Chemico-Biological Interactions 203 (2013) 72–76

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

A common mechanism for resistance to oxime reactivation of acetylcholinesterase inhibited by organophosphorus compounds

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ARTICLE INFO

Article history:
Available online 12 September 2012

Keywords:
Acetylcholinesterase
Oxime
Reactivation
Organophosphorus
Structure–activity

ABSTRACT

Administration of oxime therapy is currently the standard approach used to reverse the acute toxicity of organophosphorus (OP) compounds, which is usually attributed to OP inhibition of acetylcholinesterase (AChE). Rate constants for reactivation of OP-inhibited AChE by even the best oximes, such as HI-6 and obidoxime, can vary >100-fold between OP-AChE conjugates that are easily reactivated and those that are difficult to reactivate. To gain a better understanding of this oxime specificity problem for future design of improved reactivators, we conducted a QSAR analysis for oxime reactivation of AChE inhibited by OP agents and their analogues. Our objective was to identify common mechanism(s) among OP-AChE conjugates of phosphates, phosphonates and phosphoramidates that result in resistance to oxime reactivation. Our evaluation of oxime reactivation of AChE inhibited by a sarin analogue, *O*-methyl isopropylphosphonofluoridate, or a cyclosarin analogue, *O*-methyl cyclohexylphosphonofluoridate, indicated that AChE inhibited by these analogues was at least 70-fold more difficult to reactivate than AChE inhibited by sarin or cyclosarin. In addition, AChE inhibited by an analogue of tabun (i.e., *O*-ethyl isopropylphosphonofluoridate) was nearly as resistant to reactivation as tabun-inhibited AChE. QSAR analysis of oxime reactivation of AChE inhibited by these OP compounds and others suggested that the presence of both a large substituent (i.e., ⩾the size of dimethylamine) and an alkoxy substituent in the structure of OP compounds is the common feature that results in resistance to oxime reactivation of OP-AChE conjugates whether the OP is a phosphate, phosphonate or phosphoramidate.

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1. Introduction

The acute toxicity of organophosphorus (OP) compounds is usually attributed to their inhibition of acetylcholinesterase (AChE; E.C. 3.1.1.7) [1,2]. To mitigate the toxic effects of OP compounds, oximes were developed that were capable of reactivating OP-inhibited AChE [2]. However, reactivation of OP-inhibited AChE by even the best oximes varies significantly for AChE inhibited by different OP agents [3–6]. For example, the mono-pyridinium oxime 2-PAM is effective against human AChE inhibited by VX and sarin, but it is not effective against human AChE inhibited by VR, cyclosarin or tabun [6]. Although bis-pyridinium oximes, such as HI-6 or obidoxime, provide better reactivation against human AChE inhibited by VR and cyclosarin, tabun-inhibited human AChE is poorly reactivated by these oximes [6]. The mechanism of resistance to reactivation for these OP-AChE conjugates is not well defined [7,8], and further clarification is needed to rationally design a reactivator that is effective against a variety of OP agents [9,10].

2. Methods

2.1. Materials

Sarin, methylsarin, cyclosarin, tabun, VX, *O*-methyl isopropylphosphonofluoridate (sarin analogue), *O*-methyl cyclohexylphosphonofluoridate (cyclosarin analogue) and *O*-ethyl isopropylphosphonofluoridate (tabun analogue) were obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) and were >98% pure as determined by ^{31}P-nuclear magnetic resonance spectroscopy. Paraoxon, methyl paraoxon and methamidophos were purchased from ChemService (West Chester, PA). HI-6 dichloride and obidoxime dichloride were obtained from the Walter Reed Army Institute of Research (Washington, DC).

Recombinant human AChE was a generous gift from Nageswararao Chilukuri (US Army Medical Research Institute, Aberdeen Proving Ground, MD). This enzyme had been expressed in a Chinese Hamster Ovary cell line that was stably transfected with

Abbreviations: AChE, acetylcholinesterase; OP, organophosphorus; QSAR, quantitative structure–activity relationship; VR, *O*-isobutyl methylphosphonofluoridate.

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0009-2797/$ - see front matter Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.cbi.2012.08.024

a full-length cDNA for human AChE. The enzyme was purified by affinity chromatography on a procainamide-Sepharose 4B affinity column to a final specific activity of 345 units/nmol.

2.2. Oxime reactivation of OP-inhibited AChE

Oxime reactivation studies were performed with AChE that had been incubated for 30 min with sufficient OP agent to achieve >95% inhibition of AChE activity. Excess OP was removed by passing the OP/AChE mixture through a Sephadex G-25 PD-10 column. Oxime reactivation studies of OP-inhibited AChE (1 nM) were conducted at 25 °C in pH 7.4 phosphate buffer (50 mM) with a range of oxime concentrations from 0.5 to 1000 μM for each oxime. AChE activity was measured at 25 °C by the Ellman method [11] in 50 mM phosphate buffer (pH 7.4) using acetylthiocholine as substrate.

Analysis of the kinetics of oxime reactivation of OP-inhibited AChE was based on the scheme:

$$(EP) + (Ox) \overset{K_D}{\leftrightarrows} (EP \cdot Ox) \overset{k_r}{\rightarrow} (E) + (P - Ox)$$

where (EP) is the phosphylated enzyme, (Ox) is the reactivating oxime, (EP·Ox) is the complex formed by the phosphylated enzyme and oxime, (E) is the reactivated enzyme, (P-Ox) is the phosphylated oxime, K_D is the dissociation constant of the (EP·Ox) complex and k_r is the maximal first-order rate constant for reactivation [3]. The observed first-order rate constant for reactivation (k_{obs}) can be described by Michaelis–Menten kinetics where $k_{obs} = k_r\,(Ox)/(K_D + (Ox))$. Accordingly, all oxime reactivation studies were conducted with $(Ox) > (EP)_0$ to establish pseudo-first-order reaction conditions to simplify the determination of kinetic constants. Because $k_{r2} = k_r/K_D$ when oxime concentrations (Ox) are $<<K_D$, the second-order rate constant k_{r2} describes the reactivation of agent-inhibited AChE at the low physiological concentrations of oximes that are typically observed *in vivo*. Therefore, values for k_{r2} were used to compare oxime reactivation rate constants among OP-AChE conjugates.

2.3. Analysis of quantitative structure–activity relationships (QSAR)

QSAR for oxime reactivation were identified by analysis of linear free energy relationships between the structure of oximes and OP compounds and the ability of oximes to reactivate OP-inhibited AChE. Multiple linear regression equations were analyzed using SigmaStat for Windows, version 2.03 (Jandel Scientific, San Rafael, CA). Independent variables were considered to make statistically significant contributions to prediction of reactivation when P for the variable was <0.05.

The electronic constants of substituents (σ^{ph}) determined by Mastryukova and Kabachnik [12] were used to estimate the electronic effects of OP substituents on reactivation. The steric effects of OP substituents were evaluated by using their molecular volumes, which were estimated by the method of Connolly [13]. OP substituents were assigned to either the acyl or choline pockets of AChE based on our model of the orientation of OP structures in the active site of AChE. Our model was based on the generally reported orientation of OP substituents in the crystal structures of OP–AChE conjugates [14–16]. In these 3-D crystal structures the acyl pocket binds small OP substituents through hydrophobic bonding but not hydrogen bonding, while the choline pocket binds larger OP substituents through hydrogen bonding to a hydrogen acceptor, such as an alkoxy substituent.

3. Results and discussion

Our initial QSAR analysis was conducted with a data base for oxime reactivation of OP-inhibited human AChE that had been generated by Worek and co-workers [3–5]. This data set contained AChE reactivation data for 43 oxime/phosphonate pairs, 21 oxime/phosphoramidate pairs and 12 oxime/phosphate pairs. The best linear regression equation resulting from multiple regression analysis of oxime reactivation (k_r) of OP-inhibited AChE against a variety of structural, electronic and steric parameters was the following:

$$log(k_r) = 4.90 - 0.03V_{acyl} + 0.66(\sigma_{acyl} + \sigma_{chol}) + 0.54P_{ox} - 0.053pK_a$$

where V_{acyl} is the molecular volume of OP substituent in the acyl pocket, $\sigma_{acyl} + \sigma_{chol}$ is the sum of electronic effects of the OP substituents in the acyl and choline pockets, P_{ox} is the position of the oxime group as either ortho or para on a pyridinium ring and pK_a describes the ionization of the oxime group on a pyridinium ring.

This QSAR equation achieved a correlation coefficient (r) of 0.85 ($n = 76$). A summary of the statistical results associated with this equation is shown in Table 1. The marginal sum of squares, which is a measure of the reduction in the sum of the squared residuals contributed by each parameter, was used to quantify the contribution of each parameter in predicting k_r. The molecular volume of the OP substituent in the acyl pocket (V_{acyl}) made the greatest contribution (55% of total explained variation) toward explaining the variation in k_r, while the sum of the electronic effects of the OP substituents ($\sigma_{acyl} + \sigma_{chol}$) made a much smaller contribution (14.8%) toward explaining k_r.

Table 1
Statistical summary of QSAR equation.

Parameter	Parameter coefficient	Standard error of coefficient	P	Marginal sum of squares	% Contribution to explained variation
V_{acyl}	−0.031	0.003	<0.01	12.82	55.1
$\sigma_{acyl} + \sigma^{ch}{}_{ol}$	0.66	0.12	<0.01	3.44	14.8
P_{ox}	0.54	0.08	<0.01	4.41	18.9
pK_a	−0.053	0.011	<0.01	2.60	11.2

Based on our statistical analysis of oxime reactivation of OP-inhibited AChE, we predicted that the presence of a large OP substituent in the acyl pocket of AChE should produce a negative effect on oxime reactivation of OP-inhibited AChE (note the negative sign on the V_{acyl} coefficient in the QSAR equation) in comparison to reactivation of AChE inhibited by an OP with a methyl substituent in the acyl pocket, such as sarin. We also predicted that the molecular volume of an OP substituent in the acyl pocket would have a greater negative effect on reactivation than the electronic donation or withdrawal of the subsituent. Consequently, we predicted that the major impediment to reactivation of AChE inhibited by a phosphoramidate, such as tabun, would be the size of the OP substituent in the acyl pocket and not its electronic properties. In order to test our predictions we compared the reactivation of the OP agents and their analogues shown in Fig. 1.

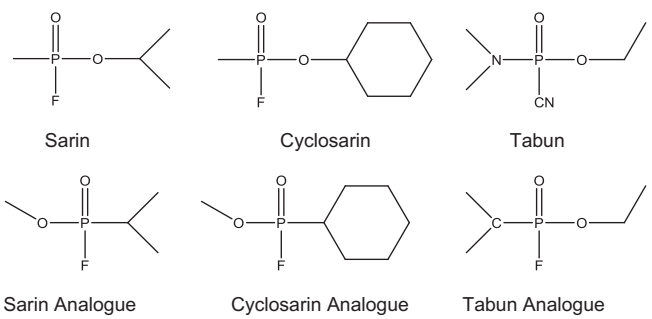

Fig. 1. Chemical structures of parent OP agents and analogues.