

Centrally acting oximes in reactivation of tabun-phosphoramidated AChE

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ARTICLE INFO

Article history:

Available online 7 September 2012

Keywords:

Oxime reactivation
Organophosphate intoxication
CNS AChE reactivation
Hydroxyiminoacetamides
Butyrylcholinesterase
Tabun

ABSTRACT

Organophosphates (OP) inhibit acetylcholinesterase (AChE, EC 3.1.1.7), both in peripheral tissues and central nervous system (CNS), causing adverse and sometimes fatal effects due to the accumulation of neurotransmitter acetylcholine (ACh). The currently used therapy, focusing on the reactivation of inhibited AChE, is limited to peripheral tissues because commonly used quaternary pyridinium oxime reactivators do not cross the blood brain barrier (BBB) at therapeutically relevant levels. A directed library of thirty uncharged oximes that contain tertiary amine or imidazole protonable functional groups that should cross the BBB as unionized species was tested as tabun-hAChE conjugate reactivators along with three reference oximes: DAM (diacetylmonoxime), MINA (monoisonitrosoacetone), and 2-PAM. The oxime RS150D [*N*-((1-(3-(2-((hydroxyimino)methyl)-1*H*-imidazol-1-yl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)benzamide)] was highlighted as the most promising reactivator of the tabun-hAChE conjugate. We also observed that oximes RS194B [*N*-(2-(azepan-1-yl)ethyl)-2-(hydroxyimino)acetamide] and RS41A [2-(hydroxyimino)-*N*-(2-(pyrrolidin-1-yl)ethyl)acetamide], which emerged as lead uncharged reactivators of phosphorylated hAChE with other OPs (sarin, cyclosarin and VX), exhibited only moderate reactivation potency for tabun inhibited hAChE. This implies that geometry of oxime access to the phosphorus atom conjugated to the active serine is an important criterion for efficient reactivation, along with the chemical nature of the conjugated moiety: phosphate, phosphonate, or phosphoramidate. Moreover, modification of the active center through mutagenesis enhances the rates of reactivation. The phosphoramidated-hAChE choline-binding site mutant Y337A showed three-times enhanced reactivation capacity with non-triazole imidazole containing aldoximes (RS113B, RS113A and RS115A) and acetamide derivative (RS194B) than with 2PAM.

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1. Introduction

Organophosphate (OP), nerve agents are small lipophilic organic molecules that readily diffuse through biological membranes and quickly reach CNS. OPs inhibit both peripheral and CNS acetylcholinesterase (AChE, EC 3.1.1.7) causing adverse effects due to the accumulation of neurotransmitter acetylcholine (ACh), which can eventually lead to death. Currently employed antidotal therapy, focused on the reactivation of inhibited AChE, is limited to the peripheral circulation because commonly used quaternary pyridinium aldoxime reactivators cannot cross the blood brain barrier (BBB) at therapeutically relevant levels due to their positive charge. We recently designed a library of more than hundred uncharged oximes that contain a tertiary amine or imidazole protonable functional group where a significant fraction of species are non-ionized at physiological pH and could cross the BBB. *N*-substituted

2-hydroxyimino acetamidoalkyl amines, though devoid of a permanent cationic charge, can efficiently reactivate the OP conjugated hAChE *in vitro* [1]. Ionization equilibria around two ionizable groups, an oxime and an amine group results in coexistence of a cationic, zwitterionic, uncharged and anionic reactivator species around physiological pH values. While zwitterionic and cationic species have the best chance of productive interaction with OP-hAChE conjugates, the uncharged species can be expected to cross the BBB delivering reactivator into CNS.

Among 135 new oximes, R194B [*N*-(2-(azepan-1-yl)ethyl)-2-(hydroxyimino)acetamide] proved to be efficient reactivator upon VX, sarin, and cyclosarin exposure [1,2]. Paraoxon- and tabun-derived conjugates with larger substituents occupying the OP-hAChE acyl pocket had different structural requirements and proved to be more refractory to reactivation [1]. Herein, we tested 29 uncharged oximes following tabun exposure along with three reference oximes: DAM (diacetylmonoxime), MINA (monoisonitrosoacetone), and 2PAM. In addition, comparative rates of reaction were investigated on phosphorylated hAChE and two hAChE mutants.

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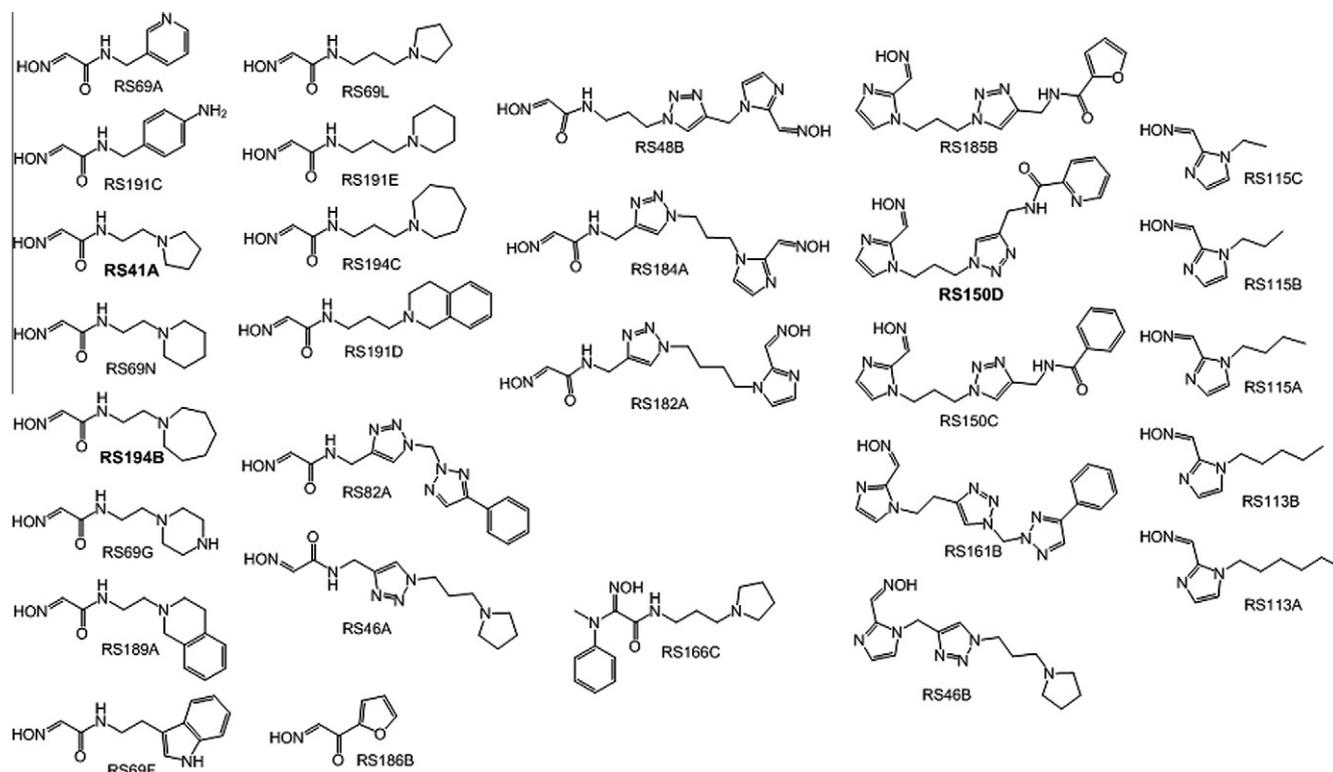


Fig. 1. Uncharged oximes that contain tertiary amine or imidazole protonable functional groups.

2. Material and methods

2.1. Chemicals

Design, synthesis, and initial functional characterization of novel oxime reactivators were reported recently [1,2]. 2PAM (2-Pyridine-aldoxime methiodide), MINA (monoisonitrosoacetone), and DAM (2,3-butanedione monoxime), acetylthiocholine iodide (ATCh) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich. Tabun [ethyl *N,N*-dimethylphosphoroamidocyanide] was purchased from the NC Laboratory, Spiez, Switzerland.

2.2. Enzyme

Highly purified monomeric hAChE was prepared as described earlier [1,3]. Purified oligomeric hBChE isolated from human plasma was kindly donated by Drs. Douglas Cerasoli and David Lenz, USAMRICD, Edgewood, MD.

2.3. Oxime reactivation assays

Phosphoramidated enzyme conjugates were prepared by incubating (0.1–1.0 μ M) enzyme with 5 μ M tabun for 30–45 min until inhibition exceeded 95%. Such inhibited enzyme was passed through a Sephadex G-50 spin column (Roche Diagnostic GmbH, Mannheim, Germany) to remove excess of unconjugated tabun. After filtration enzyme was incubated with oxime (0.05–10.0 mM) in 0.1 M sodium phosphate buffer pH 7.4 containing 0.01% BSA at 37 °C. At specified time intervals, 10 μ l aliquots were diluted 100-fold in phosphate buffer containing DTNB. Upon addition of ATCh residual enzyme activity was measured by the Ellman method [4]. Equivalent samples of uninhibited enzyme were passed through a parallel column, diluted to the same extent as the inhibition mixture, and control activity was measured in the presence of oxime at concentrations used for reactivation. Activities of control and

reactivation mixture were corrected for oxime-induced hydrolysis of ATCh. Kinetic parameters of reactivation, constants k_{+2} (maximal first order reactivation rate constant), K_{OX} (the apparent phosphorylated enzyme-oxime dissociation constant) and k_r (second-order rate constant of reactivation), were calculated from experimental data obtained in at least three separate experiments [5].

3. Results and discussion

Our recent characterization of a library of 135 uncharged, structurally diverse oximes revealed hydroxyimino acetamido alkyl amines as efficient reactivators of OP-hAChE conjugates [1,2]. Although varied oxime structures seemed to affect similarly reactivation of methyl phosphonyl OP-hAChE conjugates (derived from sarin, cyclosarin and VX), tabun-derived conjugates proved to be resistant to reactivation. Indeed, out of 29 new RS oximes (Fig. 1), three best reactivating oximes were predominately imidazole-containing aldoximes (for hAChE) and hydroxyimino acetamido amine derivatives (for hBChE) (Fig. 2). These structural features of the reactivator differ from previous studies with the methylphosphonate OPs where the opposite structure/activity relationship was observed [1]. Only RS191D, RS194B and RS194C reactivated tabun-hBChE conjugate appreciably with a maximal percentage of reactivation of 30% showing that larger moieties like azepane (RS194B and RS194C) or tetrahydroisoquinoline (RS191D) are preferable structural motives for binding to hBChE. The phosphoramidated-hAChE choline-binding site mutant Y337A showed three-times enhanced reactivation capacity with non-triazole imidazole containing aldoximes (RS113B, RS113A and RS115A) and acetamido derivative (RS194B) than with 2PAM. Bis-oxime RS182A reactivated the double mutant Y337A/F338A conjugate up to 50% with a rate similar to 2PAM.

Reactivation rates for the tabun-hAChE conjugates by the best, overall reactivator, RS150D, and other leads, RS194B and RS41A,

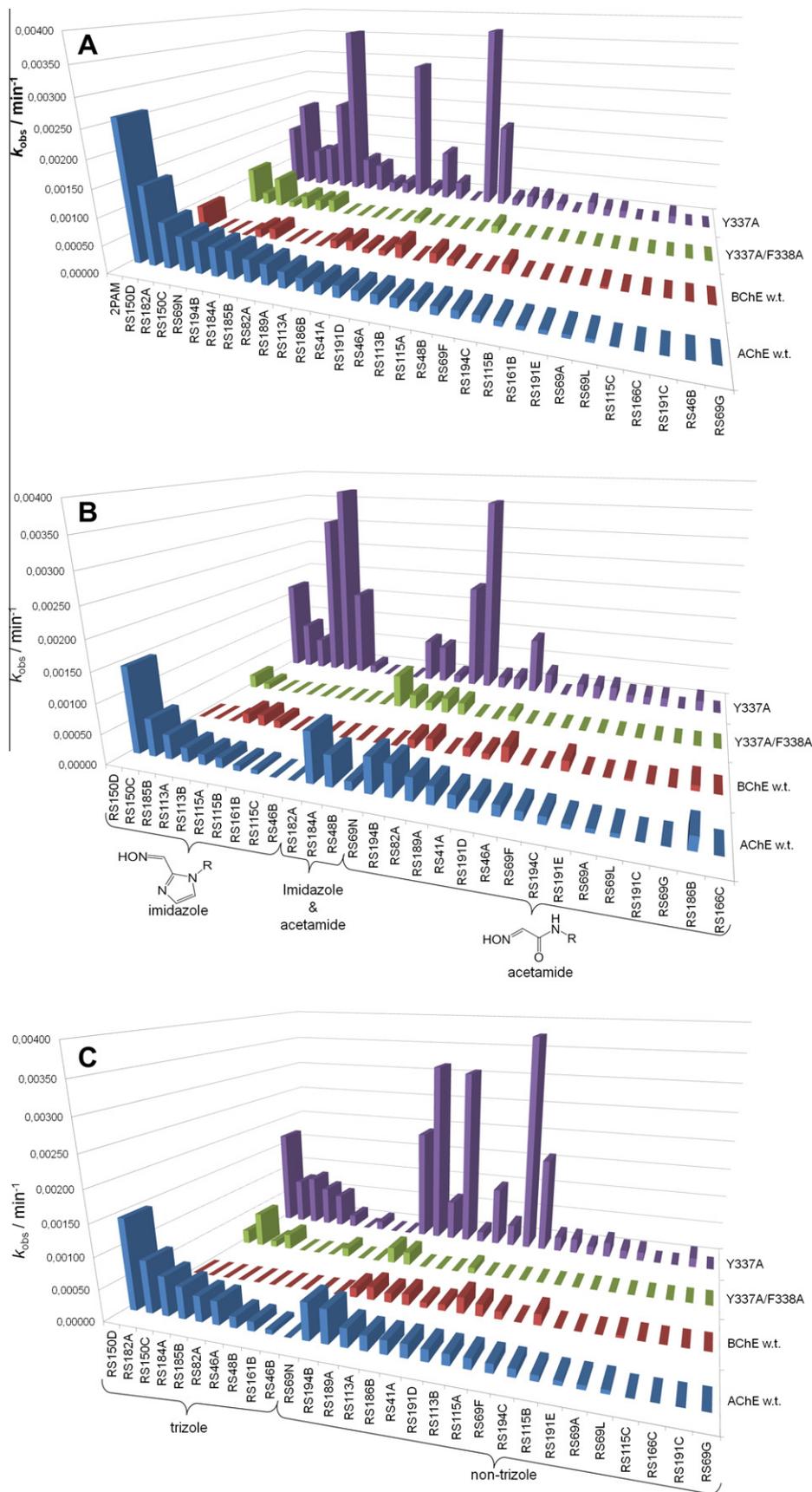


Fig. 2. Reactivation rates (k_{obs}) of tabun-inhibited hAChE, hBChE and two hAChE mutants by 1 mM oximes recorded within 22 h. Oximes are ordered by their reactivity to hAChE (A) and segregated by their general structure as imidazole, acetamide, and other oximes (B) or triazole ring in oxime structure (C).

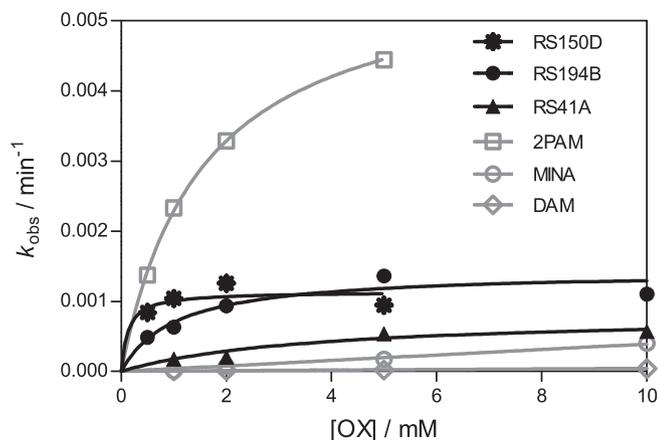


Fig. 3. Concentration dependence of oxime reactivation of tabun-inhibited hAChE.

Table 1
Detailed kinetic analysis of tabun-hAChE conjugate reactivation by varying concentrations of oximes.^a

Oxime	k_{+2}/min^{-1}	K_{OX}/mM	$k_r/\text{M}^{-1}\text{min}^{-1}$	React _{max} /%
RS194B	0.0018 ± 0.0002	1.8 ± 0.5	1.00 ± 0.29	70
RS150D	0.0015 ± 0.0001	0.41 ± 0.12	3.7 ± 1.2	70
RS41A	0.00082 ± 0.0001	3.6 ± 1.5	0.22 ± 0.10	40
2PAM	0.0058 ± 0.0006	1.5 ± 0.4	3.8 ± 1.1	70
DAM	–	–	0.0042 ± 0.0003	<10
MINA	–	–	0.039 ± 0.008	25

^a k_{+2} – the maximal first order reactivation rate constant, K_{OX} – the apparent phosphorylated enzyme-oxime dissociation constant, k_r – the second-order rate constant of reactivation, React_{max} – the maximal percentage of reactivation.

was additionally measured over a range of oxime concentrations, from 0.05 up to 10 mM, and compared to uncharged (DAM and MINA) and charged (2PAM) reference compounds (Fig. 3). While reactivation by MINA and in particular DAM was very slow, RS150D and RS194B displayed more rapid reactivation over the entire measured concentration range, but with no rate enhancement when compared to the quaternary cationic 2PAM. This suggests that the maximal reactivation rate constant for 2PAM is generally superior to other oximes, while its binding to covalent tabun-hAChE conjugates (reflected in the constant K_{OX}) is comparable to that of RS194B (Table 1). Large K_{OX} constants (i.e. low affinity) for uncharged aldoximes are consistent with AChE having preference for binding cationic ligands, even in the OP conjugated state [5]. One third of the oximes contained the triazole ring (as RS150D) showing that this heterocycle positioned some distance from the oxime group enhances tabun-hAChE conjugate reactivation (Fig. 2c). The triazole moiety was earlier observed to contribute synergistically to the total binding energy of high affinity AChE inhibitors [6]. All three RS oximes possess the oxime group positioned farther from the part of the molecule largely responsible for molecular recognition. This relationship is in accordance with our previous study conducted with VX and other OPs [1]. Optimal separation equivalent to a five- or six-atom linker is consistent with structures of all five best uncharged reactivators of tabun-hAChE conjugate. Maximum of reactivation reached 70% suggesting that reactions such as aging and reinhibition with phosphorylated oxime could have competed with reactivation. However, owing primarily to its relatively moderate affinity, RS150D was singled out as the best hAChE reactivator among these uncharged aldoximes. Interestingly it is consistent with large percentage of oximate anion calculated for RS150D at pH 7.4 (58%) vs. RS194B (0.25%) or RS41A (0.21%) [1,2]. Although several classes of novel

compounds have been suggested as promising centrally [7] or primarily peripherally [8,9] acting reactivators of tabun-hAChE conjugate, therapy following tabun exposure presents unique features for finding reactivators with enhanced reactivity and favorable pharmacokinetic properties.

4. Conclusion

Oxime RS150D was highlighted as the most promising uncharged reactivator of the tabun-hAChE conjugate, although still falls short of bispyridinium aldoximes [8,9]. We also observed that oximes RS194B and RS41A, which emerged as potential reactivators of hAChE inhibited with other methyl phosphonate OPs (sarin, cyclosarin and VX) [1], did not show such high reactivation potency for tabun inhibited hAChE. This is due to larger substituents on the phosphorus that occupy the tabun-hAChE acyl pocket in phosphorylated conjugates [5]. A limiting potency to reactivate tabun-enzyme conjugates proves that oxime access to phosphorus atom within the AChE active center gorge is an important criterion for efficient reactivation as is the organophosphorus structure itself. Although the enhancement achieved by mutation Y337A is too low for an effective catalytic scavenger [10] optimized for tabun, it confirms the importance of examining mutants for enhancing catalysis in plasma.

5. Conflicts of interest

None.

Acknowledgement

This research was supported by the CounterACT Program, National Institutes of Health, Office of the Director (NIH OD), and the National Institute of Neurological Disorders and Stroke (NINDS), Grant Number U01NS058046.

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