CHOLINESTERASE REACTIVATION IN VIVO WITH A NOVEL BIS-OXIME
OPTIMIZED BY COMPUTER-AIDED DESIGN

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ABSTRACT

Recently several bis-pyridiniumaldoximes linked by a variable-length alkylene chain were rationally designed in our laboratories as cholinesterase reactivators. Extensive in vitro tests of these oximes with acetylcholinesterase inhibited by two different organophosphate agents, echothiophate and diisopropylfluorophosphate, revealed one compound with particularly good reactivation kinetics and affinity for phosphorylated acetylcholinesterase (AChE). This compound, designated “ortho-7”, with a heptylene chain bridging two aldoximes ortho to a pyridinium ring nitrogen, was chosen for detailed comparison with the classic reactivator, 2-pralidoxime (2-PAM). In vitro, ortho-7 reactivated AChE selectively, without restoring activity of the related enzyme, butyrylcholinesterase (BChE). For in vivo studies, rats were injected with ortho-7 or 2-PAM before or after organophosphate exposure, and the activities of AChE and BChE were determined at multiple intervals in blood and solid tissues. Ortho-7 behaved nearly as well in the animal as in vitro, reactivating AChE to the same extent as 2-PAM in all peripheral tissues studied (serum, red blood cell and diaphragm), but at doses up to 100-fold smaller. Like other oxime reactivators, ortho-7 did not reactivate brain AChE after systemic administration. Nonetheless, this agent could be useful in combination therapy for organophosphate-exposure and it may provide a platform for development of additional, even more effective reactivators.
Organophosphorus anticholinesterases (OPs), including many insecticides and “nerve agents”, cause long-term inactivation of acetylcholinesterase (AChE, E.C.3.1.1.7) and butyrylcholinesterase (BChE, E.C.3.1.1.8) by phosphorylating a serine residue at the active site. Although phosphorylation of AChE is nearly irreversible under normal conditions, the enzyme can be reactivated by site-directed nucleophiles that accept a serine-bound phosphate, such as 2-pyridine aldoxime (2-PAM) (Koelle, 1963). This agent, with a quaternary nitrogen that promotes binding in the catalytic site, is now a mainstay of treatment for OP exposure. It was an early triumph of rational pharmacology based on deductions from classic structure-activity relations (Wilson and Ginsberg, 1955; Hobbiger, 1956).

Computational chemistry now enables a search for improved agents by simulated interactions with molecular groups at AChE’s catalytic center. We previously took such an approach to generate bifunctional AChE inhibitors derived from 9-amino-1,2,3,4 tetrahydroacridine (THA). Using a ligand docking program (Pang et al., 2001) and the X-ray crystal structure of AChE (Sussman et al., 1991), two potential binding sites for THA were identified: a high-affinity tryptophan residue at position 84, deep in the catalytic gorge, and a low-affinity tryptophan residue at position 279, near the AChE surface. Optimal potency was reached experimentally by linking two THA groups with a 7-carbon methylene chain capable of spanning the distance between these two proposed binding loci. This bis-THA was 10,000-fold more selective and 1000-fold more potent than the parent compound in inhibiting AChE (Pang et al., 1996). More recently we applied a similar strategy to identify new bis-oxime analogs of 2-PAM with improved potency as AChE reactivators (Pang et al., 2003). Here, in studies on OP-treated rats, we have evaluated the most promising of these agents, a compound with a 7-methylene bridge linking two aldoxime moieties ortho to a pyridinium ring nitrogen.
METHODS

Drugs and reagents

Bis-oxime derivatives, previously synthesized as described by Pang et al. (2003), were designated by the orientation of the oxime moieties relative to the ring nitrogen and by the number of methylene groups in the linker chains (e.g., “ortho-7, “para-6”). Additional drugs and reagents (from Sigma-Aldrich, St Louis MO, unless indicated otherwise) were: atropine sulfate; 1,5-bis(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide (BW284c51); pyridine-2-aldoxime methochloride (2-PAM); 4-(hydroxyiminomethyl)-1-methylpyridinium iodide (4-PAM); diisopropylfluorophosphate (DFP); ethopropazine HCl; eserine salicylate; and echothiophate iodide (Wyeth-Ayerst, Philadelphia). Stock solutions of 1M DFP were prepared in 50% ethanol, then diluted in water or 0.9% NaCl (for animal injection). The remaining drugs and experimental bis-oximes were prepared in glass-distilled water.

Enzyme assay

Cholinesterase activities were assayed by a plate-reader version of the standard spectrophotometric method (Ellman et al., 1961), using 1 mM acetylthiocholine iodide as substrate. AChE activity and BChE activity were distinguished with selective inhibitors of BChE (ethopropazine, $10^{-4}$ M) or AChE (BW284c51, $10^{-5}$ M). Blanks contained the nonselective inhibitor, eserine ($10^{-4}$ M).

Cholinesterase inhibition and reactivation in vitro

Inactivation and reactivation of cholinesterases were, with a few exceptions, tested in preparations that allowed, first, addition and removal of an OP, second, addition and removal of reactivator, and finally, enzyme assay in the absence of external agents. For studies on AChE, human red blood cells (RBCs) obtained from a single donor under an IRB approved
protocol were lysed in ice-cold water; cell membranes were then isolated by 8 to 10 cycles of repeated centrifugation (15,000 x g, 20 min) and rinsing in 10 volumes of low ionic strength buffer (10 mM Tris-HCl, pH 7.4). Readily sedimenting BChE was obtained by adsorption of human plasma (Sigma-Aldrich) onto a non-inhibitory monoclonal BChE antibody (B2-12, 10⁻⁸ M; (Brimijoin et al., 1983)) linked to Pansorbin (Calbiochem, La Jolla CA) via rabbit anti-mouse IgG (Pel-Freeze, Rogers AR). After incubation of Pansorbin-BChE for 1 hr at 37 °C with OP (10⁻⁷ M echothiophate in 50 mM sodium phosphate buffer, pH 7.4), samples were centrifuged, drug was aspirated away, and the pellets were rinsed by re-suspension in buffer, centrifugation, and aspiration. In samples treated with a reversible inhibitor, nearly 100% of the original enzyme activity was recovered after the rinsing procedure. In contrast, only 2% of the original activity remained in rinsed samples that were exposed to echothiophate (data not shown). Thus with isolated RBC membranes and antibody-immobilized BChE we could produce stably inhibited preparations that were free of OP during the incubation with reactivator (also at 37 °C). By the same means, before assay of enzyme activity, reactivator was removed by centrifugation, aspiration, and resuspension of the pellet in Tris-HCl buffer. Because rinsing was not possible with serum BChE from rat and mouse, for which antibodies were not available, reactivators were simply added to such samples 1 hr after the OP.

Animal studies

Male adult rats (generally 250-350g, Harlan Sprague Dawley, Madison WI) were used according to the NIH Guideline for Care and Use of Laboratory Animals under a protocol approved by our Institutional Animal Care and Use Committee. Three basic experiments were performed: survival tests, "real time" reactivation assays, and "end point" assays. All began with an intramuscular (i.m.) injection of the competitive muscarinic receptor
antagonist, atropine sulfate (10 mg/kg, in 0.9% NaCl), given 5 min before the OP challenge. This step was intended to minimize toxic reactions from overstimulation of muscarinic acetylcholine receptors.

In survival tests, echothiophate in various i.m. doses was given 5 min after reactivator. Cholinergic signs were monitored, including muscle twitching, defecation, salivation, and labored respiration. Experience indicated that all rats still alive at 1 hr after OP challenge would ultimately recover; therefore, this interval defined "survival."

In "real time" experiments, small quantities of venous blood were drawn serially from the tail vein after an initial i.v. injection of anticoagulant (0.5 mg of sodium heparin in 0.5 ml 0.9% NaCl). Following the baseline blood sample, the rats were given i.m. injections of atropine sulfate (10 mg/kg) and echothiophate (0.083 mg/kg). Control rats received no further treatment, but the experimental group received 2-PAM (100 µmol/kg, i.m.) or ortho-7 (1 µmol/kg, i.m.) at 30 min after echothiophate injection. Between 15 and 90 min, a total of 6 blood samples (∙100 µl each) were drawn at 15-min intervals into heparinized tubes on ice. Plasma was separated by centrifugation and frozen immediately. Pelleted RBCs (∙40 µl) were lysed by re-suspension in 10 ml ice-cold water. The membranes were collected by centrifugation and stored in 1 ml water at -80°C. Hemoglobin contents of the diluted lysates, as measured by OD405, were recorded as a basis for normalization of data. At the time of assay, plasma was thawed, clarified by centrifugation, and diluted ten-fold in 0.1 M sodium phosphate buffer, pH 8. Thawed red cell preparations were rinsed by centrifugation, aspiration, and re-suspension in 1 ml 50 mM sodium phosphate buffer, pH 7.4, with 0.2% Triton X-100 and 0.1% BSA.
“End-point” experiments measured tissue cholinesterase activities at a single time point, 1 hr after treatment with echothiophate or DFP. In all cases, atropine sulfate and oxime were given 5 min before the OP; in one experiment, oxime dosing was repeated at 15-min intervals for a total of 4 injections. Blood samples were drawn by cardiac puncture at sacrifice, allowed to clot at room temperature for 30 minutes, and centrifuged to obtain serum for storage at -80 °C. At assay, the thawed serum was handled like plasma (described above). After blood sampling, the brain and diaphragm muscle were quickly harvested, weighed, and stored at -80 °C. Later, solid tissues were thawed and homogenized (PowerGen 700, Fisher Scientific) in 10 volumes of buffer (50 mM sodium phosphate, pH 7.2). An aliquot of each sample was centrifuged (25,000g, 15 min) in order to separate cell membranes from soluble components, including enzyme inhibitors. Pellets were then diluted in the original volume of a detergent-containing buffer (50 mM phosphate pH 7.4 + 1% Triton X-100 + 0.5 M NaCl), re-homogenized, centrifuged, and divided into aliquots for assay.
RESULTS

Chemical structures of the family of bis-oximes prepared in the Pang laboratory are shown in Fig. 1, while previously determined affinities and reactivation rate constants for interactions with AChE are listed in Table 1 (Pang et al., 2003). In general, increasing chain length led to increasing affinity for AChE (as measured either by Ki or Kd) but decreasing speed of reactivation (as measured by the rate constant, k₃). The overall efficiency for AChE reactivation is described by the ratio k₃/Kd. By this definition, the most effective reactivator, representing the optimal combination of rapid action and high affinity for phosphorylated AChE, was the 7-methylene linked agent designated “ortho-7”. This compound’s advantage over 2-PAM was due to a 370-fold increase in affinity that offset a nearly 5-fold drop in the reactivation rate constant. The large increase in reactivation efficiency was associated with a 100-fold leftward shift in the dose-response curves for reactivation of RBC AChE in vitro (Pang et al, 2003). Those results clearly warranted an evaluation of ortho-7 for its ability to reactivate cholinesterases in vivo. To prepare for such studies, we carried out additional in vitro experiments to examine the effects of oximes on BChE as well as AChE.

Reactivation of AChE and BChE in vitro

RBC membranes and serum from rat, mouse or human sources were exposed sequentially to echothiophate (1 hr, 10⁻⁷ M) and reactivator (1 hr, varying doses). In preliminary experiments (data not shown), AChE was readily reactivated and the bis-oximes were much more potent than 2-PAM. BChE, on the other hand, was resistant. Thus, although, 2-PAM was able to reactivate significant amounts of BChE from all three tested species, the required dosage of oxime was much higher than for AChE. Moreover, none of the tested bis-oximes, including ortho-6 through ortho-9 and para-6 through para-9, caused any BChE reactivation.
Definitive experiments with ortho-7 and 2-PAM on human AChE and BChE were performed with a centrifugation and rinsing protocol that permitted rapid removal of inhibitor before exposure to reactivator, and removal of reactivator before assay (see Methods). Although RBC membranes were already suitable for this purpose, plasma BChE was first selectively adsorbed to a monoclonal antibody. In these experiments, after exposure to echothiophate (10^-7 M), AChE was quantitatively reactivated by 1 hr treatment with oxime in micromolar concentrations (Fig. 2). By contrast, millimolar concentrations of 2-PAM were required to restore BChE activity to 90% of control, while ortho-7 did not reactivate BChE to a measurable degree under any condition. In fact, at sufficiently high concentrations, ortho-7 caused long-term inhibition of BChE (and, to a lesser extent, AChE). This inhibition may have been irreversible since BChE activity did not recover after prolonged rinsing (Fig. 3).

*Direct oxime toxicity*

The therapeutic potential of any oxime for treatment of OP exposure is determined not only by the agent’s inherent ability to reactivate AChE but also by its direct toxicity. Before conducting protection and rescue experiments on OP-treated rats, we determined how well ortho-7 itself was tolerated. Groups of 4 to 6 rats given various doses of this compound by i.m. injection were closely monitored up to 1 hr for cholinergic signs. In doses of 1 µmol/kg and below, ortho-7 produced no clear signs of acute toxicity and no deaths. At 3 µmol/kg, frank signs emerged, chiefly nicotinic effects in the form of uncoordinated muscle twitching and fasciculation, but wheezing and difficulty in respiration were also notable. This dose was near the LD50, since 4 of 6 animals died. Doses of 5 and 10 µmol/kg uniformly caused death within 15 to 20 min, apparently from respiratory failure (n = 5 and 4, respectively). In light of
these findings, we used 1 µmol/kg as the upper limit for single or repeated doses of ortho-7 in animal studies of reactivation.

**Reactivation and rescue from OP toxicity in vivo**

Success with AChE reactivation in vitro led us to test the ability of ortho-7 to promote survival in rats challenged with i.m. echothiophate, an agent whose actions are largely confined to the periphery. In order to minimize toxicity from the OP, all rats were pre-treated with atropine, 10 mg/kg; reactivator was administered prophylactically at the same time. Most unprotected rats died after receiving echothiophate in doses above 0.15 mg/kg, which was the apparent LD50. Pretreatment with ortho-7 at 1 µmol/kg increased the estimated LD50 by 10-fold or more, but an equal dose of 2-PAM failed to promote survival (Fig. 4). Even a 10-µmol/kg dose of 2-PAM produced only a 5-fold shift in estimated LD50. On the other hand, 2-PAM at 100 µmol/kg was significantly more effective than ortho-7 at 1 µmol/kg. Because complete dose-response curves with echothiophate were not determined for all oxime treatments, we cannot calculate relative oxime potencies with precision. Nonetheless, an inspection of the data in Fig. 4 demonstrates that ortho-7 was over an order of magnitude more potent than 2-PAM in preventing death from echothiophate exposure.

It seemed likely that enzyme reactivation accounted for the improved survival in rats protected with ortho-7. To confirm this idea, rats were prepared for repeated blood sampling so we could monitor reactivation in "real time" (Fig. 5). After echothiophate treatment (0.083 mg/kg), plasma AChE and BChE activity dropped within 15 min to levels that were below the detection limit in our standard assay. Little spontaneous recovery occurred during the next 75 min, and the animals required periodic assistance with respiration. However, injection of ortho-7 (1 µmol/kg) at 30 min induced plasma AChE reactivation that was statistically...
significant by 15 min and continued steadily for 1 hr, reaching a final level of 70%.

Interestingly, cholinergic signs began to abate very early, generally within 2 min of oxime injection. A 100-fold larger injection of 2-PAM (100 µmol/kg) reactivated plasma AChE somewhat faster, at first, but enzyme recovery ceased after 15 min. As a result, the final activity measured 1 hr after 2-PAM was only about half as great as in rats given ortho-7.

Effects on RBC AChE were similar to those on plasma, except that ortho-7 acted as rapidly as 2-PAM, and again reached a plateau with twice as much AChE reactivation. As expected, ortho-7 did not reactivate plasma BChE in vivo; somewhat surprisingly, neither did 2-PAM.

**Ortho-7 reactivation effects largely confined to peripheral tissues**

Although CNS effects are a major component of OP toxicity (Inns and Leadbeater, 1983; Shih, 1993; Wolthuis et al., 1994), classical oxime reactivators like 2-PAM do not cross the blood brain barrier (Bodor and Brewster, 1983). In order to determine whether ortho-7 or another bis-oxime might be superior in this respect, we treated rats with DFP, which, unlike echothiophate, readily inactivates brain AChE after systemic administration. To promote survival, atropine (1 mg/kg) was given along with reactivator (1 µmol/kg of ortho-7 or 100 µl/kg of 2-PAM), 10 min before the challenge with DFP (2 mg/kg). One group of animals received multiple doses of reactivator (no additional atropine) at 15 min intervals. Each rat showed severe cholinergic signs, including muscle fasciculation and breathing difficulty, but even without reactivator all survived until tissues were harvested, one hour after DFP. This survival was impressive, considering that the DFP treatment reduced measured brain AChE activity by more than 95%. Fasciculation and breathing distress were reduced in rats that received reactivator, but weakness, lethargy and irritability (aggressive behavior and sensitivity to noise) persisted. Rats that received multiple doses of reactivator were similarly
compromised. This pattern contrasted with the nearly complete reversal of cholinergic signs when oxime was given to rats challenged with echothiophate, which did not inhibit brain AChE.

In line with the persistent behavioral signs despite improved motor function, “end-point” determinations of enzyme activity showed that neither 2-PAM nor ortho-7 relieved DFP-induced enzyme inhibition in brain. Even multiple doses of ortho-7 failed to restore meaningful amounts of AChE or BChE activity in that tissue (Fig. 6). In fact, such intensive treatment on its own caused a 45% inhibition of brain BChE. BChE in serum and diaphragm also resisted reactivation, as in vitro, but a single dose of ortho-7 caused 5-15% recovery of AChE activity in these tissues, and multiple doses caused 40-50% recovery. The recovering AChE activities in serum and muscle were highly correlated ($r^2 = 0.91$), indicating that blood AChE can be a reliable index of reactivation in a peripheral tissue following DFP exposure.
DISCUSSION

Kinetic properties of bis-oxime reactivators

Our rationale for producing and characterizing bis-oximes was that the presence of two quaternary ammonium groups with appropriate spacing should promote high-affinity binding to phosphorylated AChE, as explained elsewhere in more detail (Pang et al., 2003). We anticipated that the resulting gain in potency would be of practical use in reactivating the enzyme after OP exposure. Over time, and starting many years ago, a number of bis-oximes have been produced in other labs (Poziomek et al., 1958; Wilson and Ginsberg, 1958; Su et al, 1986). The most extensive series reported (Patocka and Bielavsky, 1972), comprised homodimeric compounds with chains of 1,2,3,4,5,6,8 or 10 methylenes linking two identical para-oxime molecules. In that series, the n=3 compound was the best reactivator, with a bimolecular rate constant of 3.1 x 10^{-3} M, an inhibition constant of 3.8 x 10^{-4} M, and a dissociation constant of 6.4 x 10^{-5} M. The investigators noted an inverse linear relationship in this series between chain lengths and the inhibition constants for bovine AChE. At the time of that study, the structure of AChE's active site gorge had not yet been solved, so the significance of this relationship was incompletely understood. Our results with both para and ortho bis-oximes also revealed a strong relationship between chain length and the kinetic constants that define reactivation (Table 1). Our data differ in detail from those reported earlier, but they agree in general terms where comparison is possible. It is now apparent that bis-oximes of appropriate structure, with optimal spacing between reactive centers, are much more potent than the conventional pralidoxime in reactivating "irreversibly" inhibited AChE. The optimal spacing for enhanced potency is in line with the actual distance between central and peripheral "anionic" sites in the AChE crystal structure (Sussman et al., 1991) and with
our recent results from computational models of bis-oxime/AChE complexes (Pang et al., 2003).

Both 2-PAM and ortho-7 were more effective with echothiophate-treated AChE than with DFP-treated AChE. This result might reflect re-inhibition of enzyme by phosphorylated oxime after reactivation (Schoene, 1972; Ashani et al., 1998; Luo et al., 1999). Such a phenomenon, however, would probably occur with both of the tested organophosphates. A more plausible reason for differential reactivation is that the enzyme-DFP complex "ages" more rapidly, becoming unable to relinquish its phosphate group because of a dealkylation of the alkoxy group (Main, 1979). In any event, the present observations do not suggest that available bis-oximes offer dramatic advantages in dealing with even more rapidly aging agents like Soman, crucial in the context of defense against bio-terrorism and chemical warfare. Solving that problem will require novel strategies based on the evolving understanding of the molecular architecture and structural basis for reactivation (Masson et al., 1997), as will be discussed further below.

**Selectivity of reactivation for AChE**

Many oximes are weaker reactivators of BChE than AChE (Worek et al., 1999) but it was noteworthy that our tested bis-oximes failed to restore any BChE activity in plasma samples treated with echothiophate. A possible reason for the reduced efficacy with this enzyme is that the active site of BChE is larger than that of AChE (Saxena et al. 1997) and better accommodates phosphorylated oximes that are generated during reactivation. The phosphorylated oximes can then inhibit the regenerated BChE by blocking its active site or by re-phosphorylating the newly active enzyme. As regards ortho-7, the peripheral "anionic" site of AChE that binds well with the aldoxime group (Pang et al., 2003) has evolved in BChE to
bind well with the hydrophobic butyl group but not with the hydrophilic aldoxime group. Therefore ortho-7 does not fit electrostatically at the active site of BChE and can hardly restore any BChE activity. This selectivity is consistent with our previous report that bis-THA is 10,000-times more selective for AChE than for BChE, due to the same structural difference (Pang et al., 1996). Pending a final resolution, however, and bearing in mind the limited influence of this enzyme on cholinergic homeostasis, BChE reactivation may be considered of minor importance.

Parallelism in vitro and in vivo

The present data show that the large advantage in potency of ortho-7 over 2-PAM in vitro carries over to a great extent in vivo. Given the uncertainties about the metabolism and distribution of new compounds in live organisms, this degree of parallelism could not have been assumed a priori. The results underscore the power of computer simulations with AChE for rational design of inhibitors and reactivators. Computational and kinetics studies of AChE by ourselves (Pang et al., 2003) and others (Ashani et al., 1995; Grosfeld et al., 1996) confirm that certain bis-oxime reactivators have markedly higher affinities than mono-oximes, owing to their simultaneous interactions with central and peripheral binding sites. Similar work with BChE can also contribute to our understanding of the specificity of cholinesterase inhibitors and reactivators (Radic et al., 1993).

Two unexpected findings from the in vivo studies were the early reversal of cholinergic signs and the inhibition of brain BChE by multiple systemic injections of ortho-7. We suspect that cholinergic signs begin to recede when small amounts of restored enzyme activity cause acetylcholine levels to start declining at relevant receptor sites. In short, the waning of cholinergic signs may be more sensitive than the gross level of biochemically determined
AChE activity as an index of enzyme recovery. However, the earliest blood sample in our “real time” experiment was drawn at 15 min. Hence it is possible that, at 2 min, when cholinergic signs were fading, AChE recovery might have proceeded farther than would appear from the time course in Fig. 5. As for the surprising and selective effect of ortho-7 in brain, our data suggest that this oxime did cross the blood brain barrier in doses that were too low to reactivate AChE but high enough to inhibit BChE.

**Prospects for better AChE reactivators**

Present results indicate the potential value of bis-oximes for prophylaxis or treatment of poisoning with organophosphorus anticholinesterases. Although ortho-7 exhibited toxicity at high concentrations, this toxicity may not be practically relevant since, as our data show, a sub-toxic 1 µmol/kg dose can protect animals from echothiophate in concentrations at least 10-fold higher than the LD50. The greatest need for improvement in AChE reactivation appears to be in two areas: 1) delivery of reactivator to the brain, and 2) ability to reactivate AChE after exposure to rapidly “aging” inhibitors. Strategies to improve delivery might involve design of new pyridinium aldoxime salts with reduced solvation energies, linkage to carriers that facilitate entry into the CNS, or construction of pro-drugs. Some success along these lines has been obtained with pro-2-PAM (Bodor et al., 1975; Bodor et al., 1981). As for improved effectiveness against the most problematic organophosphorus inhibitors, there has been progress in generating oxime enantiomers with more favorable stereochemistry (Taylor et al., 1999; Wong et al., 2000). Combinations of reactivating drug with a bacterial organophosphate hydrolase have yielded promising results (Dumas et al., 1990; Tuovinen et al., 1996; Ashani et al., 1998) as has the addition of enzymes or ligands to prevent re-inhibition of enzyme by phosphorylated oxime (Broomfield, 1992; Luo et al., 1998; Leader et
al., 1999; Luo et al., 1999; Kiderlen et al., 2000). Ultimately, aided by computer simulations of powerful new nucleophiles (Pang et al., 2003), one might even hope to discover reagents that will drive the de-phosphorylation of a fully aged ACHe-OP complex. Meanwhile, it is worth considering the possible benefits of administering a fast-acting mono-oxime (such as 2-PAM) together with a slower-acting but ultimately more efficient bis-oxime (i.e., ortho-7).

The continued world-wide exposure of workers to pesticides, the potential use of anticholinesterases agents in acts of war or terrorism, and the high incidence of self-inflicted poisoning by these compounds (Nigg and Knaak, 2000; Eddleston et al., 2002) all speak to a need for vigorous and sustained research efforts in this area.
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FIGURE LEGENDS

Figure 1. Chemical structure of bis-pyridinium aldoximes. Bis-oximes were previously synthesized (Pang et al, 2003) with spacer elements of methylene units (n = 2 to 9) linking the oxime moieties at the ring nitrogen, which is oriented at position 2 (ortho), 3 (meta) or 4 (para) relative to the oxime.

Figure 2. Reactivation of AChE and BChE by 2-PAM and ortho 7-bis-oxime. AChE in washed membranes from human red blood cells, and human plasma BChE bound to monoclonal antibody, were irreversibly inactivated by echothiophate. Samples were then reactivated in the absence of inhibitor, and finally assayed in the absence of oxime (see Methods). Shown is the recovery of cholinesterase activity after 1 hr treatment with the indicated concentrations of oxime. Data (means of triplicate determinations) are graphed as percentages of the activities measured in comparable preparations not exposed to organophosphate or oxime.

Fig 3. Irreversible cholinesterase inhibition by ortho-7 bis oxime. AChE on human red cell membranes and antibody-bound BChE were treated at room temperature for 1 hr with the indicated concentrations of oxime. Before assay, red cell membranes were rinsed three times, 5 min each, with > 10 volumes of 50 mM sodium phosphate, pH 7.4; BChE preparations were rinsed in the same manner, once, twice, or three times (BChE 1, BChE 2, BChE 3). Shown are means of duplicate determinations.
Figure 4. Oxime protection against lethality from echothiophate. Before challenge with i.m. organophosphate, rats received i.m. injections of atropine (10 mg/kg) and reactivator (in doses of 1, 10, or 100 µmol/kg, as indicated). Shown are the percentages of animals surviving in each treatment group (n = 4 to 10 per group, 130 rats overall).

Figure 5. Real-time reactivation after echothiophate. Tail veins were cannulated for repeated collection of small blood samples over a 90 min period. After a baseline sample was drawn, echothiophate (0.083 mg/kg, i.v.) was administered followed 30 min later by reactivator (2-PAM, 100 µmol/kg, or ortho-7, 1 µmol/kg, i.m.). Shown are the AChE activities in red blood cells (top) and the AChE and BChE activities in plasma (bottom). Data are means ± SEM from groups of 3 to 4 rats each. * (significantly different from group receiving other reactivator, p < 0.05).

Figure 6. Protecting tissue cholinesterases from DFP. Naïve and ortho-7-pretreated rats were challenged with DFP (2 mg/kg, i.m). Some rats received only oxime pretreatment (1-ox); others received 3 more oxime injections at 15-min intervals (4-ox). Comparable groups received 1 or 4 doses of ortho-7 without DFP. Tissues were harvested 1 hr after DFP exposure for assay of BChE and AChE. Data (means ± SEM) are percentages of the mean values from control rats not exposed to organophosphate or oxime. † (significantly different from control, p < 0.05); †† (significantly different from control, p < 0.01); ** (significantly different from DFP only, p < 0.001).
Table 1. Kinetic Constants for Oxime Reactivators vs AChE

<table>
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<th>compound</th>
<th>n</th>
<th>Kᵢ (µM)</th>
<th>Kᵣ (µM)</th>
<th>k₃ (min⁻¹)</th>
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- number of methylene groups used to tether two pyridiniumaloximes.

Inhibition constants (Kᵢ) indicate oxime affinities for free AChE; dissociation constants (Kᵣ) indicate affinities for phosphorylated AChE; reactivation rate constants (k₃) indicate intrinsic reactivities with phosphorylated AChE; the ratios k₃/Kᵣ indicate overall reactivator efficiency. Data are from Pang et al. (2003).
bis-pyridinium aldoximes

"para"

"meta"

"ortho"

Figure 1 JPET 53405
Fig 2 JPET 53405

Percent reactivation

AChE

BChE

Oxime concentration (M)

-10 -8 -6 -4 -2

2 PAM
ortho-7
Fig 3 JPET 53405
Fig. 4 JPET 53405
Time (min) after OP

Cholinesterase activity (% control)

RBC

Plasma

oxime

Fig 5 JPET 53405

AChE

BChE

+ 2-PAM

+ ortho-7

+ 2-PAM

+ ortho-7