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# A structure–activity analysis of the variation in oxime efficacy against nerve agents ☆

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ABSTRACT

A structure–activity analysis was used to evaluate the variation in oxime efficacy of 2-PAM, obidoxime, HI-6 and ICD585 against nerve agents. In vivo oxime protection and in vitro oxime reactivation were used as indicators of oxime efficacy against VX, sarin, VR and cyclosarin. Analysis of in vivo oxime protection was conducted with oxime protective ratios (PR) from guinea pigs receiving oxime and atropine therapy after sc administration of nerve agent. Analysis of in vitro reactivation was conducted with second-order rate contants ($k_{r2}$) for oxime reactivation of agent-inhibited acetylcholinesterase (AChE) from guinea pig erythrocytes. In vivo oxime PR and in vitro $k_{r2}$ decreased as the volume of the alkylmethylphosphonate moiety of nerve agents increased from VX to cyclosarin. This effect was greater with 2-PAM and obidoxime (>14-fold decrease in PR) than with HI-6 and ICD585 (<3.7-fold decrease in PR). The decrease in oxime PR and $k_{r2}$ as the volume of the agent moiety conjugated to AChE increased was consistent with a steric hindrance mechanism. Linear regression of log (PR-1) against log ($k_{r2}$·[oxime dose]) produced two offset parallel regression lines that delineated a significant difference between the coupling of oxime reactivation and oxime protection for HI-6 and ICD585 compared to 2-PAM and obidoxime. HI-6 and ICD585 appeared to be 6.8-fold more effective than 2-PAM and obidoxime at coupling oxime reactivation to oxime protection, which suggested that the isonicotinamide group that is common to both of these oximes, but absent from 2-PAM and obidoxime, is important for oxime efficacy.

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## Introduction

The acute toxicity of organophosphorus (OP) nerve agents is usually attributed to their inhibition of acetylcholinesterase (AChE; E.C. 3.1.1.7), an enzyme that terminates the action of acetylcholine in the nervous system (Taylor, 2006; Maxwell et al., 2006). The inhibition of AChE produces an increase of acetylcholine at cholinergic synapses resulting in a variety of cholinergic effects – miosis, increased tracheobronchial and salivary secretions, bronchoconstriction, bradycardia, muscle fasciculations and seizures – and culminating in death by respiratory failure (Ballantyne and Marrs, 1992). Current medical treatment for OP nerve agents consists of a regimen of atropine to counteract the accumulation of acetylcholine, benzodiazepines to treat seizures, and oximes to reactivate OP-inhibited AChE (Aas, 2003). Since the discovery that mono-pyridinium oximes were effective reactivators of OP-inhibited AChE (Wilson and Ginsburg, 1955) a number of mono-pyridinium and bis-pyridinium oximes have been synthesized and tested for their efficacy (Hobbiger, 1963; Schoene, 1980; Kuca and Patocka, 2004; Kokshareva et al., 2005; Jun et al., 2008). However, no oxime has been identified that is effective against all OP nerve agents (Dawson, 1994; Kassa, 2002; Kuca et al., 2006), and thus, a number of different oximes, such as 2-PAM, obidoxime or HI-6, have been adopted by different countries for treatment of OP poisoning (Aas, 2003). Regardless of whether efficacy was evaluated by in vitro or in vivo investigations (Schoene et al., 1983; Calic et al., 2006), structure–activity relationships for oxime efficacy are poorly understood (Su et al., 1983, 1986; Kuca et al., 2006) because oxime reactivation has a complex dependency on the nucleophilicity and orientation of the oxime as well as on the structure of the OP-AChE conjugate (Ashani et al., 1995; Wong et al., 2000; Kovarik et al., 2004).

With respect to their structures current military nerve agents (i.e., VX, sarin, VR, cyclosarin, soman and tabun) are all alkylmethylphosphonates except for tabun, and consequently most of the research to explain the variation in oxime reactivation of nerve agent-inhibited AChE has been conducted with alkylmethylphosphonate agents. In spite of this focus on reactivation of alkylmethylphosphonate-inhibited

*Abbreviations:* AChE, acetylcholinesterase; 2-PAM, 2-[(hydroxyimino)methyl]-1-methylpyridinium chloride; obidoxime, 1,1′-(oxydimethylene)bis-[4-(hydroxyimino) methyl] pyridinium dichloride; HI-6, 1-[[[4-(aminocarbonyl)-pyidinio]-methoxy]-methyl]-2-[(hydroxyimino)methyl] pyridinium dichloride; ICD585, 1-[[4-(aminocarbonyl)-pyidinio]-trimethylene]-2-[(hydroxyimino)methyl] pyridinium dichloride; VX, O-ethyl-S-(2-(diisopropylamino)-ethyl)-methylphosphonothioate; VR, O-isobutyl-S-(2-(diethylamino)-ethyl)-methylphosphonothioate; sarin, isopropylmethylphosphonofluoridate; cyclosarin, cyclohexylmethylphosphonofluoridate; μmol, micromoles.

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AChE, soman is the only alkylmethylphosphonate for which a definitive mechanism has been developed that explains the resistance of an OP-AChE conjugate to oxime reactivation. This mechanism is based on the chemical principle that oxime reactivation occurs by the nucleophilic attack of oximate anions on OP-AChE conjugates (Wilson et al., 1992). The mechanism for resistance to reactivation of soman-inhibited AChE has been attributed to a rapid aging process (Worek et al., 2004) in which dealkylation of the soman-AChE conjugate occurs after soman inhibition of AChE (Benschop and Keijer, 1966). The anionic methylphosphonic acid-AChE conjugate that results from aging is no longer susceptible to oxime reactivation because of charge repulsion between the anionic oximate and methylphosphonic acid groups (Barak et al., 1997). This aging mechanism does not explain the resistance to oxime reactivation of AChE inhibited by VX, sarin, VR or cyclosarin because these OP-AChE conjugates do not age as quickly as soman (Worek et al., 2004).

As a result of recent studies investigating reactivation of AChE mutants, a steric mechanism has been proposed to explain the resistance to oxime reactivation of AChE that has been inhibited by some non-military alkylmethylphosphonates (Wong et al., 2000; Kovarik et al., 2004; Taylor et al., 2007). This mechanism suggests that the resistance to oxime reactivation of these alkylmethylphosphonate-AChE conjugates is due to the steric hindrance encountered by the reactivating oxime. Because of the similarity between the structures of these non-military alkylmethylphosphonates and military nerve agents, we hypothesized that this steric hindrance mechanism might also explain the variation in oxime reactivation of AChE inhibited by nerve agents whose resistance to oxime reactivation cannot be explained by the aging mechanism.

Therefore, the purpose of our current study was to perform a structure–activity analysis of the differences in oxime efficacy against VX, sarin, VR and cyclosarin. These agents form a homologous series of alkylmethylphosphonates with enough variation in molecular size to test the hypothesis that variation in oxime efficacy against military nerve agents can be influenced by the steric effects of their alkyl substituents. Our analysis was designed (1) to utilize both in vivo oxime protection data and in vitro reactivation data, (2) to maximize the size of the data sets for analysis by combining existing data from the literature with new data generated for this study and (3) to conduct a structure–activity analysis of both nerve agents and oximes.

The group of oximes evaluated in this study (Fig. 1) consisted of 2-PAM and obidoxime, which are licensed for treatment of nerve agent poisoning (Eyer and Worek, 2007), HI-6, which is being considered for regulatory approval (Aas, 2003) and ICD585, which has been considered for advanced development (Saxena et al., 2008). Thus, these oximes are some of the most successful oximes that have been developed for treatment of nerve agent intoxication (Saxena et al., 2008). As a consequence of their success in the drug development process, a more extensive data base existed for these oximes than for other less efficacious oximes that could not be included in our current structure–activity analysis because of the absence of essential data.

In spite of the existence of in vivo oxime protection data for many of these oximes in a variety of animal models, the only animal data that was included in our structure–activity analysis was derived from protection studies in guinea pigs. The greatest number of in vivo evaluations of oxime protection has been generated in small mammals such as mice, rats and guinea pigs (Dawson, 1994). However, the presence of high levels of plasma carboxylesterase Es-1 (Kadner et al., 1992) in mice and rats precludes a comparison of protective ratios generated in rats and mice with protective ratios generated in guinea pigs, which have very low levels of plasma carboxylesterase. Plasma carboxylesterase is the major determinant of the species variation in median lethal doses ($LD_{50}$) for nerve agents in unprotected animals (Maxwell et al., 1987; Maxwell, 1992). Since an oxime protective ratio is the ratio of the nerve agent $LD_{50}$ in oxime-protected animals divided by the nerve agent $LD_{50}$ in unprotected animals, variations in plasma carboxylesterase create variable $LD_{50}$ values for nerve agents across species, which leads to variation in the protective ratio for an oxime in different species (Maxwell and Brecht, 1991). To eliminate the complications that interspecies variation would have introduced into our structure–activity analysis, only guinea pig data were used in our analysis because guinea pigs contain low levels of plasma carboxylesterase, a characteristic that they share with humans (Li et al., 2005).

## Methods

*Animals.* Male Hartley guinea pigs (Crl:(HA) BR COBS) weighing 250–400 g were obtained from Charles River Laboratories (Kingston, NY), quarantined upon arrival and screened for evidence of disease for a minimum of 5 days. Animals were allowed free access to food and water before and after administration of nerve agents or drugs. Animal rooms were maintained at 20–22 °C and 50% relative humidity with at least ten complete air changes per hour. All animals were on a 12-h light/dark full spectrum lighting cycle with no twilight. All animal procedures described in this report were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, Publication No. 85-23, 1996), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended in an AAALAC, international accredited facility.

*Chemicals.* VX, sarin, VR and cyclosarin were obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) or the German Ministry of Defence and were >98% pure as determined by $^{31}$P-nuclear magnetic resonance spectroscopy. Pralidoxime chloride (2-PAM) and obidoxime dichloride were purchased from Ayerst

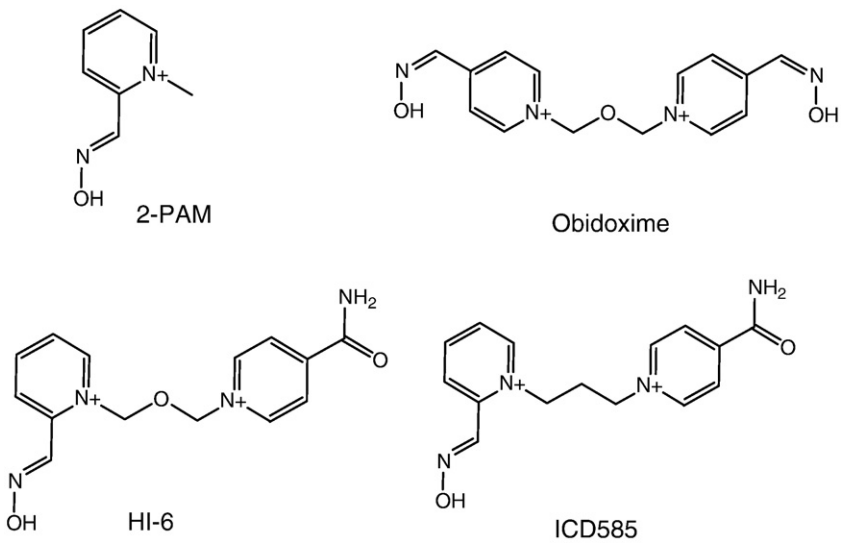



**Fig. 1.** Structures of oximes used in this study.

Laboratories (New York, NY) and Duphar (Amsterdam, Netherlands), respectively. HI-6 dichloride and ICD585 dichloride were obtained from the Walter Reed Army Institute of Research (Washington, DC). Atropine sulfate was purchased from Sigma Chemicals (St. Louis, MO).

*Preparation of erythrocyte ghosts.* Hemoglobin-free erythrocyte ghosts were prepared by a modification of the method of Dodge et al. (1963) as described by Worek et al. (2004). Briefly, heparinized blood from guinea pigs was centrifuged (3000 ×g) for 10 min and the plasma was removed. The erythrocytes were washed three times with two volumes of 0.1 M phosphate buffer (pH 7.4) and the washed packed erythrocytes were diluted in twenty volumes of hypotonic 6.7 mM buffer (pH 7.4) to facilitate hydrolysis. The hemolyzed erythrocytes were then centrifuged (50,000 ×g) for 30 min at 4 °C. The supernate was removed and the pellet was resuspended in hypotonic phosphate buffer for further washing. After two additional washes in hypotonic phosphate buffer the pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) and the erythrocyte ghosts were concentrated by centrifugation (100,000 ×g) for 30 min at 4 °C. The AChE activity in the washed erythrocyte ghosts was adjusted to 4–5 U/ml by dilution with 0.1 M phosphate buffer (pH 7.4) and aliquots of this preparation were stored at −60 °C until use. Prior to use in inhibition and reactivation studies, aliquots of the erythrocyte ghosts were homogenized on ice by three 5-s pulses at 30-s intervals with an ultrasonic homogenizer.

*Measurement of AChE activity.* AChE activity was measured spectrophotometrically at 37 °C by a modification of the method of Ellman et al. (1961) as described by Worek et al. (2004). The assay mixture contained 0.45 mM acetylthiocholine as substrate and 0.3 mM dithionitrobenzoic acid (DTNB) as chromogen in 0.1 M phosphate buffer (pH 7.4).

*Reactivation of agent-inhibited AChE.* Inhibition and reactivation of erythrocyte ghost AChE was conducted at pH 7.4 and 37 °C by the method of Worek et al. (2002). Briefly, erythrocyte ghosts were incubated for 15 min with adequate amounts of VX, sarin, VR or cyclosarin to produce 95–98% inhibition of AChE. For oxime/agent combinations that produced high rates of reactivation the kinetics of oxime reactivation were determined by the continuous procedure of Kitz et al. (1965). An aliquot of the agent-inhibited AChE (10 µl) was added to a cuvette containing oxime in 0.1 M phosphate buffer (3.0 ml), DTNB (0.1 ml), and acetylthiocholine (0.05 ml), and AChE activity was measured continuously for 5 min. To reduce the effect of oxime-induced inhibition of AChE the maximal concentrations used were 30 µM for HI-6 and ICD585 and 100 µM for 2-PAM and obidoxime. For oxime/agent combinations that produced low rates of reactivation the kinetics of oxime reactivation were determined by a discontinuous procedure (Wang and Braid, 1967; Worek et al., 1999), which allowed use of oxime concentrations up to 5 mM. In the discontinuous procedure a larger aliquot of agent-inhibited AChE (60 µl) was incubated for specified time intervals up to 10 min with smaller volumes of oxime (2 µl) and 0.45 mM acetylthiocholine (1 µl), and AChE activity was measured by transferring 10 µl of the incubation mixture to cuvettes containing DTNB in phosphate buffer. In both the continuous and discontinuous procedures 6–10 different concentrations of oximes were evaluated and AChE activities were individually corrected for spontaneous and oxime-induced hydrolysis of acetylthiocholine.

*Kinetics of oxime reactivation.* Oxime reactivation of phosphonate-inhibited AChE can be described by the following scheme:

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

where (EP) is the phosphonylated enzyme, (Ox) is the reactivating oxime, (EP·Ox) is the complex formed by the phosphonylated enzyme and oxime, (E) is the reactivated enzyme and (P-Ox) is the phosphonylated oxime (Worek et al., 2004). Accordingly, oxime reactivation studies were conducted with $(\mathrm{Ox}) >> (\mathrm{EP})_0$ to establish pseudo-first-order reaction conditions to simplify the determination of kinetic constants.

Observed first-order rate constants ($k_{obs}$) for reactivation at each oxime concentration were determined for high rates of reactivation from the continuously measured velocity of AChE activity, using the method of Kitz et al. (1965). Observed first-order rate constants for low rates of reactivation were determined by the discontinuous procedure of Wang and Braid (1967), using linear regression of the equation

$$\ln \quad (v_0 - v_t)/(v_0 - v_i) = -k_{obs}t$$

where $v_0$ is the activity of the control enzyme, $v_i$ is the activity of the inhibited enzyme and $v_t$ is the activity of the reactivated enzyme at any time ($t$).

The dissociation constant ($K_D$) of the phosphonylated enzyme oxime complex and the maximal first-order rate constant ($k_r$) for reactivation were determined by non-linear regression of the equation

$$k_{obs} = k_r(\mathrm{Ox})/(K_D + (\mathrm{Ox}))$$

in which reactivation is described by Michaelis–Menten kinetics.

The second-order reactivation rate constant ($k_{r2}$) to describe reactivation when $(\mathrm{Ox}) << K_D$ was calculated by the equation

$$k_{r2} = k_r/K_D.$$

*In vivo oxime protection.* In vivo protection of oximes against nerve agents was determined from nerve agent dose-lethality curves of guinea pigs receiving oxime (145 µmol/kg for 2-PAM, HI-6 and ICD585; 40 µmol/kg for obidoxime) and atropine (50 µmol/kg) or saline by im injection 1 min after sc administration of a nerve agent. Drugs and nerve agents were administered as solutions in saline with injection volumes <1 ml/kg. Lethality was assessed at 24 h. $LD_{50}$ values and 95% confidence intervals were determined by probit analysis (Feder et al., 1991) of mortality fractions with at least five doses of each nerve agent and up to 6 animals per dose. An oxime's in vivo protection was expressed as a protective ratio (PR), which is the ratio of the nerve agent $LD_{50}$ in oxime/atropine-treated animals divided by the nerve agent $LD_{50}$ in untreated animals.

## Results

### *In vivo oxime protection*

Table 1 is a compilation of PR values from the current study and also from the literature describing the protection provided by atropine and oximes against nerve agents in guinea pigs. The dose of atropine (46–50 µmol/kg) in all studies was a dose that maximizes protection when used in conjunction with oximes in guinea pigs and was sufficient to control the cholinergic effects of nerve agents and to terminate agent-induced seizures (Shih and McDonough, 2000). Oxime doses in Table 1 are essentially constant at 130–145 µmol/kg for 2-PAM, HI-6 and ICD585 and at 40–56 µmol/kg for obidoxime. Obidoxime was tested at a lower dose than the other oximes because of its greater toxicity (Dawson, 1994). The protection (i.e., PR) achieved by an oxime against nerve agents varied among agents. In vivo protection by 2-PAM against nerve agents varied 14.2-fold with PR values ranging from 36.8 against VX to 2.6 against cyclosarin. A similar pattern was observed with obidoxime where PR values varied 14.5-fold between VX and cyclosarin. Less variation in oxime protection was observed with HI-6 and ICD585. Oxime protection by HI-6 and ICD585 varied only 2.5-fold and 3.7-fold, respectively, between the highest and lowest PR values. In general, the PR values for each oxime against nerve agents decreased in the order VX ≥ sarin > VR > cyclosarin, although there are examples (e.g., HI-6 against VX and sarin; ICD585 against VR and cyclosarin) where this order was reversed.

### *In vitro oxime reactivation*

Kinetic studies of oxime reactivation of nerve agent-inhibited guinea pig AChE were conducted with a range of oxime concentrations in order to determine both $K_D$ and $k_r$ for in vitro reactivation (Fig. 2). Table 2 is a compilation of the in vitro reactivation rate constants for

**Table 1**
Comparison of oxime protection against nerve agents in guinea pigs

| Oxime | Agent | Oxime dose (µmol/kg) | Protective ratio (PR)[a] | Reference for PR |
|---|---|---|---|---|
| 2-PAM | VX | 145 | 36.8 (27.9–46.1) | Maxwell et al. (2006) |
| 2-PAM | Sarin | 145 | 22.7 (17.4–29.5) | Maxwell et al. (2006) |
| 2-PAM | VR | 145 | 6.28 (5.22–7.35) | Current paper |
| 2-PAM | Cyclosarin | 145 | 2.6 (2.1–3.5) | Maxwell et al. (2006) |
| Obidoxime | VX | 40 | 58 (23–150) | Inns and Leadbeater (1983) |
| Obidoxime | Sarin | 40 | 59 (37–94) | Inns and Leadbeater (1983) |
| Obidoxime | VR | 40 | 13.6 (9.0–20.8) | Current paper |
| Obidoxime | Cyclosarin | 56 | 4 (No Limits) | Lundy et al. (1992) |
| HI-6 | VX | 130 | 66 (32–140) | Inns and Leadbeater (1983) |
| HI-6 | Sarin | 130 | 76 (44–130) | Inns and Leadbeater (1983) |
| HI-6 | VR | 145 | 39.8 (32.7–48.3) | Current paper |
| HI-6 | Cyclosarin | 145 | 31.1 (19.0–51.1) | Current paper |
| ICD585 | VX | 145 | 47.6 (31.7–71.8) | Current paper |
| ICD585 | Sarin | 145 | 33.3 (23.3–47.5) | Current paper |
| ICD585 | VR | 145 | 12.7 (9.8–18.3) | Current paper |
| ICD585 | Cyclosarin | 145 | 19.1 (10.7–34.2) | Current paper |

[a] PR values, where PR=(agent $LD_{50}$ in drug-treated group)/(agent $LD_{50}$ in saline-treated group), were determined in guinea pigs that received oxime and atropine (im) 1 min after receiving nerve agent (sc). The $LD_{50}$ values for nerve agents in saline-treated guinea pigs were 8.9 (8.6–9.1) µg/kg for VX, 43.5 (41.1–46.1) µg/kg for sarin, 11.3 (10.5–12.1) µg/kg for VR and 54.4 (49.2–63.7) µg/kg for cyclosarin. Values in parentheses are 95% confidence intervals.

oxime reactivation of AChE inhibited by different nerve agents. The second-order rate constants ($k_{r2}$) for oxime reactivation varied 111-fold for 2-PAM and 236-fold for obidoxime and generally decreased in the order VX≥sarin>VR>cyclosarin. Less variation in oxime reactivation was observed with HI-6 and ICD585. Oxime reactivation by HI-6 and ICD585 varied only 2.4-fold and 5.4-fold, respectively, between the highest and lowest $k_{r2}$ values. The small variation among the $k_{r2}$ values for HI-6 appeared to be a random variation about a fixed value. The somewhat larger variation among the $k_{r2}$ values for ICD585 decreased in the order sarin>VX>VR>cyclosarin, which was nearly the same order that was observed with 2-PAM and obidoxime.

Inasmuch as $k_{r2}=k_r(1/K_D)$ the contributions of oxime reactivity ($k_r$) and oxime affinity ($1/K_D$) to the variation in the $k_{r2}$ values for reactivation were also evaluated in our analysis. In Table 3 the contributions of oxime affinity and oxime reactivity are compared for their effect on the difference in reactivation $k_{r2}$ values between an agent-AChE conjugate that is easily reactivated, VX-inhibited AChE, and an agent-AChE conjugate that is difficult to reactivate, cyclosarin-inhibited AChE. Oxime affinity had a much greater effect on the 128-fold decrease in $k_{r2}$ values for 2-PAM reactivation between VX-inhibited AChE and cyclosarin-inhibited AChE. Affinity for 2-PAM decreased 33-fold, while reactivity only decreased 3.9-fold. For obidoxime, the 209-fold decrease in reactivation $k_{r2}$ values between VX-inhibited AChE and cyclosarin-inhibited AChE was primarily the result of a difference in reactivity, which decreased 32-fold, rather than oxime affinity, which decreased 6.6-fold. For HI-6, the variations in oxime affinity and reactivity were approximately equal, but they changed in opposite directions to produce no net effect on $k_{r2}$ when reactivations of VX-inhibited AChE and cyclosarin-inhibited AChE were compared. For ICD585, the entire decrease in $k_{r2}$ between reactivation of VX-inhibited AChE and cyclosarin-inhibited AChE was due to a decrease in affinity because no change was observed in oxime reactivity.

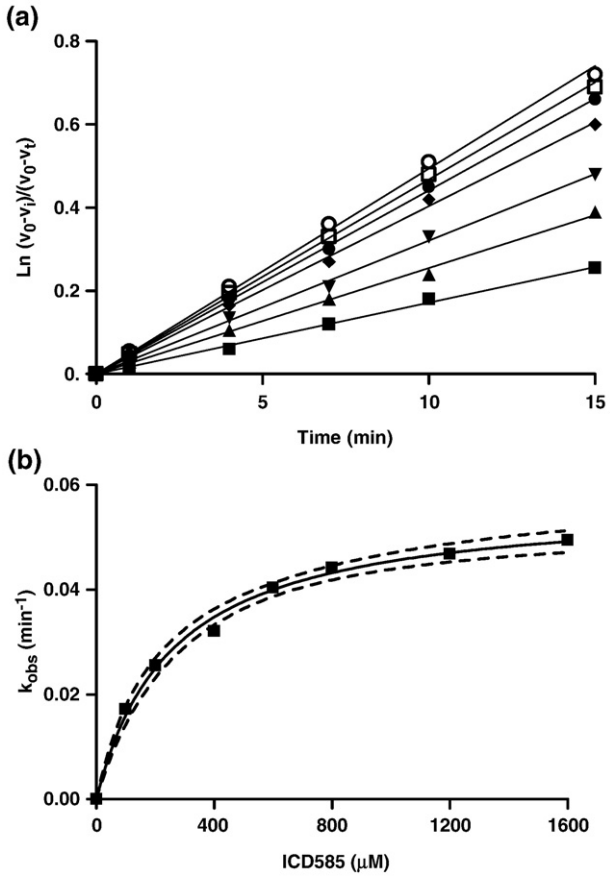



**Fig. 2.** Reactivation of sarin-inhibited AChE by ICD585. (a) $k_{obs}$ values for reactivation with 100 (■), 200 (▲), 400 (▼), 600 (♦), 800 (●), 1200 (□) and 1600 (○) μM ICD585 were calculated from AChE activities at designated elapsed times for reactivation by linear regression of ln $(v_0-v_i)/(v_0-v_t)$ against time. (b) Secondary plot of $k_{obs}$ against concentration of ICD585. Data points for $k_{obs}$ were taken from values determined in panel (a). Fitted line was calculated using the equation $k_{obs}=k_r(Ox)/(K_D+(Ox))$ which was used to determine $K_D$ and $k_r$ by non-linear regression. Values for $K_D$ and $k_r$ are found in Table 2. 95% confidence limits are indicated by (---).

**Table 2**
Reactivation rate constants for oximes against nerve agent-inhibited AChE from guinea pig erythrocytes

| Oxime | Agent | $K_D$ (μM)[a] | $k_r$ ($min^{-1}$)[a] | $k_{r2}$ ($mM^{-1}$ $min^{-1}$) |
|---|---|---|---|---|
| 2-PAM[b] | VX | 27.0 | 0.090 | 3.3 |
| 2-PAM[b] | Sarin | 14.8 | 0.040 | 2.7 |
| 2-PAM | VR | 405±78 | 0.041±0.005 | 0.10 |
| 2-PAM[b] | Cyclosarin | 896 | 0.023 | 0.026 |
| Obidoxime[b] | VX | 184 | 2.30 | 12.5 |
| Obidoxime[b] | Sarin | 23.1 | 0.33 | 14.3 |
| Obidoxime | VR | 277±35 | 0.054±0.003 | 0.19 |
| Obidoxime[b] | Cyclosarin | 1211 | 0.071 | 0.059 |
| HI-6[b] | VX | 449 | 0.061 | 0.14 |
| HI-6[b] | Sarin | 261 | 0.085 | 0.33 |
| HI-6 | VR | 561±89 | 0.078±0.006 | 0.14 |
| HI-6[b] | Cyclosarin | 713 | 0.14 | 0.19 |
| ICD585 | VX | 304±53 | 0.033±0.003 | 0.11 |
| ICD585 | Sarin | 247±67 | 0.059±0.005 | 0.24 |
| ICD585 | VR | 408±51 | 0.037±0.004 | 0.091 |
| ICD585 | Cyclosarin | 750±108 | 0.034±0.004 | 0.045 |

[a] X±S.E. of rate constants determined using 6–10 oxime concentrations.
[b] Rate constants taken from Worek et al. (2002).

*Steric effects on oxime reactivation and oxime protection*

The steric influence that differences in the structures of VX, sarin, VR and cyclosarin might contribute to the variation of in vivo oxime protective ratios and in vitro oxime reactivation rate constants was examined by estimating the differences in the molecular volumes of these OP agents. Inasmuch as their leaving groups are cleaved from these agents when they inhibit AChE to form alkylmethylphosphonate-AChE conjugates, the only differences in the molecular volumes of the alkylmethylphosphonate moieties of these OP-AChE conjugates result from the differences in their alkyl substituents. The molecular volumes of these alkyl substituents, which are presented in Table 4, varied > 2-fold between the smallest and largest substituents.

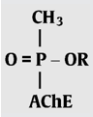
The effects of changes in the molecular volumes of the alkyl substituents of alkylmethylphosphonate agents on both oxime protection (PR) in guinea pigs and on oxime reactivation ($k_{r2}$) are shown in Fig. 3. Protective ratios are presented as PR-1 because a PR of 1 means that an oxime has no effect on agent $LD_{50}$. For 2-PAM and obidoxime both PR-1 and $k_{r2}$ had a similar pattern of dependency on molecular volume. There was a plateau where there was no effect of molecular volume as the volume of the alkyl substituent of the agent increased from 45.8 $Å^3$ for ethyl (VX) to 63.3 $Å^3$ for isopropyl (sarin) followed by a decrease of PR-1 and $k_{r2}$ as the molecular volume increased to 80.8 $Å^3$ for isobutyl (VR) and 104.7 $Å^3$ for cyclohexyl (cyclosarin). For HI-6 and ICD585 there was less of an effect of molecular volume on PR-1 and $k_{r2}$ than was observed with 2-PAM and obidoxime. For HI-6 the increase in molecular volume of alkyl substituents produced an oscillation around an average value with little decrease of either PR-1 or $k_{r2}$. For ICD585

**Table 3**
Ratio of rate constants for oxime reactivation of agent-inhibited AChE (VX-AChE/cyclosarin-AChE)

| Oxime | Affinity ($1/K_D$)[a] | Reactivity ($k_r$)[a] | Second-order rate constant ($k_{r2}$)[a] |
|---|---|---|---|
| 2-PAM | 33 ↓ | 3.9 ↓ | 128 ↓ |
| Obidoxime | 6.6 ↓ | 32 ↓ | 209 ↓ |
| HI-6 | 1.6 ↓ | 2.2 ↑ | No Change |
| ICD585 | 2.4 ↓ | No change | 2.4 ↓ |

[a] Ratios were calculated from rate constants in Table 2.

**Table 4**
Molecular volumes of alkyl substituents of nerve agent-AChE conjugates




| Agent | Alkyl substituent (R) | Molecular Volume of R (Å$^3$)[a] |
|---|---|---|
| VX | Ethyl | 45.8 |
| Sarin | Isopropyl | 63.3 |
| VR | Isobutyl | 80.8 |
| Cyclosarin | Cyclohexyl | 104.7 |

[a] Estimated by the method of Connolly (1985).

the increase in molecular volume produced a trend toward a decrease of PR-1 and $k_{r2}$, but the decrease was overlaid by oscillations similar to those observed with HI-6.

*Coupling of oxime reactivation and oxime protection*

The correlation between in vivo oxime protection expressed as PR-1 and in vitro oxime reactivation expressed as $k_{r2}$ is illustrated in a full logarithmic plot in Fig. 4. Although the dose of each oxime that was used to evaluate in vivo oxime PR values in guinea pigs was held essentially constant for all agents, the same dose was not used for all oximes. The dose of obidoxime used to determine PR values was 40–56 μmol/kg, whereas the dose for the other oximes was 130–145 μmol/kg. The relationship between in vivo protection and in vitro reactivation for those oximes (i.e., 2-PAM, HI-6 and ICD585) that were tested at the 130–145 μmol/kg dose are shown in Fig. 4a. The points representing the values for PR-1 and $k_{r2}$ for each nerve agent/oxime combination separated into two populations described by two parallel lines that were offset from each other. One data set was composed of the data describing oxime efficacy by 2-PAM, and the second data set was composed of the data describing oxime efficacy by HI-6 and ICD585.

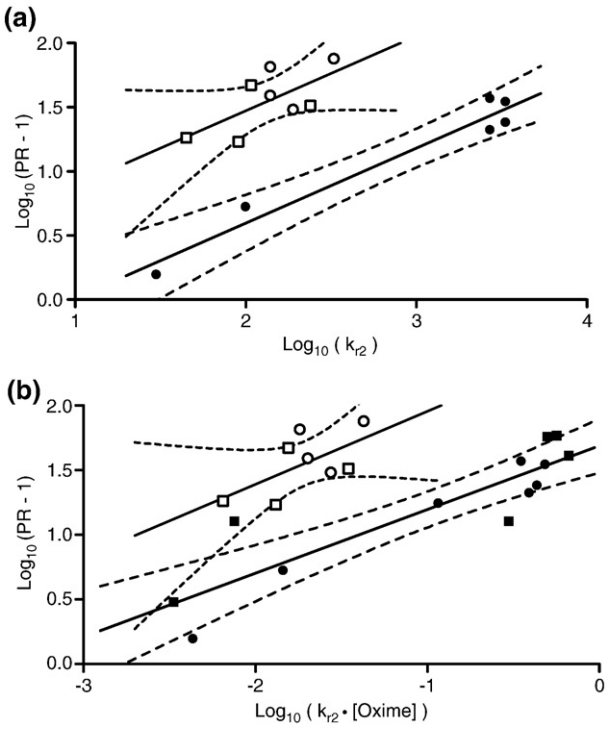



**Fig. 4.** Correlation of oxime protection and oxime reactivation with (a) constant doses of oxime and (b) variable doses of oxime. Data points represent HI-6 (○), ICD585 (□), 2-PAM (●) and obidoxime (■). 95% confidence limits are indicated by (---).

The slopes of these lines were indistinguishable with slopes of 0.59 and 0.58 for the 2-PAM and HI-6/ICD585 lines, respectively. No data points for the 2-PAM data set overlapped into the 95% confidence boundaries of the HI-6/ICD585 data set, and none of the HI-6/ICD585 data points overlapped into the 95% confidence boundaries of the 2-PAM

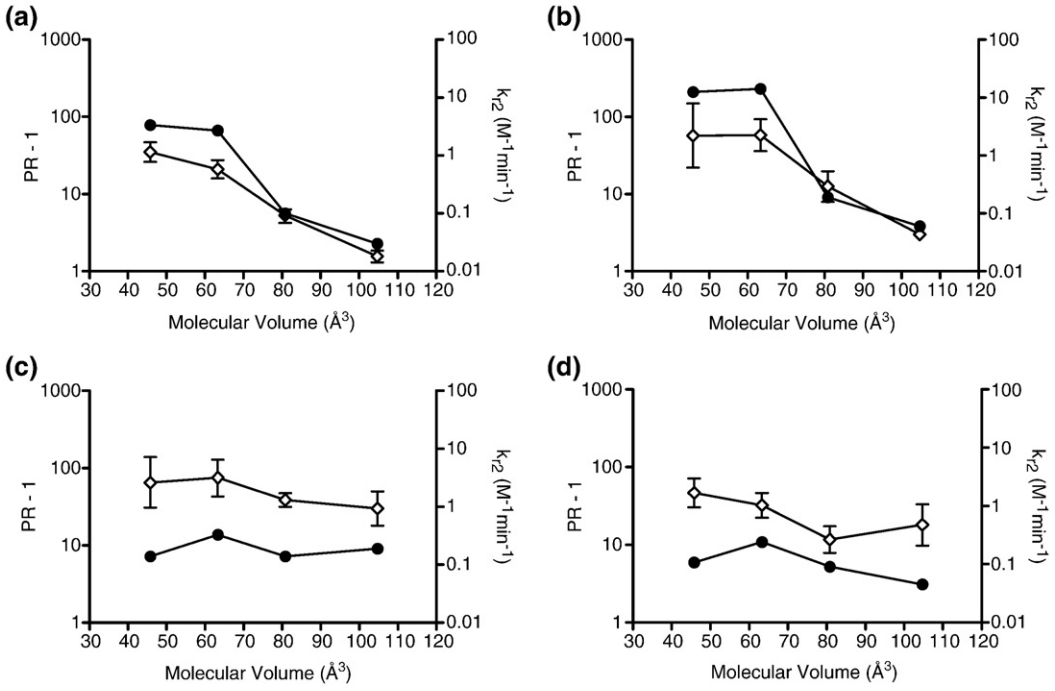



**Fig. 3.** Effect of molecular volume of alkyl substituents of alkylmethylphosphonate nerve agents on oxime protection and oxime reactivation by (a) 2-PAM, (b) obidoxime, (c) HI-6 and (d) ICD585. Oxime protection (◊) is presented as PR-1 and oxime reactivation (●) is presented as $k_{r2}$. Values for PR and $k_{r2}$ were taken from Tables 1 and 2, respectively. Molecular volumes of alkyl substituents were taken from Table 4.

**Table 5**
Additional data for oxime protection against nerve agents in guinea pigs

| Oxime | Agent | Oxime dose (μmol/kg) | Protective ratio (PR)[a] | Reference for PR |
|---|---|---|---|---|
| 2-PAM | VX | 130 | 25 (14–44) | Inns and Leadbeater (1983) |
| 2-PAM | Sarin | 130 | 38 (27–52) | Inns and Leadbeater (1983) |
| 2-PAM | Sarin | 43 | 18.5 (12.2–28.2) | Fleisher et al. (1970) |
| Obidoxime | Sarin | 47 | 41.6 (29.0–59.6) | Fleisher et al. (1970) |
| Obidoxime | Sarin | 21 | 13.6 (11.4–16.1) | Fleisher et al. (1970) |

[a] PR values were determined in guinea pigs that received oxime and atropine (im) 1 min after receiving nerve agent (sc). Values in parentheses are 95% confidence intervals.

data set. The offset between the two lines was estimated from the difference in the *y*-intercepts of the 2-PAM and HI-6/ICD585 lines. Inasmuch as the difference between the two *y*-intercepts on this $\log_{10}$–$\log_{10}$ plot was 0.88 the mean linear displacement between these two populations of data points was $10^{0.88}=7.59$. This indicated that HI-6/ICD585 achieved 7.6 times greater in vivo protection than 2-PAM for a given degree of in vitro oxime reactivation when all oximes were administered at an essentially constant dose.

To increase the number of data points for our evaluation of the correlation of oxime protection and oxime reactivation, we normalized the $k_{r2}$ data by multiplying it by the oxime dose used to determine the PR values in guinea pigs. This permitted an evaluation of the correlation of reactivation and protection with oximes administered at doses other than the 130–145 μmol/kg used for 2-PAM, HI-6 and ICD585. Thus, the obidoxime data generated at 40–56 μmol/kg (see Table 1), as well as additional data from the literature with other doses of 2-PAM and obidoxime (see Table 5), could be included in the evaluation. The points representing the values for PR-1 and ($k_{r2}$·[oxime dose]) for each nerve agent/oxime combination are presented in a full logarithmic plot in Fig. 4b. The data points for these agent/oxime combinations again separated into two populations that are described by two offset but parallel lines. One data set is composed of the data describing oxime efficacy by 2-PAM and obidoxime, and the second data set is composed of the data describing oxime efficacy by HI-6 and ICD585. The slopes of these lines were similar with slopes of 0.49 and 0.57 for the 2-PAM/obidoxime and HI-6/ICD585 lines, respectively. Only one point of the 2-PAM/obidoxime data set overlapped into the 95% confidence boundaries of the HI-6/ICD585 data set. No points in the HI-6/ICD585 data overlapped into the 95% confidence boundaries of the 2-PAM/obidoxime data set, which reinforced the conclusion that 2-PAM/obidoxime data set and the HI-6/ICD585 data set described different relationships between oxime reactivation and oxime protection. Inasmuch as the difference between the two *y*-intercepts on this $\log_{10}$–$\log_{10}$ plot was 0.83 the mean linear displacement between these two populations of data points was $10^{0.83}=6.76$. This indicated that the HI-6/ICD585 achieved 6.8 times greater in vivo protection than 2-PAM/obidoxime for a given degree of in vitro oxime reactivation across a range of oxime doses.

## Discussion

### *Structure–activity analysis of the variation in oxime efficacy*

Although the variation of in vivo efficacy of oximes against nerve agents and the absence of a single oxime that provides effective treatment against a broad spectrum of nerve agents have been noted by many authors (Aas, 2003; Dawson, 1994; Kassa, 2005; Jokanovic and Stojilkovic, 2006), the explanation for this variation has remained elusive. Structure–activity analyses of the data from either in vivo oxime protection assessments or in vitro reactivation studies have not provided a comprehensive explanation for the variation in oxime efficacy against the structural diversity of military nerve agents. In our current study we addressed this problem by performing a structure–activity analysis of oxime efficacy against the series of alkylmethylphosphonate-AChE conjugates resulting from inhibition of AChE by VX, sarin, VR and cyclosarin. These alkylmethylphosphonate nerve agents vary primarily in the molecular volume of their alkyl substituents. We excluded tabun from our analysis because a phosphoramidate nerve agent is quite different in its electronic properties from alkylmethylphosphonate nerve agents. We also excluded soman from the analysis, even though it is an alkylmethylphosphonate, because its alkyl substituent (i.e., pinacolyl) produces aging with a half-life that is 30–1000 times faster than the aging rates of VX, sarin, VR or cyclosarin (Worek et al., 2004). The alkylmethylphosphonates that were included in our structure–activity analysis possessed similar electronic properties (Hansch et al., 1995) and aging rates (Worek et al., 2004) but varied in the volume of their alkyl substituents in a graded homologous manner.

The design of our structure–activity analysis of oxime efficacy (i.e., focusing on alkymethylphosphonate nerve agents, including only agent/oxime combinations for which both in vivo and in vitro efficacy measurements were available, and using only guinea pig data) imposed some limitations on the general application of the conclusions of the study. One limitation was that our study contained only four nerve agents and four oximes, which was not a large number of compounds with which to perform a structure–activity analysis of both nerve agents and oximes. In an attempt to address any deficiency in the numbers of agents and oximes in the study, we evaluated multiple descriptors of oxime efficacy (i.e., PR, $k_{r2}$, $k_r$, $K_D$) for each agent/oxime combination in order to assess the consistency of possible structure–activity relationships across several indicators of efficacy. Fortunately, the large range of the observed measurements for all descriptors of oxime efficacy aided us in identifying structure–activity relationships. In vivo oxime efficacy against nerve agents as measured by PR values varied 29-fold from 2.6 to 76 among the agent/oxime combinations. In vitro oxime reactivation rate constants varied >300-fold for $k_{r2}$ (0.045 to 14.3 $mM^{-1}$ $min^{-1}$), 100-fold for $k_r$ (0.023 to 2.3 $min^{-1}$) and 32-fold for $K_D$ (23.1 to 750 μM). However, these large ranges of oxime efficacy in combination with the relatively small number of oximes and agents in our study meant that our structure–activity analysis identified only major structure–activity relationships. Inasmuch as these structure–activity relationships were identified from oxime efficacy data restricted to guinea pigs, the extrapolation of these relationships to other species, such as humans, should be done with caution because oxime reactivation of AChE from guinea pigs and humans have been reported to differ significantly (Worek et al., 2002).

### *Steric effects on oxime efficacy*

The oxime protection data in guinea pigs reported in our study confirmed previous assessments that oxime protection varies dramatically against different military nerve agents (Aas, 2003; Dawson, 1994; Kassa, 2002). In our study oxime protection against VX, sarin, VR and cyclosarin in guinea pigs appeared to vary primarily as a result of steric effects. As the molecular volume of the alkyl substituents of the nerve agents increased beyond the size of an isopropyl group, oxime protection decreased, particularly with 2-PAM and obidoxime. Inasmuch as 2-PAM was designed to bind in the choline-binding subsite of the AChE active site (Wilson et al., 1992) and the alkyl substituents of nerve agents have been shown to bind in the choline-binding subsite (Hornberg et al., 2007), it is likely that steric hindrance by alkyl substituents occurred in the choline-binding subsite. If the size of choline (114 $Å^3$) provides a rough estimate of the molecular volume of the choline-binding subsite and alkyl substituents larger than the isopropyl substituent (63.3 $Å^3$) produced steric hindrance for 2-PAM binding, then steric hindrance occurred when >55% of the choline-binding subsite was occupied by the alkyl substituent of an alkylmethylphosphonate-inhibited AChE. If 2-PAM with a molecular volume of 101 $Å^3$

normally occupies most of the choline-binding subsite, occupancy of >55% of the choline-binding subsite by an alkyl substituent would be expected to hinder 2-PAM binding. The isobutyl group of VR and the cyclohexyl group of cyclosarin with molecular volumes of 80.8 $Å^3$ and 105 $Å^3$ could also occupy 71% and 92%, respectively, of the choline-binding site leaving little opportunity for 2-PAM to bind in this site.

In addition to the decrease of oxime protection in guinea pigs as the volume of alkyl substituents increased, support for the steric hindrance of alkyl substituents also appeared when the rate constants for oxime reactivation were examined. The second-order reactivation rate constants ($k_{r2}$) for 2-PAM reactivation decreased in the same pattern as the decrease observed with 2-PAM protection in guinea pigs, where the decrease occurred when the volume of the alkyl substituent of the alkylmethylphosphonate inhibitor exceeded the size of an isopropyl group. The primary cause of this decrease in $k_{r2}$ was a progressive decrease in the affinity of 2-PAM for OP-inhibited AChE as the alkyl substituent increased in volume. 2-PAM reactivity was much less affected by changes in the volume of the alkyl substituent. Major reductions in 2-PAM affinity with only minor changes in reactivity were consistent with a simple steric mechanism where alkyl substituents hindered the binding of 2-PAM to the choline-binding subsite.

Although the magnitude of the steric effects on oxime protection in guinea pigs and on $k_{r2}$ values for reactivation was similar for 2-PAM and obidoxime, the underlying mechanisms for these effects were different. While the steric effect of alkyl substituents produced a much greater effect on affinity than reactivity for 2-PAM, the steric effect on reactivity was greater than the effect on affinity for obidoxime. This observation highlights an essential difference between the binding of 2-PAM, a mono-pyridinium oxime, and obidoxime, a bis-pyridinium oxime. Bis-quaternary ligands bind to AChE by binding to both the choline-binding subsite and a peripheral binding site at the rim of the active site gorge (Harel et al., 1993). If the affinity of obidoxime to the AChE peripheral binding site is unaffected by alkylmethylphosphonate inhibition, this additional binding site may reduce the detrimental effect of steric hindrance on the net binding of oxime to AChE. However, even with adequate oxime affinity at the peripheral binding site, other steric effects may still occur that reduce reactivation by steric displacement of the angle of attack for an oxime. Oxime reactivation is especially sensitive to any displacement of the angle of attack for an oxime because the mechanism for reactivation is a nucleophilic attack in which the oxygen of serine, the phosphorus of the OP inhibitor and the attacking oximate anion must be in line to optimize reactivation (Ashani et al., 1995). X-ray crystallographic studies of AChE have indicated that the site of conjugation for OP nerve agents is at the base of a narrow 18–20 Å deep active site gorge (Kovarik et al., 2004). The spatial constraints of this narrow gorge produce an OP site of conjugation with limited angles of access for the attacking oxime. When OP compounds with larger substituents inhibit AChE they can displace the angle of attack of an oxime even if the oxime binds to a peripheral binding site. The importance of this displacement of the angle of oxime attack has been demonstrated by evaluating the reactivity and affinity of bis-pyridinium oximes with mutants of AChE that have enlarged active sites. After conjugation with large OP inhibitors, mutants of AChE with enlarged active sites exhibited 10-fold greater oxime reactivity than wild-type AChE (Wong et al., 2000). This enhanced reactivity was attributed to the more favorable angle of oxime attack that occurred with the reduced steric constraints of a larger AChE active site because it occurred in the absence of changes in oxime affinity (Taylor et al., 2007).

Steric effects on oxime protection in guinea pigs and oxime reactivation with HI-6 and ICD585 were much smaller than the steric effects observed with obidoxime and 2-PAM. The smaller steric effect on protection and reactivation with HI-6 and ICD585 compared to the steric effect observed with 2-PAM presumably results from the ability of these oximes to bind to the peripheral binding site when the alkylmethylphosphonates impede the binding of oximes to the choline-binding subsite. However, the differences between the magnitude of the steric effects observed with HI-6 and ICD585 and the steric effects observed with obidoxime are more problematic, since all three oximes are bis-pyridinium and thus possess the ability to bind to the peripheral binding site. The steric effects on affinity observed with these bis-pyridinium oximes were small, similar in magnitude and resulted in decreases in affinity as alkyl substituents increased in size. The steric effects on reactivity observed with these bis-pyridinium oximes differed among the oximes with a large decrease of reactivity (i.e., 32-fold) for obidoxime and a small increase or no effect on reactivity for HI-6 and ICD585, respectively, as alkyl substituents increased in size. It is likely that the differences that were observed among the steric effects with obidoxime, HI-6 and ICD585 were dependent on whether the oxime moiety was in the 4-position on the pyridinium ring, as it is with obidoxime, or in the 2-position, as it is with HI-6 and ICD585. The steric displacement of the angle of attack for obidoxime resulted in a much less favorable angle of attack and a 32-fold reduction in reactivity, while a similar steric displacement of HI-6 and ICD585 produced a minor (i.e., <2.2-fold) effect on reactivity.

### *Coupling of oxime reactivation and oxime protection*

Our analysis of the relationship between oxime reactivation and oxime protection supported the conclusion that there is a difference between the coupling of oxime reactivation and oxime protection for HI-6 and ICD585 and the coupling of these same two processes with 2-PAM and obidoxime. If we assume that oxime reactivation is the sole mechanism of oxime protection, then HI-6 and ICD585 were 6.8 times more effective in coupling oxime reactivation to oxime protection (i.e., survival) than were 2-PAM and obidoxime. While it has been generally believed that the superior in vivo protection of HI-6 in comparison to other oximes is the result of its greater ability to reactivate, that viewpoint ignores the coupling of oxime reactivation to oxime protection. Our study confirmed previous observations that HI-6 is a better reactivator than the other oximes in our study, but it also demonstrated that there is an advantage of HI-6 and ICD585 compared to 2-PAM and obidoxime that is separate from any differences between these pairs of oximes in their ability to reactivate OP-inhibited AChE. One possibility for the greater protection provided by HI-6 and ICD585 might be the direct pharmacological effects that have been observed with some oximes in addition to their ability to reactivate (Jokanovic and Stojilkovic, 2006). The difficulty with this explanation is that there is no correlation between the rank order of oxime protection observed in our study and the rank order of the ability of oximes to produce direct pharmacological effects at either the neuromuscular junction (Tattersall, 1993) or in the central nervous system (Oydvin et al., 2005). Thus, there is no obvious mechanism at this time for the enhanced oxime protection provided by HI-6 and ICD585. However, our study does indicate that the enhanced protection is due to the isonicotinamide group that is common to both HI-6 and ICD585 and is absent from 2-PAM and obidoxime. This isonicotinamide group was originally incorporated into the structure of bis-pyridinium oximes to reduce their toxicity (Oldiges and Schoene, 1970), but our study suggests that it may also improve the efficacy of oximes.

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