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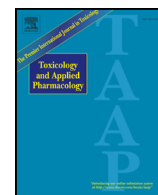

Quaternary and tertiary aldoxime antidotes for organophosphate exposure in a zebrafish model system

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ABSTRACT

The zebrafish is rapidly becoming an important model system for screening of new therapeutics. Here we evaluated the zebrafish as a potential pharmacological model for screening novel oxime antidotes to organophosphate (OP)-inhibited acetylcholinesterase (AChE). The k_i values determined for chlorpyrifos oxon (CPO) and dichlorvos (DDVP) showed that CPO was a more potent inhibitor of both human and zebrafish AChE, but overall zebrafish AChE was less sensitive to OP inhibition. In contrast, aldoxime antidotes, the quaternary ammonium 2-PAM and tertiary amine RS-194B, showed generally similar overall reactivation kinetics, k_r, in both zebrafish and human AChE. However, differences between the K_{ox} and k_2 constants suggest that zebrafish AChE associates more tightly with oximes, but has a slower maximal reactivation rate than human AChE. Homology modeling suggests that these kinetic differences result from divergences in the amino acids lining the entrance to the active site gorge. Although 2-PAM had the more favorable in vitro reactivation kinetics, RS-194B was more effective antidote in vivo. In intact zebrafish embryos, antidotal treatment with RS-194B rescued embryos from OP toxicity, whereas 2-PAM had no effect. Dechorionation of the embryos prior to antidotal treatment allowed both 2-PAM and RS-194B to rescue zebrafish embryos from OP toxicity. Interestingly, RS-194B and 2-PAM alone increased cholinergic motor activity in dechorionated embryos possibly due to the reversible inhibition kinetics, K_i and αK_i, of the oximes. Together these results demonstrate that the zebrafish at various developmental stages provides an excellent model for investigating membrane penetrant antidotes to OP exposure.

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Introduction

Organophosphates (OPs) are a class of compounds that have been used both as insecticides and as chemical warfare agents (Soreq and Seidman, 2001). The toxicity of OPs is primarily due to their inhibition of acetylcholinesterase (AChE) through phosphorylation of the active site serine (Wilson and Bergmann, 1950; Aldridge and Reiner, 1972; Taylor et al., 1995). The inhibition of AChE leads to excessive stimulation of cholinergic neurons in both the central and peripheral nervous systems causing a sequelae of symptoms including seizures, muscle fasciculation, excessive salivation, gastrointestinal hyperactivity, and in the case of acute poisoning, death (Taylor et al., 1965; Balali-Mood and Saber, 2012). Thus OP poisoning represents a significant health threat due to agricultural exposure risks (Bouchard et al., 2011), intentional poisonings (Aardema et al., 2008), and acts of terrorism (Ohbu et al., 1997).

Current treatments for OP poisoning include muscarinic anticholinergic drugs, anticonvulsant drugs, and cholinesterase-reactivating agents (Antonijevic and Stojiljkovic, 2007; Masson, 2011). Cholinesterase-reactivating agents include pyridinium aldoximes, such as 2-PAM, TMB-4, LüH-6, and HI-6 (Taylor, 2011; Balali-Mood and Saber, 2012). Oximes accelerate the hydrolysis of phosphorylated AChE via nucleophilic attack, allowing for enzyme reactivation and restoration of enzyme activity (Taylor et al., 2007). Antidotal treatment with oximes has been shown to improve clinical outcomes after OP poisoning (Kusic et al., 1991; Willems et al., 1993; Ohbu et al., 1997; Balali-Mood and Shariat, 1998; Thiermann et al., 1999). However, existing oximes are not equally effective against all classes of OPs (Antonijevic and Stojiljkovic, 2007). Even within a single class of methylphosphonate or alkylphosphonate agents, considerable variation of oxime antidote efficacy exists.

A more significant issue with currently available pyridinium aldoximes is their inability to cross the blood–brain barrier (BBB) due to the cationic quaternary nitrogen (Shih et al., 2010). Current clinically approved oxime antidotes for OP poisoning have limited BBB penetrance, with brain levels reaching only 3–10% of plasma levels (Shrot

Abbreviations: AChE, acetylcholinesterase; hAChE, human acetylcholinesterase; zAChE, zebrafish acetylcholinesterase; ATCh, acetylthiocholine; BBB, blood–brain barrier; BSA, bovine serum albumin; CPO, chlorpyrifos oxon; DDVP, dichlorvos; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); hpf, hours post-fertilization; OP, organophosphate.

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et al., 2009). Consequently, these antidotal treatments do not protect against OP-induced CNS neurotoxicity leading to chronic neurological issues (Okumura et al., 2005). Therefore, the development of BBB penetrant antidotes is of paramount importance. To address this problem, several compounds with increased BBB penetrance have been developed. The uncharged Pro-2-PAM was shown to have increased BBB penetrance and improved CNS outcomes in soman exposed guinea pigs (Demar et al., 2010), but it had limited efficacy against sarin, cyclosarin, or VX induced CNS toxicity (Boskovic et al., 1980; Shih et al., 2011). Several neutral and ionizable amine compounds have been developed with strong reactivation kinetics and a non-ionized fraction that should cross the BBB (Mercey et al., 2011, 2012a,2012b; Sit et al., 2011; Renou et al., 2013; Kliachyna et al., 2014). A promising new compound is the N-substituted 2-hydroxyiminoacetamido alkylamine, RS-194B, a zwitterionic oxime with an ionizable tertiary nitrogen with a comparable pKa to that of the oxime group (Radic et al., 2012, 2013). RS-194B has in vitro kinetic parameters superior or comparable to 2-PAM in human AChE (Radic et al., 2012, 2013), and has been shown to be therapeutically effective in mice in which it exhibited a relatively high degree of BBB penetration alongside low toxicity (Radic et al., 2012). Mice treated with RS-194B before and after VX, or sarin exposure recovered better than those exposed to 2-PAM (Radic et al., 2012). However, further in vivo testing in other animal species is needed to confirm RS-194B's efficacy and to better understand what modifications could be made to improve its utility as an antidote.

A promising new model system in which to screen BBB penetrant antidotes is the zebrafish (*Danio rerio*). Zebrafish embryos have already become a popular model to study behavioral and physiological influences of OPs and other compounds due to their rapid development, transparent embryo and larvae, and genetic and physiological similarities to mammalian vertebrates (Linney et al., 2004; Fraysse et al., 2006; Peterson et al., 2008; Selderslaghs et al., 2010; Watson et al., 2014). Specifically, the amino acid sequence of zebrafish AChE (zAChE) is 62% identical to mammalian AChE and key residues in the catalytic triad, acyl binding pocket, and choline binding pocket appear to be conserved (Bertrand et al., 2001). In addition, there is no evidence for a zebrafish gene encoding butyrylcholinesterase, and a significant majority of ACh hydrolytic activity seems to be from zAChE, as opposed to other esterases (Bertrand et al., 2001; Kuster, 2005). This simplifies the study of AChE in the zebrafish. However, some catalytic differences between zAChE and mammalian AChE are evident. While k_{cat} for zAChE seems to be half that of mammalian AChE, zAChE is 5–10 fold more sensitive to peripheral anionic site inhibitors (Bertrand et al., 2001). It has been proposed that this increased sensitivity may result from substitution of tyrosine for phenylalanine at position 70 (Bertrand et al., 2001). Considering these differences, it is important to ensure that interactions between zAChE and OP inhibitors and their oxime reactivators resemble those observed in human AChE (hAChE) sufficiently to warrant the use of zebrafish as an in vivo model.

Here we assess the suitability of zebrafish as a model organism for the in vivo evaluation of novel oxime antidotes as AChE reactivators. Specifically, we conducted a detailed structural and kinetic comparison of zebrafish and human AChE. We compared the reactivation kinetics of zebrafish and human diethylphosphoryl and dimethylphosphoryl AChE conjugates by a novel and established oxime reactivators, RS-194B and 2-PAM respectively. Additionally, we evaluated the in vivo antidotal efficacy of RS-194B and 2-PAM in zebrafish embryos exposed to CPO and DDVP. These studies affirmed that the zebrafish is a good pharmacological model for toxicity endpoints and efficacy screening of newly developed oximes or nucleophiles.

Materials and methods

In silico modeling of zAChE. Sequence alignments of hAChE (P22303) and zAChE (Q9DDE3) were performed using the public UniProt protein database. Using an optimized sequence alignment and the three dimensional crystal structure of hAChE, UniProt's modeling tools generated a hypothetical three-dimensional structure for zAChE. Both the hypothetical zAChE structure and the known crystal structure of hAChE were visualized and superimposed using Discovery Studio Visualizer versions 3.5 and 4.0 (Accelrys, San Diego).

Chemicals. Chlorpyrifos oxon (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphate), dichlorvos (2,2-dichlorovinyl dimethyl phosphate), and 2-PAM (2-pyridine aldoxime methyl chloride) were purchased from Sigma-Aldrich (St. Louis MO, USA) and stored until use at −20 °C. The novel oxime reactivator RS-194B was synthesized and characterized as described (Sit et al., 2011; Radic et al., 2012). Stock solutions of chlorpyrifos oxon (CPO) and dichlorvos (DDVP) were prepared in methanol. Stock solutions of 2-PAM were prepared in 0.1 M sodium phosphate buffer, pH 7.4, and RS-194B was dissolved in a small amount of HCl and diluted with 0.1 M sodium phosphate buffer for in vitro studies as previously described (Radic et al., 2011) and methanol for in vivo studies. CPO forms a representative diethylphosphoryl and DDVP forms a representative dimethylphosphoryl AChE conjugate.

Zebrafish in vivo assay. Adult zebrafish were maintained at 28 °C on a 14 h light:10 h dark photoperiod. Animals were maintained according to the NIH Office of Animal Health and Laboratory Welfare. One day prior to spawning, 5–8 adult fish were transferred to a spawning tank. Embryos were collected 1–2 h post-fertilization (hpf) and transferred to 100 mm Petri dishes containing 25 mL of egg water (50 mg sea salt/L) and incubated at 28 °C on the same photoperiod as the adults. At 12 hpf, embryos were exposed to either OP (2 μM CPO or 100 μM DDVP) or vehicle control. These OP concentrations were chosen since preliminary studies showed that they caused a statistical increase in spontaneous movements without significant embryo mortality. Methanol concentrations did not exceed 0.1%. At 24 hpf, embryos were transferred to new 100 mm Petri dishes with 25 mL of egg water and either 100 μM 2-PAM, 100 μM RS-194B, or vehicle control. To examine the effect of the chorion on oxime efficacy, embryos were dechorionated prior to exposure to oxime or vehicle control using fine forceps. Spontaneous movements, defined as flexing or side to side motion of the trunk or tail, were then sampled in triplicate for ten randomly selected embryos in each condition as described previously (Watson et al., 2014). At 48 hpf, embryo survival was evaluated for dechorionated embryos. For in vivo studies, a minimum of ten replicates per experimental condition were measured for a minimum of three independent trials. Results were expressed as means ± SEM and analyzed by one-way ANOVA followed by Tukey post-hoc tests. A $P < 0.01$ was considered statistically significant.

Enzyme preparation. Recombinant human acetylcholinesterase (hAChE) was prepared as previously described (Cochran et al., 2011; Sit et al., 2011). Zebrafish protein homogenates were prepared from commercially purchased adult zebrafish. All experiments were carried out using procedures according to the NIH Office of Animal Health and Laboratory Welfare. Euthanized specimens were ground to a fine powder under liquid nitrogen using a mortar and pestle, and suspended in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.01% bovine serum albumin (BSA) and 1% Triton X-100. The sample was sonicated briefly and spun at 10,000 *g* for 10 min at 4 °C. The supernatant containing zAChE was used to examine inhibition and reactivation kinetics.

In vitro organophosphate inhibition assays. For both hAChE and zAChE, activities were measured using a spectrophotometric assay (Ellman et al., 1961) at 25 °C in 0.1 M sodium phosphate buffer, pH 7.4, with 0.01% BSA, 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 1.0 mM acetylthiocholine (ATCh). Final concentrations of Triton X-100 in the samples were at or below 0.001%. We compared the inhibition kinetics for zAChE to hAChE for a representative diethyl-OP (CPO) and

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