to the WT. However, the double mutant showed the most significant effect with both the activity stimulation and the inactivation (**Fig. 7 and Table 8**).

The second set of mutants aimed to examine the interaction of helix H2 with rHDL (**Fig. 6B**). We produced the following mutants: Y190A, W194A, Y190A/W194A, L198W and L198Y. The Ala mutants show significant decrease in their activity stimulation, with the double mutant show almost no activity stimulation (**Fig. 7 and Table 8**). These results are in-line with the

suggested model of the interaction between PON1 and HDL. The L198 mutants aimed to obtain improvement in both stabilization and activity stimulation. Unlike the L198Y that show decrease in both stabilization and activity stimulation, for the L198W mutant the activity stimulation is improved compared to WT (**Fig. 7 and Table 8**). This suggests that L198 might play a role in the binding to the HDL and that further mutational analysis in this position is needed (potential mutants might include L198F, L198I and L198A). Currently, we are designing mutants based on key positions on helix H1, that are aimed at increasing significantly the stability of the PON1/HDL complex to allow longer half-life time in blood.

We are also developing assays to detect changes in affinity of rePON1 for HDL.

Key Research Accomplishments

- Design and characterization of the best variants of library VXG6
- Design and generation of library VXG7
- Quantitative protocol to measure the "thio effect"
- Validation of the model for association of PON1 with HDL, and elucidation of the factors involved in the stimulation of PON1 activity produced by this association
- Development of a cell-free expression system for generation of the PON1/HDL complexes
- Identification of PON1 residues at H1and H2 that are associated with increased stability and enhancement of the catalytic activity.

Conclusions

The effort to generate VX-hydrolyzing rePON1 variants should be continued in order to increase k_{cat}/K_m values to the levels required to qualify a PON1 variant as a VX scavenger. Data from measurements of k_{cat}/K_m and the "thio effect" will be utilized to improve the docking models of V agents into the PON1 scaffold. The validation of the PON1-HDL association model at the molecular level, and the identification of residues on H1 and H2 that are involved in association and stimulation of PON1 activity will be used in future studies to improve both stability in the circulation and the enhancement of catalytic activity.

Appendices

Table 1. Kinetic parameters of the most improved variants for VX hydrolysis in library VXG6-3.

Amino Acid	Rinkoo's PON1a	3.4 6bG12	3.1 617F7	2.6 637C1	1.9 617F4	1.7 610H5	1.6 633A6	1.3 637G1	1.3 636A3	
49	D	D	D	D	D	E	D	D	D	
55	L	I	V	L	I	I	I	V	I	
67	S	T	S	S	T	T	T	S	T	
69	L	V	V	V	V	V	V	V	V	
70	K	N	N	N	N	N	N	N	N	
71	Y	M	M	M	M	M	M	M	M	
72	P	E	P	P	E	P	E	P	P	←
97	V	A	A	A	A	A	A	A	A	
106	T	T	T	T	T	T	A	T	T	
111	S	T	S	T	S	S	T	S	T	
115	H	W	W	W	W	W	W	W	W	
134	H	R	R	R	R	R	R	R	R	
137	S	S	H	S	S	S	S	S	S	
166	S	S	N	A	S	A	N	N	N	
192	K	Q	Q	Q	Q	Q	Q	Q	Q	
193	S	Y	S	S	S	S	S	S	S	←
222	F	M	M	M	M	M	M	M	M	
240	L	L	L	M	L	M	M	L	L	
293	Y	F	F	F	F	Y	F	F	F	
332	T	S	S	S	S	S	S	S	S	
		15	13	13	13	14	17	12	14	
	purified	*	*	*	*	*				
DTNB assay	kcat/KM [M-1min-1]	2.06E+05	8.10E+04	1.17E+05	1.55E+05	1.43E+05	S.D (0.07-0.24) ^5			
DTNB assay -(slow)	kcat/KM [M-1min-1]	-	-	1.50E+04	-	2.10E+04	S.D (0.2-0.3) ^4			
Enzymatic assay	kcat/KM [M-1min-1]	2.54E+05	2.08E+05	ND	ND	ND	S.D (0.17-0.21) ^5			

* Variants that were purified and characterized kinetically using either the DTNB or the enzymatic AChE assay for VX hydrolysis.

** Arrows point to new residues that were incorporated in the current round of directed evolution.

Table 2. The catalytic activities of the best variants from rounds 4, 5 and 6 towards various OP substrates.

k_{cat}/K_m, x10^6 $M^{-1}min^{-1}$

Round	Variant	VX	GF	GB	EMP(fast)	EMP (slow)
VXR4	1-4B9	0.15±0.028	0.87±0.18	0.71±0.025	ND	ND
VXR5	2G7	0.29±0.055	0.47±0.04	0.39±0.03	138±22	15±0.7
VXR6	b-G12	0.25±0.21	0.97±0.07	0.48±0.09	70.1±0.1	7.8±0.2

EMP is the VX surrogate, $CH_3P(O)(OEt)$-O-coumarin, and the fast and slow components are attributed to the presence of the two enantiomers in the racemic EMP.

Table 3: Measurements of the "thio-effect" on enzymatic activity for the best rePON1 variants from round 5 (VXG5).

Variant	$CH_3P(O)OEt$-O-coumarin		$CH_3P(O)OEt$-S-phenyl		O/S	
	fast	slow	fast	slow	fast	slow
2G7	13.8±2.2	1.5±0.07	0.026±0.0005	0.0068±0.0002	530	220
2C5	16.9±0.14	1.3±0.02	0.0047±0.0018 0.0036±0.0001	0.0028±0.0009 0.0036±0.0001	3595 4690	464 361
1B9	6.3±0.78	0.71±0.09	0.032±0.004	0.0058±0.001	197	122
1G6	5.91±0.97	0.97±0.03	0.020±0.0014	0.0062±0.0001	295	156
8C4	5.70±0.56	1.37±0.02	0.021±0.0013	0.0064±0.0002	271	214

The fast and slow isomers were characterized by fitting the experimental data to a bi-exponential association equation, and reflect the 1:1 distribution of the S and R enantiomers.

Table 4. Measurements of the "thio-effect" on enzymatic activity for the best rePON1 variants from round 6 (VXG6).

Variant	$CH_3P(O)OEt$-O-coumarin		$CH_3P(O)OEt$-S-phenyl		O/S	
	fast	slow	fast	slow	fast	slow
bG12	7.07±0.1	0.78±0.02	0.0063±0.0035	0.00275±0.0015	1120	283
7F7	18.7±6.0	1.31±0.1	0.0089±0.0017	0.00635±0.003	2101	208
10H5	4.75±1.8	1.28±0.06	0.0225±0.0007	0.005±0.0007	211	256
7F4	5.60±0.28	0.64±0.05	0.0056±0.0003	0.00415±0.0023	1000	154
7C1	3.9±0.02	1.04±0.03	0.0186±0.0037	0.00515±0.0006	210	202

The fast and slow isomers were characterized by fitting the experimental data to a bi-exponential association equation, and reflect the 1:1 distribution of the S and R enantiomers.

Table 5. Residues used for site-directed targeted mutagenesis in VXG7 libraries.

Library	residue	PON family alignment
1	S52	A,T,P
2	P114	P,G,S,A
	I117	I,L,M
3	V167	V,L,I,M,C,A
	D169	D,G,N,Q,A
4	D221	D,A,T,S,L,Y,H,N,C,V
5	A223	A,P,G,N,Q,T
	G225	G,S,C,M,A
6	V268	V,P,L,A,C,Q
	N270	N,K,Q
7	S331	S,V,A,T,I
8	S332	S,I,L,V,F,T,A,Y,C,Q
	V333	V,I,N,S,T,A,C

* Substituting residues in red were added because of similar physical and chemical properties.

Table 6. Sequences and catalytic efficiencies of improved variants from round 7.

rePON1	Amino Acid	rePON1	167-mix1-F5	114-2-C1	223-1-C3	268-2-E8	52-2-G6	52-2-H12	52-1-H6
GAC	49	D	D	D	D	D	E	D	D
TCT	52	S	S	S	S	S	A	S	A
TTG	55	L	I	I	I	I	I	I	V
TCC	67	S	T	T	T	T	S	T	T
TTA	69	L	V	V	V	V	V	V	V
AAG	70	K	N	N	N	N	N	N	N
TAT	71	Y	M	M	M	M	M	M	M
CCT	72	P	E	E	E	E	E	E	E
GTG	97	V	A	A	A	A	A	A	A
ACA	106	T	T	T	A	A	A	A	A
TCA	111	S	T	T	T	T	T	T	T
CAT	115	H	W	W	W	W	W	W	W
ATT	117	I	I	M	I	I	I	I	I
CAT	134	H	R	R	R	R	R	R	R
AGT	166	S	S	A	A	N	N	N	S
GAC	169	D	N	D	D	D	D	D	D
AAA	192	K	Q	Q	Q	Q	Q	Q	Q
TTT	222	F	M	M	M	M	M	M	M
TTG	240	L	L	L	L	M	L	M	L
TAC	293	Y	F	F	F	F	F	F	F
ACG	332	T	S	S	S	S	S	S	S
			15	16	16	17	17	17	16
	kcat/KM [min-1M-1]	Fast DTNB	5.0±1.1x10^5	4.75±0.21x10^5	4.4±0.28x10^5	5.6±0.56x10^5	2.4±0.4x10^5	1.2±0.2x10^5	0.3±0.06x10^5
	for VX with DTNB	Slow DTNB	3.45±1.06x10^5	2.85±0.49x10^5	2.4±0.7x10^5	0.76±0.06x10^5	2.4±0.4x10^5	1.2±0.2x10^5	0.3±0.06x10^5

* Fast DTNB isomer = Toxic S_p-VX isomer.

Table 7. The "thio-effect" in selected round 7 clones

Variant	$CH_3P(O)OEt$-O-coumarin		$CH_3P(O)OEt$-S-phenyl		O/S	
	fast	slow	fast	slow	fast	slow
167-X1-A6	0.61±0.011	0.18±0.033	0.016±0.0005	ND	38	
167-X1-F5	5.0±0.51	2.48±0.29	0.043±0.014	0.030±0.005	116	83
268-2-E8	17.5±2.73	1.43±0.032	0.032±0.001	0.0058±0.0006	546	246
223-1-C3	5.60±0.14	0.73±0.051	0.022±0.0006	0.0102±0.005	254	72
114-2-C1	7.63±0.37	0.91±0.059	0.028±0.0015	0.014±0.0016	272	65

Table 8: Lipo-lactonase activity stimulation (TBBL) of WT rePON1 and its mutants with various rHDL concentrations.

Variant	K_{app}(μM)	V_{max} (%)[a]
WT	0.58±0.07	406±11
Y185A	0.68±0.1	484±19
F186A	2.4±0.32	331±15
Y185A/F186A	2.9±0.7	183±17
Y190A	1.3±0.3	268±17
W194A	0.46±0.13	188±10
Y190A/W194A	3±1	152±18
L198W	0.63±0.09	457±15
L198Y	1.3±0.2	359±13

Relative to absence of HDL

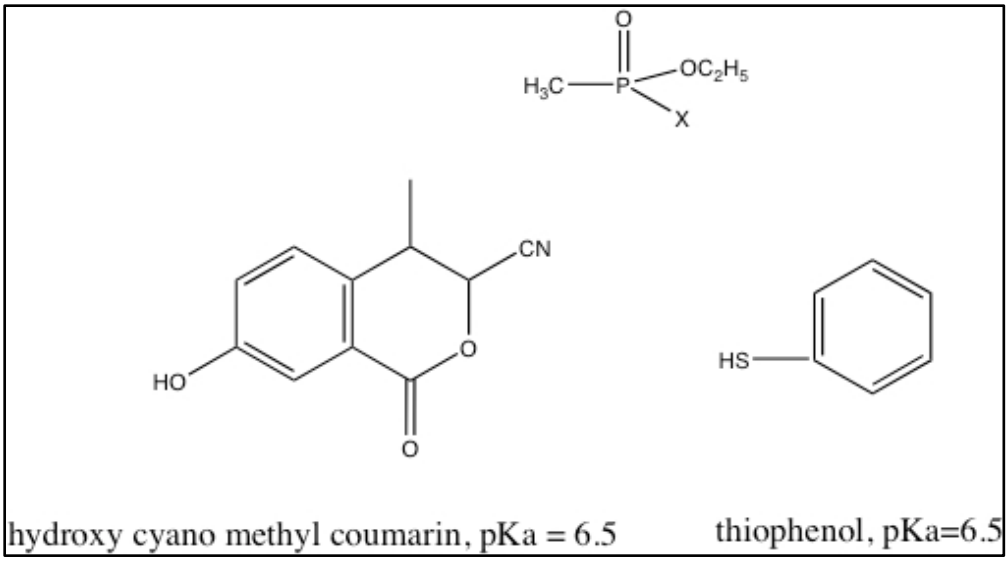

Figure 1. Structures of the thio and oxo analogues of VX that were synthesized.
The coumarin and thiophenyl analogs of VX were synthesized and used as substrates to examine the effects of a thiol-based leaving group vs. an oxo-based leaving group on enzymatic hydrolysis (see also Tables 3, 4). The two leaving groups have the same pKa.

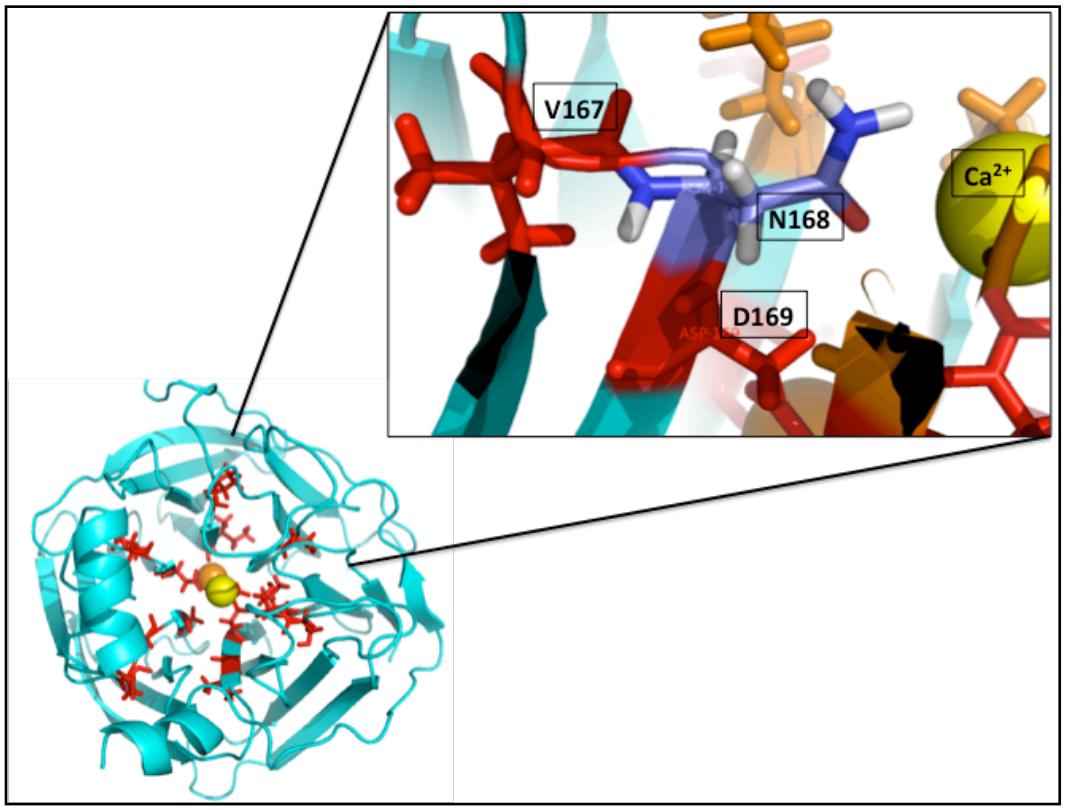

Figure 2. Positions targeted for substitution in VXG7 libraries. Bottom left, the structure of PON1 in cyan, the structural calcium atom is highlighted as an orange sphere and the catalytic calcium as a yellow one. Red colored residues were targeted for substitution in one of the 8 libraries tested in this round of directed evolution. **Top right**, a magnification of the residues flanking the active site residue N168. These were targeted for directed substitution in library #3 of VXG7.

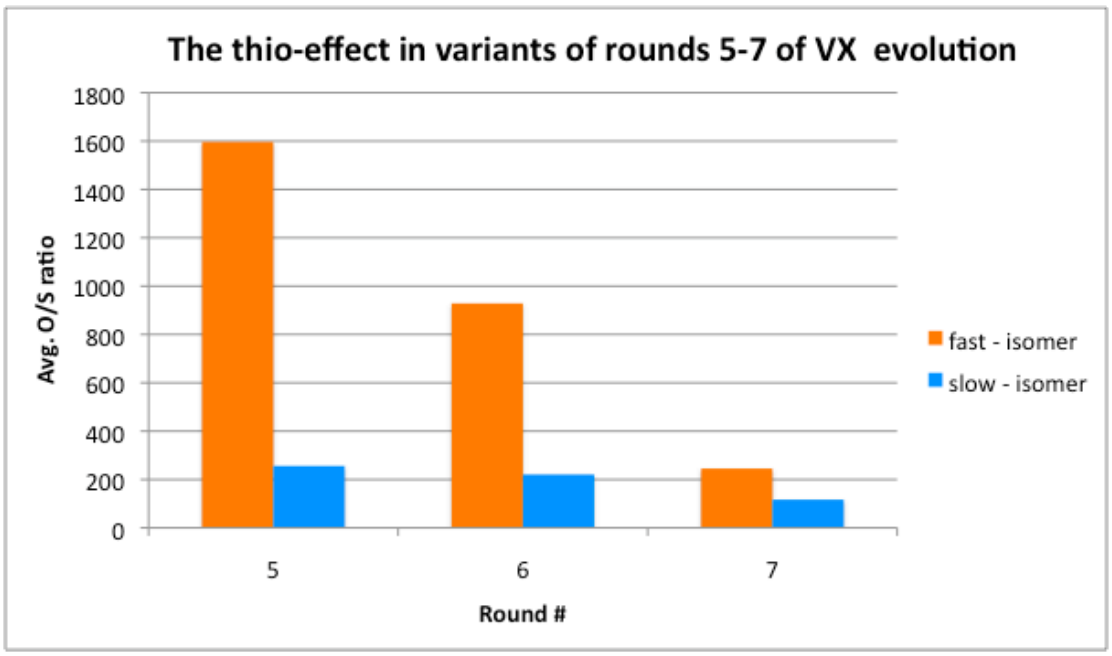

Figure 3. The average "thio-effect" of improved variants from round 5-7.
The average "Thio-effect" (i.e. the ratio of a variant's catalytic efficiency with EMP-coumarin versus it's efficiency with EMP-S-phenyl), for the best variants purified from rounds 5, 6 and 7 is displayed for both their fast and slow isomers.

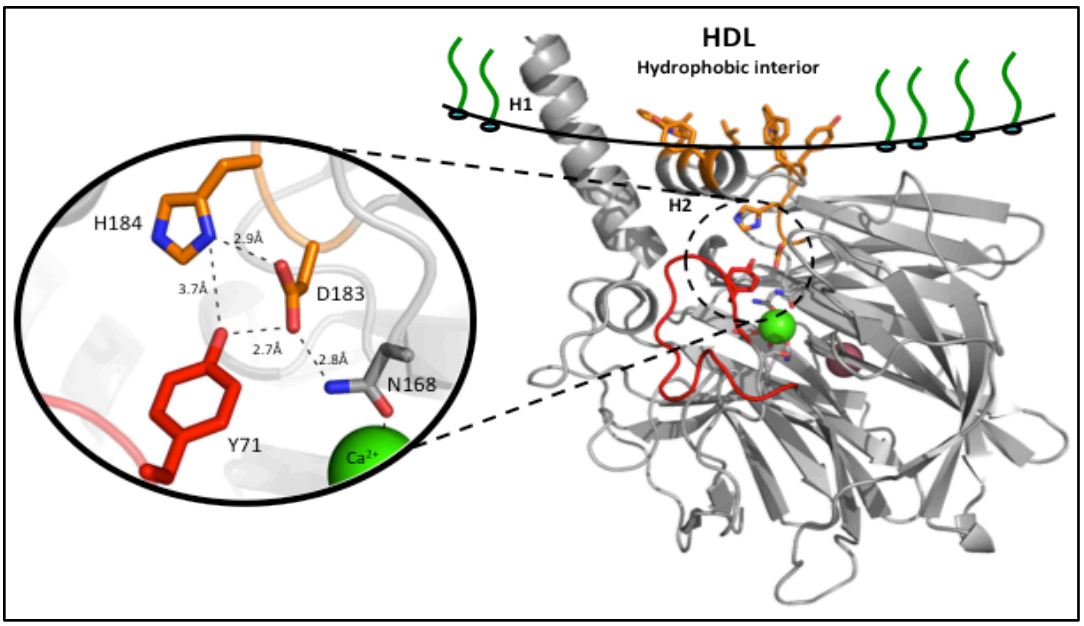

Figure 4: We hypothesize that the H2 helix and its connecting loops, the H1 helix, and the flexible loop residues, are together responsible for the structural changes that result in stimulation of activity when the PON1 is bound to HDL.

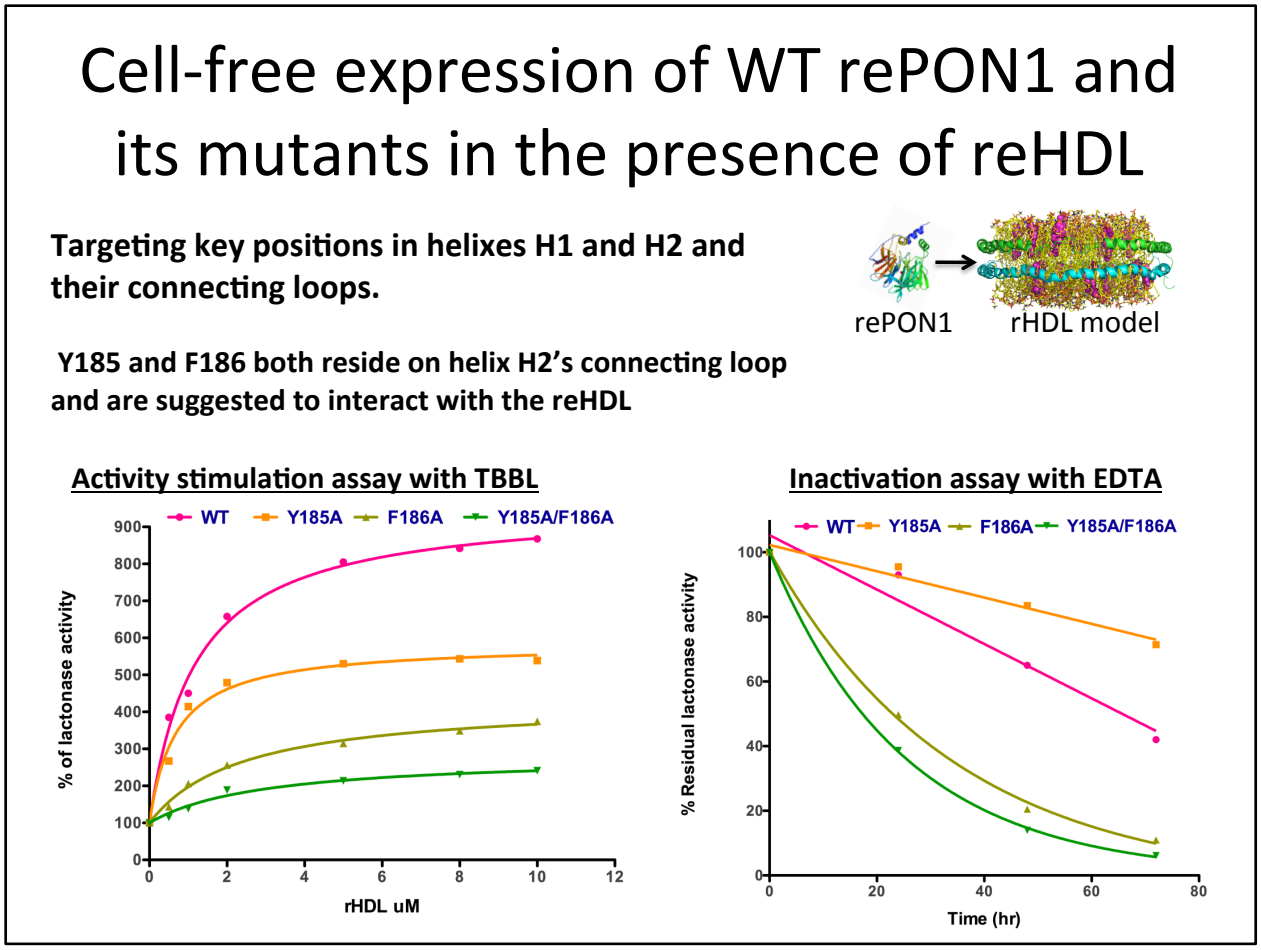

Figure 5: Initial data from the cell-free expression of PON1 mutants in presence of HDL. Activity stimulation is measured with the substrate TBBL and stability is monitored by thermal inactivation in presence of EDTA (for more details see Fig 8 below)

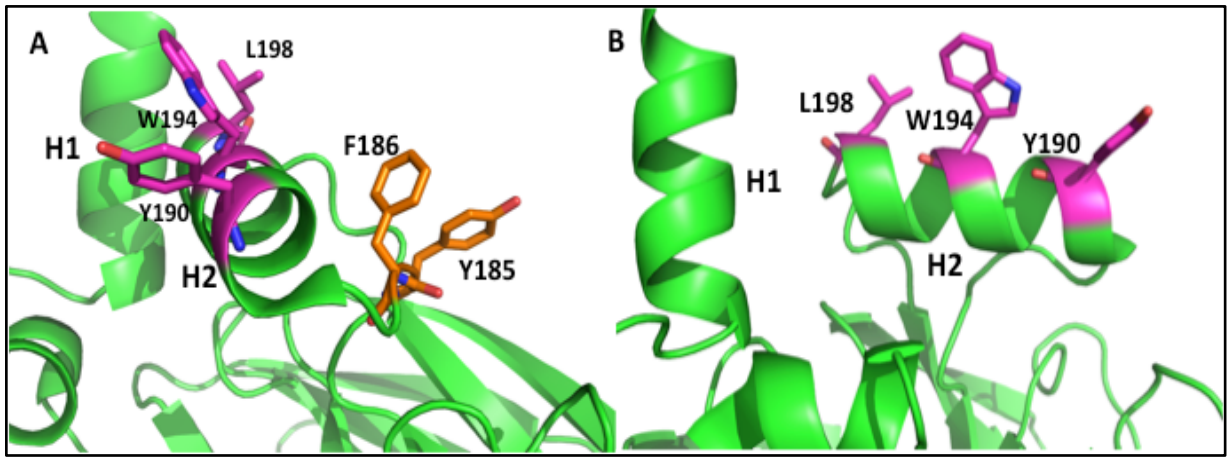

Figure 6: The selected residues that were subjected to mutagenesis that reside on helix H2 and its connecting loop.

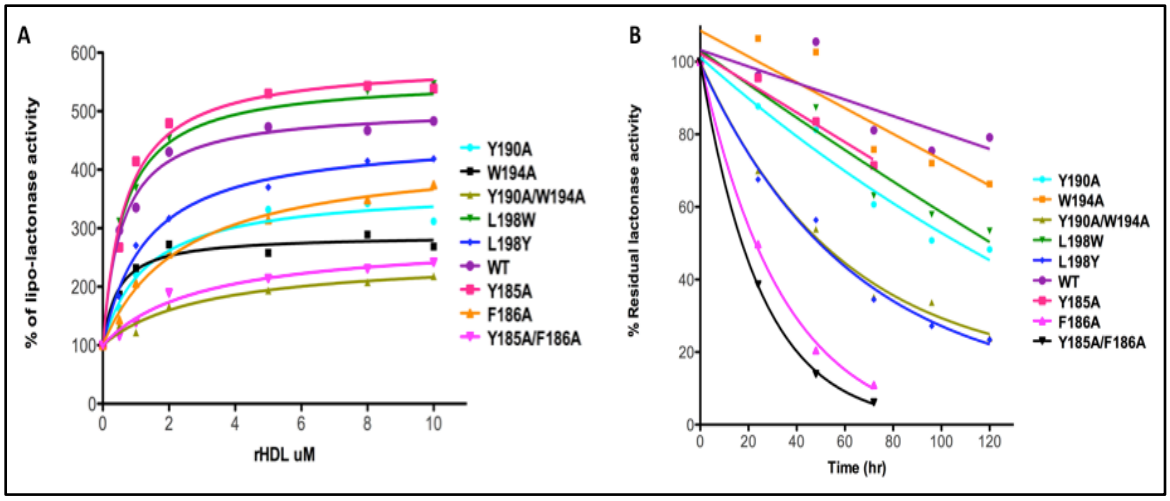

Figure 7: (A) lipo-lactonase activity stimulation analysis (TBBL 0.25mM) of WT and it mutants in the presence of various rHDL concentrations (rHDL 0-10µM; TBBL 0.5mM). (B) Inactivation (15mM EDTA and incubation at 37°c) analysis of WT and its mutants in the presence of 10µM rHDL.