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Testing of novel brain-penetrating oxime reactivators of acetylcholinesterase inhibited by nerve agent surrogates

Janice E. Chambers [a,*,1], Howard W. Chambers [b,1], Edward C. Meek [a], Ronald B. Pringle [a]

[a] Center for Environmental Health Sciences, College of Veterinary Medicine, P.O. Box 6100, Mississippi State University, Mississippi State, MS 39762-6100, USA
[b] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, P.O. Box 9775, Mississippi State University, Mississippi State, MS 39762-9775, USA

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ABSTRACT

A critical need for combating the effects of organophosphate (OP) anticholinesterases, such as nerve agents, is the current lack of an effective oxime reactivator which can penetrate the blood–brain barrier (BBB), and therefore reactivate inhibited acetylcholinesterase (AChE) in the brain. Our laboratories have synthesized and have initiated testing of novel phenoxyalkyl pyridinium oximes (patent pending) that are more lipophilic than currently approved oximes. This is a preliminary report on these novel oximes which have been tested *in vitro* in rat brain homogenates with highly relevant surrogates for sarin (phthalimidyl isopropyl methylphosphonate; PIMP) and VX (nitrophenyl ethyl methylphosphonate; NEMP). The oximes demonstrated a range of 14–76% reactivation of rat brain AChE *in vitro*. An *in vivo* testing paradigm was developed in which the novel oxime was administered at the time of maximal brain AChE inhibition (about 80%) (1 h) elicited by nitrophenyl isopropyl methylphosphonate (NIMP; sarin surrogate). This paradigm, with delayed administration of oxime to a time when brain AChE was starting to recover, was designed to minimize reactivation/reinhibition of peripheral AChE during the reactivation period which would decrease the availability of the surrogate for entry into the brain; this paradigm will allow proof of concept of BBB penetrability. The initial studies of these oximes *in vivo* with the sarin surrogate NIMP have indicated reactivation of up to about 25% at 30 min after oxime administration and substantial attenuation of seizure behavior from some of the oximes. Therefore these novel oximes have considerable potential as brain-protecting therapeutics for anticholinesterases.

1. Introduction

The organophosphate (OP) anticholinesterase class includes the nerve agents and some insecticides which are potent and persistent inhibitors of acetylcholinesterase (AChE) in both the central and peripheral nervous systems. The inhibition of AChE from exposure to high levels of OP anticholinesterases leads to the accumulation of the neurotransmitter acetylcholine in synapses and neuromuscular junctions and thus hypercholinergic activity that results in excitotoxicity, seizures, brain damage and long-term behavioral aberrations, including cognitive deficits [1]. Therefore, the protection of the military and the public from the consequences of potential OP poisoning is of considerable importance in order to restore both the physiological functions and cognitive functions that are impacted by nerve agent poisoning.

At present the approved therapy for OP anticholinesterase poisoning is the administration of a pyridinium oxime, such as 2-PAM in the United States, which can reactivate the inhibited AChE and restore its function. However, because 2-PAM cannot effectively cross the blood–brain barrier (BBB), it reactivates AChE appreciably only in the peripheral nervous system and it fails to prevent excitotoxicity and subsequent brain damage [2,3]. Most other known oximes under development in the US and in Europe are also ineffective at crossing the BBB [4,5]. A centrally-active oxime reactivator that could attenuate or prevent seizures, and consequently attenuate or prevent neuropathology, would constitute a substantial improvement for protecting people from the long-term effects of OP's.

Our laboratories have been involved for many years in the study of OP anticholinesterases. Because our laboratories are not authorized to use live nerve agents, we have developed and described some highly relevant surrogates for sarin and VX that have the

Abbreviations: AChE, acetylcholinesterase; ANOVA, analysis of variance; BBB, blood–brain barrier; DMSO, dimethyl sulfoxide; K_{ow}, octanol–water partition coefficient; NEMP, nitrophenyl ethyl methylphosphonate; NIMP, nitrophenyl isopropyl methylphosphonate; OP, organophosphate; 2-PAM, pralidoxime chloride; PIMP, phthalimidyl isopropyl methylphosphonate.

* Corresponding author. Tel.: +1 (662) 325 1255; fax: +1 (662) 325 1031.
E-mail addresses: chambers@cvm.msstate.edu (J.E. Chambers), hwc2@msstate.edu (H.W. Chambers), emeek@cvm.msstate.edu (E.C. Meek), rbp62@msstate.edu (R.B. Pringle).

[1] Contributed equally to this manuscript.

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leaving group of these nerve agents substituted with a different chemical moiety [6]. We have also used a sarin surrogate originally described by Ohta et al. [7]. Thus these surrogates leave AChE inhibited by the same chemical moiety as from sarin or VX, and are highly suitable chemicals to study the efficacy of potential reactivators. We have used these nerve agent surrogates to produce seizure behavior in experimental animals. Our laboratories have produced a series of novel phenoxyalkyl oximes (patent pending) that were designed to be more lipophilic than traditional oximes and are hypothesized to have more likelihood to cross the BBB and therefore be effective reactivators in the central nervous system. This paper constitutes a preliminary report on this series of novel oximes with respect to their *in vitro* efficacy as AChE reactivators with nerve agent surrogates, their lipophilicity and initial data on *in vivo* efficacy as reactivators. Because of space constraints in the present paper, synthesis methods and further chemical and biological characterization of the oximes will be discussed in subsequent publications.

2. Materials and methods

2.1. Materials

2.1.1. Nerve agent surrogates

Two sarin surrogates and a VX surrogate have been synthesized and characterized in our laboratories [6]. The sarin surrogate that has been used in *in vitro* reactivation studies is phthalimidyl isopropyl methylphosphonate (PIMP), which is unstable in aqueous solution, degrading within about 15 min; PIMP has been used in these *in vitro* efficacy studies to prevent appreciable reinhibition of reactivated AChE. Nitrophenyl isopropyl methylphosphonate (NIMP), originally described by Ohta et al. [7], is stable in aqueous solution and so was used *in vivo*. The VX surrogate is nitrophenyl ethyl methylphosphonate (NEMP) [6].

2.1.2. Novel oxime reactivators

The novel oximes, which are substituted phenoxyalkyl pyridinium oximes, have incorporated lipophilic moieties with the expectation that the charge of the quaternary ammonium will be, in part, counterbalanced by the lipophilic moiety that will impart to the oxime an ability to cross the BBB. Concurrently, there is the expectation that substantial reactivating ability of the pyridinium moiety will be retained. The general structure of this oxime series is shown in Fig. 1 and the specific substitutions are listed in Table 1.

2.1.3. Animals

Adult male Sprague Dawley-derived rats were used as the animal model for *in vitro* and *in vivo* screening of the oximes. All animal protocols received prior approval from the Mississippi State University Animal Care and Use Committee.

2.2. Methods

2.2.1. Lipophilicity

Octanol–water partition coefficients (K_{ow}'s) were determined by standard methods as an index of lipophilicity.

2.2.2. In vitro reactivation screening

The screening of novel oximes for reactivation potential was conducted with PIMP and NEMP with homogenates of rat brain (40 mg/ml in 50 mM Tris HCl buffer, pH 7.4 at 25 °C) serving as the AChE source. A concentration of surrogate (in ethanol) was determined that yields about 80% rat brain AChE inhibition in 15 min at 37 °C. These concentrations were determined to be 175 nM for PIMP and 56 nM for NEMP; therefore these surrogates are potent anticholinesterases, suitable for testing reactivation efficacy of oximes.

Following a 15 min incubation of the rat brain homogenate with PIMP or NEMP, the oxime in DMSO:ethanol (1:1) at 100 μM was added for an additional 30 min incubation for reactivation. The reaction mix was then diluted to 1 mg brain wet weight/ml, and AChE was assayed spectrophotometrically using 1 mM acetylthiocholine as the substrate in a discontinuous 15 min assay by our modification of the Ellman et al. [8] method [9]. The inhibition and reactivation were calculated compared to solvent controls. The novel oximes were screened using at least three replications for each oxime. The criterion for bringing each oxime forward for *in vivo* screening was at least 40% reactivation in the *in vitro* screening.

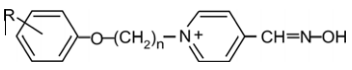

Fig. 1. Generic structure of substituted phenoxyalkyl pyridinium oximes.

2.2.3. In vivo reactivation screening

A dosage of the NIMP was determined that yielded a peak brain AChE inhibition of about 80%, a high sublethal dosage that did not require atropine or other therapies for survival. NIMP was administered ip in a DMSO vehicle to insure rapid absorption. The dosage was 0.325 mg/kg and the time of maximal inhibition was 1 h, with AChE activity showing some recovery after the 1 h peak [6]. At the time of maximal inhibition the oxime was administered at 0.1 mmol/kg in DMSO im; this dosage is approximately the molar equivalent of the approved dosage of 2-PAM. Vehicle and surrogate only groups were also tested. The rats were observed for signs of toxicity, including seizure behavior. Rats were sacrificed at 30 min following administration of the oxime to test brain AChE activities. Brain AChE inhibition and reactivation were calculated compared to the corresponding controls.

3. Theory

The theory behind this project is that the positive charges of the quaternary ammonium in the pyridinium ring of most oxime reactivators that restrict BBB penetration can be counterbalanced, at least in part, by the lipophilic characteristics within these novel oxime chemistries, thereby enhancing their likelihood of penetrating the BBB.

4. Results and discussion

4.1. In vitro reactivation results

The novel oximes tested displayed a range of 14–76% reactivation of rat brain AChE with both surrogates, and the patterns of efficacy were similar with PIMP and NEMP (Table 1). For comparison, brain AChE reactivation was assessed with the two surrogates tested with two well-known oximes, 2-PAM and TMB-4; 2-PAM yielded 91% reactivation and TMB-4 yielded 98% reactivation.

4.2. Lipophilicity

The novel oximes displayed a 3-order of magnitude range in lipophilicities, as indicated by K_{ow} values, which ranged from 0.011–2.244. The novel oximes were all appreciably more lipophilic than traditional oximes, with 2-PAM having a K_{ow} of 0.006 and TMB-4 having a K_{ow} of <0.001.