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Characterization of asymmetric fluorogenic phosphonates as probes for developing organophosphorus hydrolases with broader stereoselectivity

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ABSTRACT

Organophosphorus hydrolases (OPH) such as mammalian plama paraoxonase (PON1) detoxify asymmetric toxic organophosphorus (OP) nerve agents by preferentially hydrolyzing the less toxic P(+) optical isomer. In order to develop new OPHs with broader stereoselectivity we have prepared a series of asymmetric fluorogenic organophosphonates (Flu-OPs). Such Flu-OPs may serve as molecular probes for screening large libraries of OP hydrolases during directed evolution. Flu-OPs were prepared as methylphosphonates (MPs) diesters containing either ethyl (E), isopropyl (I), cyclohexyl (C) or pinacolyl (P) groups that are structural congeners of the nerve agents VX, sarin, cyclosarin and soman, respectively. The second ester bond was formed with fluorescent moieties that are either 3-cyano-4-methyl-7-hydroxy coumarin (MeCyC) or 1,3-dichloro-7-hydroxy 9,9-dimethyl-9*H*-acridin-2-one (DDAO). To further characterize the Flu-OPs as surrogates of their respective nerve agents, we have studied the reactivation of Flu-OP-inhibited AChE using 2-PAM and toxogonin (TOX). AChE was 90–95% inhibited by all Flu-OPs (0.36–0.9 (M) and then was reactivated by either 2-PAM or TOX. TOX caused a more rapid reactivation than 2-PAM with the following rank order; EMP > IMP > CMP. TOX was also shown to be a better reactivator than 2-PAM for AChE inhibited by the nerve agents VX and cyclosarin. PMP-AChE could not be reactivated by either TOX or 2-PAM, similarly to aging of PMP-AChE formed by inhibition with soman.

Racemic CMP-MeCyC was used for screening two new PON1 variants from a neutral library of PON1. These multiple mutation variants include replacement of active site amino acid residues. Neither mutation in these new variants appeared in PON1 variants previously discovered by directed evolution using symmetric Flu-OP. Detoxification rate of cylcosarin by these new PON1 variants was rather slow indicating the need to further screen PON1 clones using optically active Flu-OPs. Therefore, we have separated enzymatically the P(−) enantiomer of CMP-MeCyC and determined its 98% purity using chiral HPLC.

1. Introduction

Enzymatic detoxification of nerve agents such as soman and cyclosarin (GF) by systemic administration of certain OP hydrolases could markedly reduce their toxicity. It was demonstrated that bacterial phosphotriesterases (PTE) parathion hydrolase isolated from *Pseudomonas* sp. could be used successfully as prophylactic treatment for paraoxon and tabun poisoning in mice [1,2]. For instance, 15 and 22 μg of parathion hydrolase per mouse conferred a protective ratio of 3.94 and 5.65, respectively, against tabun poisoning, without any post-exposure treat-

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ment [1,2]. The high-density lipoprotein (HDL) associated paraoxonase (PON1) protects against poisoning by the OP insecticide chlorpyrifos in mice [3]. Chlorpyrifos is transformed in vivo by liver cytochrome P450 oxidation into its oxo metabolite chlorpyrifos-oxon (ChPo). ChPo is an excellent substrate of PON1 exhibiting higher conversion rate than paraoxon, as we have previously demonstrated [4]. Further, interspecies differences in PON1 activity correlate well with experimentally observed LD_{50} values of toxic OPs [5]. The generation of phosphonyl-oxime intermediate was demonstrated in vitro following reaction of oximes such as obidoxime with nerve agents such as VX and sarin [6]. The use of organophosphate acid anhydrolase (OPAA) markedly reduced the re-inhibition of AChE by phosphonyl-oxime generated by the interaction of sarin, VX and soman with obidoxime [6]. It was further demonstrated that certain synthetic phosphoryl-oximes, especially those phosphoryl-oxime conjugates that contain a 4-pyridinium aldoxime moiety, are potent inhibitors of AChE and good substrates of bacterial phosphotriesterases (PTEs) [7,8].

The relatively high catalytic efficiency of human PON1 toward sarin and soman and the possibility to re-inject it in humans renders PON1 a suitable candidate for medical countermeasure against nerve agents poisoning [9]. It was estimated that another 10-fold increase in wild type human PON1 phosphotriesterase activity would be sufficient to provide substantial in vivo protection against CW nerve agents such as sarin [10]. We have recently demonstrated relative higher in vitro activity of PON1 compared to bacterial OP hydrolase (OPH) and squid DFPase toward cyclosarin and soman [4]. The hydrolytic activity of recombinant mammalian PON1 toward cyclosarin was 7- and 9-fold higher than that of squid DFPase and *Pseudomonas diminuta* OPH, respectively (at 0.03 mg/ml enzyme, 10 μM cyclosarin, $k_{obs} = 25.4 \times 10^{-3}$, 3.8×10^{-3} and 2.7×10^{-3} min^{-1}, respectively). PON1 was also more active than DFPase and OPH in detoxifying soman, with 4-fold higher rate (at 0.03 mg/ml enzyme, 10 μM soman, $k_{obs} = 7.5 \times 10^{-3}$, 1.8×10^{-3} and 1.7×10^{-3} min^{-1}, respectively) [4]. Furthermore, it was noted that certain directly evolved PON1 variants display enhanced hydrolysis of cyclosarin, soman, DFP, chlorpyrifos-oxon (ChPo) and parathiol, the phosphorothiolate analog of parathion [4]. Detoxification of GF with PON1 single mutation variants V346A and L69V was ~10-fold faster than wt PON1 [4]. The variant V346A also exhibited 9-fold higher activity toward soman. The variant L69V exhibited 100-fold faster hydrolysis of DFP than wt PON1 [4]. The active site H115W mutant exhibited 270–380-fold enhanced hydrolysis of P—S bond in parathiol. Thus, we have identified primarily three key positions in PON1 that caused significant enhancement in OP hydrolysis: Leu69, Val346 and His115 [4]. These PON1 variants were screened and selected during enhanced evolution process by using a symmetric fluorogenic OP: DEP-CyC [11]. However, GF is a racemic mixture of its S and R optical isomers configured around the phosphorus (P) atom [P(−) and P(+) optical isomers]. Soman is the mixture of four optical isomers consisting of two pairs of diastereo-isomers having two chiral centers: one on the phosphorus atom (P) and a second on the asymmetric carbon (C) atom of the pinacolyl group [P(−)C(+), P(−)C(−), P(+)C(+) and P(+)C(−) stereo-isomers]. Benschop et al. have found that the pair of soman diastereo-isomers which are configured with the (−) symmetry on the P atom [P(−)C(+/−)] are 20–150-fold more toxic than the P(+)C(+/−) pair of diastereo-isomers [12,13]. It was also noted that *P. diminuta* OPH preferentially hydrolyzes the less toxic P(+) optical isomers of cyclosarin [14] as well as those of *p*-nitrophenol analogues of sarin and soman [15]. We have examined the stereoselectivity of enzymatic hydrolysis of cyclosarin, soman and the sarin analogue *O*-isopropyl-*O*-*p*-nitrophenyl methylphosphonate (IMP-pNP) [4]. It was demonstrated that the less toxic P(+) optical isomer of cyclosarin and IMP-pNP and the less toxic diastereo-isomers of soman (P(+)C(+), P(+)C(−)) were hydrolyzed by PON1 more rapidly than their respective P(−) isomers [4]. Since enzymatic detoxification of the more toxic P(−) stereo-isomer is significantly slower than that of its P(+) counterpart, it is of interest to search for hydrolases with reversed or broader stereoselectivity.

2. Materials and methods

2.1. Reactivation of OP-inhibited AChE

The synthesis of fluorogenic organophosphonates (Flu-OPs) shown in Fig. 1 was described previously [16]. AChE used in these experiments is recombinant human AChE, a kind gift of Dr. A. Shafferman, IIBR. The concentrations of Flu-OPs used for 95–97% inhibition of AChE were: CMP-MeCyC 0.7 μM, EMP-MeCyC 0.6 μM, IMP-MeCyC 0.5 μM, PMP-MeCyC 1 μM, CMP-DDAO 0.8 μM, EMP-DDAO 0.3 μM, IMP-DDAO 0.8 μM and PMP-DDAO 1 μM.

2-Pyridine aldoxime methchloride (2-PAM) and toxogonin dichloride (Tox) were used for the reactivation of AChE inhibited by Flu-OPs. When AChE was inhibited by Flu-OPs that contain DDAO as leaving group – Tox was used at 0.5 mM and 2-PAM at 1 mM. The reactivation of OP-AChE conjugates formed by Flu-OPs that contain MeCyC as leaving group was conducted using the following oxime concentrations: 0.5 mM of Tox and 2-PAM with CMP-MeCyC, 0.1 mM Tox and 0.5 mM 2-PAM with EMP-MeCyC and 0.5 mM Tox and 0.1 mM 2-PAM with IMP-MeCyC. No reactivation of AChE was detected for PMP-AChE at all oxime concentrations used. All experiments were preformed in 50 mM sodium phosphate with 0.1% bovine serum albumin, pH 7.5, 25 °C.

2.2. Enzymatic separation of P(−) stereo-isomer of CMP-MeCyC from its racemic mixture

The P(−) isomer of CMP-MeCyC was separated from the racemic mixture of CMP-MeCyC (2 mM) using wt PON1 (0.6 mg/ml) added for 1.5–2 h incubation (25 °C). This treatment caused 96 ± 1% hydrolysis of the P(+) isomer ($n = 6$) as evidenced by increase in absorption of released *p*-nitrophenol at 400 nm and chiral HPLC analysis. The PON1-treated solution (30 ml) was extracted three times with chloroform (15–20 ml). The combined chloroform extract was evaporated to dryness and purified by preparative TLC. The purified compound was dissolved in methanol then diluted with phosphate buffer (50 mM, pH 7.5) to give the concentrations needed for measuring the inhibi-