Biology Evolves to Fight Chemistry

Romas Kazlauskas[1,*]
[1]University of Minnesota, Department of Biochemistry, Molecular Biology and Biophysics and The Biotechnology Institute, 1479 Gortner Avenue, Saint Paul, MN 55108, USA
*Correspondence: rjk@umn.edu
DOI 10.1016/j.chembiol.2012.04.004

A human enzyme variant, PON1-G3C9, accidentally catalyzes the hydrolysis of organophosphorus chemical weapons. In this issue of *Chemistry & Biology*, Goldsmith and coworkers describe a new PON1 variant with improved hydrolysis by several hundred fold; enough that it may protect animals from a toxic dose.

Insects have evolved resistance to organophosphorus insecticides through evolution, which involved the death of countless insects (e.g., Newcomb et al., 1997). Organophosphorus insecticides inactivate acetyl cholinesterase (AcChE), a key enzyme in nerve function. Nerve agents (Figure 1) are similar organophosphorus compounds that are highly toxic to humans and have been manufactured as chemical weapons. In 1995, a terrorist group killed thirteen people and injured fifty by releasing the nerve agent sarin (or GB) in the Tokyo subway. Although in principle humans could evolve resistance to these nerve agents, the entire process would lead to countless deaths and is absolutely unacceptable in myriad ways. Therefore, there is a need for alternative strategies to address this issue, and one of the acceptable alternatives is the laboratory evolution of a human enzyme that can detoxify these compounds. The work of Goldsmith et al. (2012),discussed in this issue *of Chemistry & Biology*, is an important step toward this goal.

G. Schrader at IG Farben discovered the first series (now called the G-series; Figure 1) of organophosphorus chemical warfare agents during insecticide research starting in 1936. Toxins in this series are volatile and react quickly with water because the phosphorus contains a good leaving group (usually fluoride). Because they hydrolyze in the environment, they are non persistent. R. Ghosh at Imperial Chemical Industries in England discovered the second series (V-series) starting in 1954, also while researching insecticides. These compounds are more toxic, but also less volatile, making skin contact the primary hazard. The substituted aminoethanethiol leaving group makes them less reactive with water and therefore they persist in the environment.

The organophosphorus toxins react quickly with the active site serine of AcChE to form a covalent link, but the cleavage of this adduct is slow leading to an inactivated enzyme. The tetrahedral geometry of the phosphonyl serine adduct (as compared to the trigonal geometry of the acetyl enzyme intermediate) sterically hinders the attack of water and orients the leaving group in a non-optimal orientation.

Two current defenses against nerve agents use a stoichiometric approach using chemicals. Noncovalent AcChE inhibitors (e.g., pyridostigmine bromide) block the access of nerve agents to the active site. Strong nucleophiles, like oximes, react with phosphonyl-AcChE complex to cleave the phosphonyl group and reactivate the enzyme. These approaches still involve inhibition of the AcChE, which causes serious side effects (review Doctor and Saxena, 2005).

A third approach uses a sacrificial enzyme, human butyryl cholinesterase (HuBChE), to react with the nerve agent instead of AcChE. By combining this approach with a strong nucleophile, the HuBChE can inactivate more than one molecule of nerve agent, but this approach still requires a large, ~200 mg, dose of protein (Lenza et al., 2007).

Insect resistance to organophosphorus insecticides uses a catalytic approach where one enzyme detoxifies many toxin molecules (Marino, 2007). A similar approach may be possible for providing humans with protection strategy. Several phosphotriesterases, also known as organophosphorus acid anhydride hydrolases, have been reported including a two-zinc metalloenzyme (PTE, likely a lactonase) from a soil bacterium, a two-manganese metalloenzyme (likely a proline peptidase) from a halophilic pond bacterium, and two two-calcium metalloenzymes, PON1, found in liver and blood (likely a lactonase) from human serum, and diisopropyl-fluorophosphatase from squid. In each case, the biological role of the enzyme is something else, and the organophosphorus activity is a promiscuous or accidental ability.

The organophosphorus toxins are racemic mixtures where the enantiomers differ in configuration at phosphorus. The enantiomer with the S_P configuration is more toxic, so this enantiomer is the target for any detoxification, but enantioselectivity

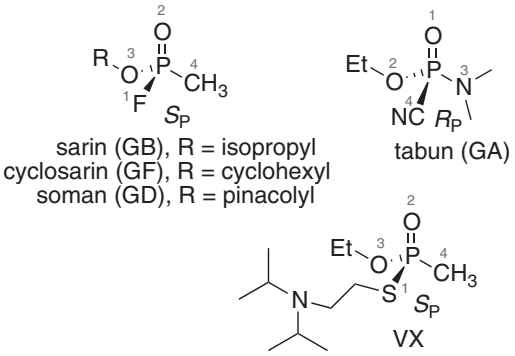

Figure 1. Structures of several G-series organophosphorus toxins as well as the structure of VX
The toxins are racemic mixtures, but the enantiomer shown is the more toxic one (S_P; tabun is an exception due to a different priority of the substituents). The ideal detoxifying enzyme should be stable, favor hydrolysis of this more toxic enantiomer and accept all of these substrates. The numbering ranks the substituents to assign the configuration of the phosphorus stereocenter.

should not be perfect because the less toxic enantiomer also needs to be degraded. Unfortunately, the four organophosphorus hydrolases all favor hydrolysis of the less toxic R_P enantiomers (e.g., Harvey et al., 2005). Tsai et al. (2010) previously reversed the enantioselectivity of PTE toward the G-series nerve agents and nerve agent analogs by rational engineering of the active site. The enantioselectivity changed 80,000-fold from 760-fold in favor of the less toxic enantiomer to 110-fold in favor of the more toxic enantiomer. The reaction rate for sarin (GB) with the engineered enzyme was fast (k_{cat}/K_M = 1.4 × 10^4 $M^{-1}s^{-1}$). Two limitations of PTE as a drug are poor stability and that it is a bacterial protein, which will likely cause an immune response if injected into the bloodstream. Melzer et al. (2009) also engineered the squid enzyme to reverse the enantioselectivity to favor the more toxic enantiomer and achieved an impressive rate of k_{cat}/K_M = 2.3 × 10^5 $M^{-1}s^{-1}$ for sarin (GB) and 4.9 × 10^5 $M^{-1}s^{-1}$ for cyclosarin (GF). These values correspond to a 5.5- and 29-fold increase over wild-type.

To minimize the problem of immune response, Goldsmith et al. (2012) focused on the human enzyme PON1. This enzyme is also unstable, but recombination of homologous enzymes from rabbit, human, mouse, and rat yielded variant re-PON1-G3C9, which is more stable and more easily expressed in *Escherichia coli* (Aharoni et al., 2004). This variant is closest to rabbit enzyme (95% amino acid sequence identity) and is 86% identical with the human enzyme. Presumably, this close relationship to the human enzyme will minimize any immune response, but this hypothesis has not been tested. The two limitations of the enzyme are slow rate [k_{cat}/K_M = 2 × 10^3 $M^{-1}s^{-1}$ toward (S_P)-cyclosarin (GF); the units here are in $M^{-1}s^{-1}$ for easy comparison to other work; the paper lists values in $M^{-1}min^{-1}$] and preference for the less toxic enantiomer.

In an earlier work, Gupta et al. (2011) used directed evolution to increase the rate of hydrolysis 135-fold toward the more toxic enantiomer (S_P)-cyclosarin reaching an impressive value of k_{cat}/K_M = 3 × 10^5 $M^{-1}s^{-1}$. The directed evolution involved screening toward a less toxic and more easily monitored analog (a coumaryl leaving group in place of fluoride). The reaction rate toward the S_P enantiomer of this analog increased by an impressive factor of ~10^5, but this increase toward the true substrate, cyclosarin, was a more modest 135-fold. This improved variant could protect mice from a lethal dose of the coumaryl analog, but the protection against cyclosarin was not tested. The evolved variant 2D8 has five amino acid substitutions all in the substrate-binding site.

Now, Goldsmith et al. (2012) use directed evolution to expand the substrate range to three other G-series organophosphorus toxins and even includes the slow, but detectable, hydrolysis of VX from the less reactive V series of nerve agents. The researchers first tested the variants identified previously (Tsai et al., 2010) and found that they already had a broader substrate range. The current work improved the rates further by 3- to 340-fold. These improvements also reversed the enantioselectivity to an analog of cyclosarin such that hydrolysis of the toxic enantiomer is now favored.

Using the X-ray crystal structure (Harel et al., 2004), the researchers targeted eight positions in the active site for mutagenesis. Complete randomization would create approximately 20^8 or 26 billion variants, which is far more than is possible to screen. The researchers used four different strategies to limit the number of variations, but still screened only a small fraction of possible mutants. For example, in round 1, only some amino acid substitutions were allow at the eight positions so that only ~22,000 variations were possible. Of these, they screened only 2,000, so better variants may still be found.

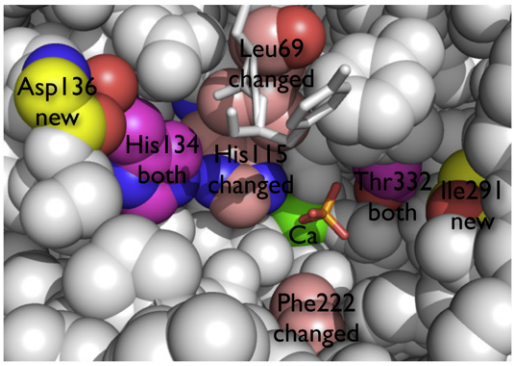

Figure 2. Active site structure of calcium-dependent phosphotriesterase PON1-G2E6, which is similar to starting variant PON1-G3C9, showing the locations of the amino acid substitutions

The final variant after four rounds of directed evolution contains eight amino acid substitutions. Two of these substitutions (labeled both) are identical to the substitutions found previously. Three of the substitutions are in new locations (labeled new; one location (Leu55) cannot be seen in this view). Three of the substitutions are different substitutions in at previously mutated locations (labeled changed). Catalysis occurs at the calcium ion shown in green, which also contains a bound phosphate anion (sticks representation). Two amino acid residues (Lys70, Tyr71) in a flexible loop are shown in sticks representation to allow a better view of the active site.

The final mutant (Figure 2) catalyzes hydrolysis of soman (GD) and cyclosarin (GF) at k_{cat}/K_M = 7 × 10^5 $M^{-1}s^{-1}$, which the researchers predict to be fast enough to detoxify them in animals. Hydrolysis of tabun (GA) and sarin (GB) are about an order of magnitude slower, but they are less toxic so this slower rate may be enough for detoxification. The variant also catalyzes the hydrolysis of VX k_{cat}/K_M = 2–4 $M^{-1}s^{-1}$, which is very slow, but may be a good starting point for further evolution.

The key features of this work are the use of a mostly human enzyme and the large, several hundred-fold increases in the achieved rate. Still left to do is to show that this enzyme can protect animals against nerve agents. The researchers showed that enzyme retains >75% activity in a blood sample for 24 h, but immune response and stability to proteases must still be tested. If successful, the availability of potent detoxifying agents would render this class of chemical weapons less effective.

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Selective Inhibition of USP7

Robert Menard[1,*] and Traian Sulea[1]
[1]Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, QC, H4P 2R2, Canada
*Correspondence: robert.menard@cnrc-nrc.gc.ca
DOI 10.1016/j.chembiol.2012.04.001

The deubiquitinating enzyme USP7 is an emerging oncology and antiviral target. Reverdy et al., in this issue of *Chemistry & Biology*, disclose the first small-molecule inhibitor selective for USP7, which recapitulates its knockdown in cancer cells and hence demonstrates the therapeutic feasibility of USP7 inhibitors.

Covalent modification of cellular proteins by ubiquitin (Ub) represents a highly versatile posttranslational mechanism that underpins proteasomal degradation and regulates a diverse array of cellular processes (Komander et al., 2009; Reyes-Turcu et al., 2009). Defects in ubiquitin-based control mechanisms have been implicated in the pathogenesis of numerous disease states including cancer, chronic diseases, and viral infections (Lindner, 2007; Singhal et al., 2008). Hence, components of the ubiquitin system emerge as attractive drug targets, reinforced by the approval of the proteasome inhibitor Velcade in oncology. Protein ubiquitination is a dynamic and reversible process controlled by the coordinated action of multiple ubiquitin-conjugating and deubiquitinating enzymes (DUBs). The human genome contains nearly 100 DUBs, which can be divided in five structural classes. USP7, which is the enzyme targeted for inhibitor discovery by Colland and coworkers (Reverdy et al., 2012), is part of the ubiquitin-specific proteases (USP) class, a group of cysteine proteases comprising approximately 60 members. Several studies have linked USP7 to diseases, particularly cancer, and the enzyme is considered a very promising target for drug discovery (Nicholson and Suresh Kumar, 2011).

A major hurdle in the discovery of inhibitors for cysteine proteases having the papain-like fold is achieving inhibitory selectivity. This is due to relatively shallow substrate-binding sites lined with residues well conserved over large families of homologous enzymes. Moreover, high-throughput screening (HTS) hits have acceptable inhibitory potency but often do not display the desired level of selectivity, requiring further optimization. Nonetheless, HTS campaigns have led to several active site-directed small-molecule USP inhibitors. A cyano-indenopyrazine derivative (HBX 41,108) was disclosed as a reversible uncompetitive low μM inhibitor of human USP7 of modest selectivity against several DUBs and other cysteine proteases (Colland et al., 2009; Reverdy et al., 2012). Another compound was found to inhibit the proteaseome-activated form of human USP14 in μM range but to inhibit IsoT (human USP5) only 20-fold weaker (Lee et al., 2010). Several structurally unrelated inhibitors of human USP1 were discovered in μM range but inhibited at least one other human USP with similar potencies (Chen et al., 2011). Other recent efforts in the field have lead to compounds with often limited assessment of selectivity. Perhaps the most successful selective inhibition of a USP was achieved for the PLpro of the SARS coronavirus. Small-molecule, non-covalent, substrate-binding-site-directed inhibitors of this viral USP were discovered in the μM range potency and with excellent selectivity profile (Ratia et al., 2008). This high selectivity was likely afforded by the relative divergence of the viral USP from human USPs both structurally and functionally. In all these cases, the inhibitors were tested against a very limited set of USPs or DUBs.

In this issue of *Chemistry & Biology*, Reverdy et al. (2012) report 9-chloro derivatives of amidotetrahydroacridine as USP7 inhibitors in the μM range, identified from an in vitro enzymatic HTS. Importantly, these compounds show good selectivity for USP7 when tested against other human USPs and DUBs. An interesting and commendable aspect is the use of an activity-based molecular probe (HAUbVS) that can label active cellular USPs, giving access to a large repertoire of USPs therefore permitting a thorough evaluation of inhibitory selectivity. The selectivity data obtained in vitro on cell lysates using HAUbVS indicate that the inhibitor (HBX