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Novel bifunctional hybrid small molecule scavengers for mitigating nerve agents toxicity

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ABSTRACT

The antidotal treatment of organophosphates (OP) nerve agents (NA) poisoning is based on anticholinergics (e.g. atropine) combined with oxime reactivators (e.g. 2PAM) of acetylcholinesterase (AChE). This treatment is symptomatic and does not degrade the OP. New small-molecule OP scavengers were developed as bifunctional hybrids. Their molecular design was based on combining a nucleophile that directly degrades OP with a moiety that reactivates OP-inhibited AChE. The OP degrading moiety is either benzhydroxamic acid (BHA) or 4-pyridinehydroxamic acid (4PHA) coupled via $-(CH_2)_n-$, (n = 1 or 3) to 2PAM. Three newly synthesized oxime-hydroxamate hybrids: 2PAMPr4PHA, 2PAMMeBHA and 2,4-DiPAMMeBHA were found to detoxify sarin, cyclosarin and soman in solution at 3–10-fold faster rate than 2PAM and to reactivate OP-AChE *in vitro*. 2PAMPr4PHA displayed 18-fold faster reactivation than 2-PAM of cyclosarin-inhibited HuAChE ($k_r = 3.6 \times 10^2$ vs. 0.2×10^2 $M^{-1}min^{-1}$, respectively, 37 °C). These hybrids inhibited AChE reversibly, $IC_{50} = 16–48$ μM, thereby decreasing the inhibition rates by OPs. The LD_{50} (im) of 2PAMPr4PHA, 2PAMMeBHA and 2,4DiPAMMeBHA are >568, 508 and >506 μmol/kg in rats and 144, 203 and >506 μmol/kg in guinea pigs. The rate of blood ChE recovery by the hybrids administered either pre- or post-exposure to $0.8xLD_{50}$ sarin was comparable or faster than 2PAM. Antidotal efficacy of 2PAMPr4PHA, 2PAMMeBHA and 2,4DiPAMMeBHA administered with atropine, as pre-treatment to sarin in rats (im), yielded protection ratios (PR) 11.6, 11.5 and 4.7, respectively, vs. 5.5 with 2PAM. Post-treatment against various OPs in rats and guinea-pigs yielded PRs higher or similar to that of 2 PAM. Our *in vivo* data indicates that some hybrids may serve as efficient small molecule scavengers for mitigating the toxicity of OP NAs.

1. Introduction

The inhibition of acetylcholinesterase (AChE) by organophosphorus (OP) chemical warfare (CW) nerve agents (NAs) and agricultural OP pesticides, causes excessive accumulation of acetylcholine (ACh) in cholinergic synapses. If left untreated, OP poisoning leads to dose-dependent toxic signs including tremor, hyper secretion, seizures, respiratory depression and eventually death. Various quaternary oxime reactivators such as 2PAM and toxogonin (obidoxime), combined with anticholinergics such as atropine and scopolamine have been used to treat acute poisoning by OP nerve agents [1,2]. Seizures are often controlled by the administration of GABA receptor agonists such as diazepam or midazolam [3].

The currently available combination of antidotes alleviate significantly the symptoms of OP intoxication but are not able to degrade OPs and thereby incapable to diminish their level in blood and tissues. Consequently, the re-appearance of toxic signs in

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; OP, organophosphates; NA, nerve agents; PR, protection ratio; NP, no protection; 2-PAM, 2-pyridine aldoxime methochloride; 4PHA, 4-pyridine hydroxamic acid; BHA, benzhydroxamic acid; i.m, intra muscular.

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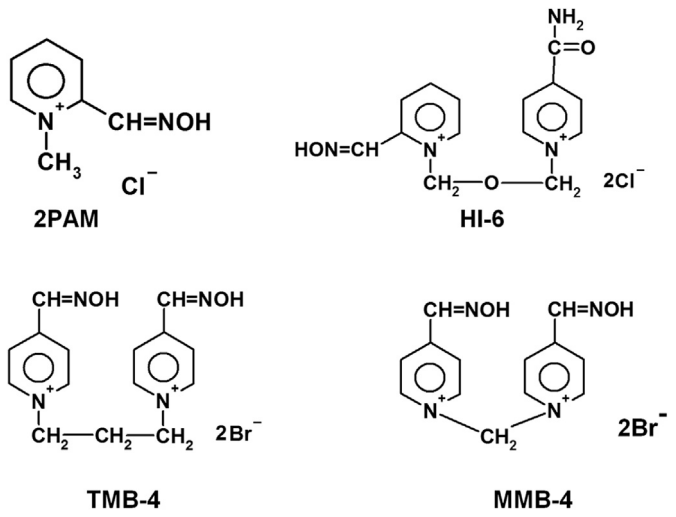
severe OP intoxications requires continuous medical support such as respiratory ventilation and repeated administration of antidotes. Such continuous medical attendance and treatment of battlefield CW casualties as well as mess casualties during a civilian chemical terror act may create a heavy logistic burden on the medical personnel [4]. Various bio-scavengers that could sequester toxic OPs thus preventing their blood ChE inhibitions, were developed during the last three decades. The physiologically disrupting irreversible inhibition of AChE by OPs was utilized to develop AChE [5,6] as well as its non-physiological isoenzyme butyrylcholinesterase (BChE) [7], as effective stoichiometric bioscavengers of OP NAs *in vivo*. However, using stoichiometric enzyme scavengers constitutes an excessive pharmaceutical payload. For instance, AChE with molecular weight (MW) of 80 kD (D = Dalton) and BChE with MW 360 kD were used to sequester low molecular weight OPs such as sarin or VX of MW 140D or 267D, respectively.

Consequently, enzymes that could catalytically hydrolyze OPs were developed as catalytic bioscavengers. These phosphotriesterases (PTEs) include OP acid anhydrolases (OPAA) from bacterial sources [8,9], OP hydrolases (OPH) from bacteria [10] and mammalian sources e.g. serum paraoxonase1 PON1 [11], and diisopropyl fluorophosphatase (DFPase) isolated from the squid giant axon (*Loligo vulgaris*) [12,13]. Some PON1 variants developed by directed evolution displayed marked hydrolytic activity on NAs bearing a P—F bond (e.g. sarin, cyclosarin) [14]. Further, using computational libraries combined with enhanced evolution, a more than three orders of magnitude improvement in the rate of hydrolysis of V-type NAs by bacterial PTE was demonstrated [15]. The outstanding achievements in the enhancement of the detoxification kinetics by catalytic bioscavengers measured *in vitro* were also corroborated by successful *in vivo* antidotal efficacy in OP NA-challenged animals [16,17]. However, some bio-pharmaceutical issues concerning protein therapy remained unresolved. Catalytic protein bio-scavengers are limited in their route of administration (no oral preparations), they still may create excessive payload by their large mass per volume of administered solution with MW ~40 KD, and may induce immunological response upon repeated administration unless chemically modified. Small molecule scavengers may be used to overcome most of these disadvantages and be pharmaceutically compatible with current small molecule therapy.



Scheme 1. Chemical structure of quaternary oximes 2-PAM, HI-6, TMB-4 (trimedoxime) and MMB-4 (methoxime).

Quaternary pyridinium oxime reactivators of OP-inhibited AChE that were developed in the early 1950's [18] provided the first efficient reactivation of diisopropyl fluorophosphate (DFP)-and Tetraethyl pyrophosphate (TEPP)-inhibited AChE by 2-pyridine aldoxime methiodide (2PAM, Scheme 1). Efficient reactivation of sarin inhibited AChE by 2PAM was later demonstrated [19]. The first bisquaternary pyridinium oximes TMB-4 (trimedoxime) (Scheme 1) and some of its congeners such as MMB-4 (methoxime) (Scheme 1) demonstrated a faster rate of reactivation of sarin-inhibited AChE than 2PAM [20,21]. The second generation of bis-quaternary oximes termed H-oximes [22], such as HI-6 (Scheme 1) demonstrated high antidotal efficacy in the treatment of non-human primates exposed to sarin, soman and tabun [23,24]. The development of mono- and bis-quaternary oximes and their efficacy in the treatment of OP poisoning was reviewed extensively by Worek and Thiermann [2].

It was suggested that 2PAM and other oximes could be used for both reactivation of OP-AChE conjugates by displacing the OP moiety from the enzyme active site as well as reactive nucleophiles directly reacting with OPs [25]. Mono- and bis-pyridinium oximes (e.g. 2PAM, Toxogonin and HI-6) were indeed examined kinetically as direct scavengers of OP NAs *in vitro* [26,27]. However, the rate of the direct reaction of these oximes with OP NAs were found too slow for significant detoxification of OP poisoning *in vivo* [26]. Notably, Benzhydroxamic acid (BHA), N-methyl 4-Pyridine hydroxamic acid (N—Me 4-PHA), N-methyl 2-PHA and some of their structural analogues were found to be more active than their respective pyridinium oximes as nucleophiles toward OP NAs causing their degradation under physiological conditions [28—30]. By contrast, hydroxamic acid combined with quaternary pyridinium, the structural homologs of 2-PAM and 4-PAM, were very poor reactivators of sarin-inhibited AChE compared to their oxime analogues [31,32].

The efficacious reactivation property of quaternary oximes, as well as the relatively rapid rate of direct reaction of aromatic hydroxamates with OPs, served as a basis for our design of new hybrid molecules. Our synthetic approach was based on linking either BHA or 4PHA, capable of hydrolyzing directly the OP agent, to 2PAM moiety via short hydrocarbon bridge $(CH_2)_n$ (n = 1 or 3) (Scheme 2). These double headed hybrids were expected to elicit bifunctional activity that will both diminish the level of toxic OP NAs in blood with concomitant reactivation of OP-inhibited AChE *in situ*. Since these hybrid compounds include a positively charged quaternary pyridinium moiety (Scheme 2), it may enhance the affinity of the whole molecule to AChE by guiding the hybrid molecule to the negatively charged active-site region [1]. One important advantage of covalently combined moieties compared to a mixture of two separate compounds, is the unitary pharmacokinetics of these two complementary activities (scavenging and reactivation) *in vivo*. Uniform rates of absorption, distribution, metabolism and elimination (ADME) of a hybrid compound are preferable to two distinct active pharmaceutical ingredients, each displaying its own ADME pattern. This bifunctional drug concept has recently been applied to novel therapy of chronic obstructive pulmonary disease (COPD) that is presently under clinical trials [33]. The hybrid drug combines β_2-adrenergic receptor agonist with muscarinic receptor antagonist in one small molecule. One of the benefits of this approach includes avoiding the problem of formulating two different drugs in one pill or inhaler [33]. In the course of our studies 22 new oxime-hydroxamate hybrids were synthesized of which three compounds were selected for further *in vivo* studies: 2PAMPr4PHA, 2PAMMeBHA and 2,4DiPAMMeBHA (Scheme 2).