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Reactivation potency of fluorinated pyridinium oximes for acetylcholinesterases inhibited by paraoxon organophosphorus agent

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ABSTRACT

For the purpose of developing new oxime reactivators of acetylcholinesterases (AChE) that have been inhibited by organophosphorus agents, emphasis was given to the finding that the lipophilic nature of fluorinated compounds is responsible for their enhanced transport across the blood brain barrier (BBB). As a result, we have designed and synthesized the fluorinated oxime derivatives, which quantum mechanical calculations suggest should have a greater lipophilicity and BBB permeability than their non-fluorinated analogs. Among the compounds explored in this study, **4** was found to have the highest potency for reactivation of paraoxon-inhibited housefly (HF) AChE.

A number of organophosphorus compounds that serve as nerve agents and insecticides (e.g., parathion and malathion) are cholinesterase inhibitors and, in particular, acetylcholinesterase (AChE) inhibitors. Electrophilic phosphorous atoms in these agents are reactive toward nucleophilic attack by the serine hydroxyl group in the acetylcholinesterases. The toxicity of these organophosphorus agents is a consequence of the fact that the resulting, covalently inactivated phosphorylated enzymes resist hydrolysis. The inhibition of AChE increases the amount of neurotransmitter acetylcholine at central and peripheral sites of the nervous system and causes excessive stimulation of muscarinic and nicotinic receptors.[1] The inhibited AChE can be reactivated by using selective nucleophilic substances, such as pyridinium oximes.[2] Oxime-induced reactivation is the primary therapeutic antidote for poisoning by organophosphorus warfare agents and insecticides. As a result, the development of oxime reactivators has received great attention during the last several decades.

These efforts, some of which have been carried out in our laboratory, have led to the discovery of a variety of pyridinium oximes,[3] several of which are in clinical use currently (e.g., pralidoxime, obidoxime and HI-6, Fig. 1).[4] However these oximes are not ideal reactivators of inhibited AChEs, primarily because they have limited blood brain barrier (BBB) penetration. Even though the mean BBB penetration of 2-PAM is approximately 10%, determined by using an in vivo rat microdialysis technique with HPLC/UV,[5] BBB penetration remains a key issue in the development of the oxime type AChE reactivators.

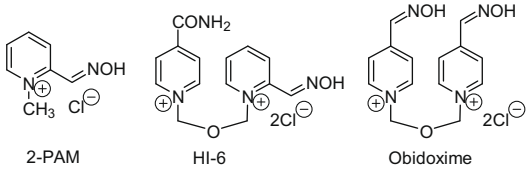

Figure 1. Structures of AChE reactivators.

Lipophilicity is a key molecular parameter in medicinal chemistry,[6] especially in the development of central nervous system (CNS) active drugs that require BBB permeability. Biological absorption and distribution are largely controlled by the ionization state of a drug that leads to a balance between lipophilicity and hydrophilicity. Enhanced lipophilicity can lead to an increase in the measured binding free energy by making partitioning between the polar aqueous solution and the less polar receptor site more favorable.

Fluorinated compounds are frequently encountered in modern medicinal chemistry and many of these substances are highly effective drugs.[7] Commonly, fluorine is introduced to block a metabolically labile site in the molecule. Another beneficial property associated with fluorine substitution is an increase in BBB permeability due to changes in lipophilicity. The results of studies probing the effect of replacing hydrogen by fluorine on lipophilicity show that a single H/F exchange raises the log*D* value by approximately 0.25.[8] Thus, we hypothesized that introduction of fluorine

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at heterocyclic ring positions of pyridinium oximes might represent a viable strategy for the enhancement of liphophilicity, BBB permeability, and potency for inhibited AChE reactivation.

In order to assist in the design of ideal fluorinated pyridinium oximes, computer aided calculations of lipophilicity were performed. Ab Initio Hartree–Fock (HF) and density functional B3LYP calculations using the Gaussian 03 package were carried on pyridinium oximes **1-3** (Fig. 2) first in order to measure and compare electron densities. The conformations of **1–3** were optimized at the HF/6-31+G(d) level. The conductor-like polarizable continuum model (CPCM) SCRF method, which is implemented in the Gaussian 03 package to consider solvation effect, was also used. To derive a hydrophilicity index (Eq. (1)), we used the electron densities as parameters to assess the degrees to which atoms in the compound resemble those in water.[9]

$$\text{Hydrophilic index} = \sum_{\rho a<\rho \mathrm{H_2O}} \rho a/\rho \mathrm{H_2O} + \sum_{\rho a>\mathrm{H_2O}} \left(1 - \frac{\rho a \mathrm{HB}}{\rho \mathrm{H_2O}}\right) - \sum \rho a \mathrm{HC}/\rho \mathrm{H_2O} \quad (1)$$

In equation 1, ρa represents the electron densities of individual atoms in the compound, ρH2O represents the electron density of water, HB refers to potential hydrogen-bonding atoms (O, N, F) and to hydrogen atoms bonded to O or N, and HC refers to hydrogen atoms bonded to carbon. The electron densities of hydrogen atoms were compared with the electron density of hydrogen atoms in water regardless of their origin (0.457) and the electron densities of O, N, or F atoms were compared with the electron density of the oxygen atom of water (7.868). By using this relationship, the hydrophilic indexes of **1–3** were determine to be 2.53, −0.2 and −0.33, respectively (Table 1), where the lower value corresponds to lower hydrophilicity and more liphophilicity. These results suggest that the fluorinated compounds **2** and **3** are more liphophilic than the non-fluorinated substance **1**. Consequently, **2** and **3** should be more able to penetrate the Brain Blood Barrier (BBB) as compared to **1**. The aqueous solubilities and BBB penetrations for three compounds were also calculated by using Accelrys/ADME prediction software as shown in Table 1.

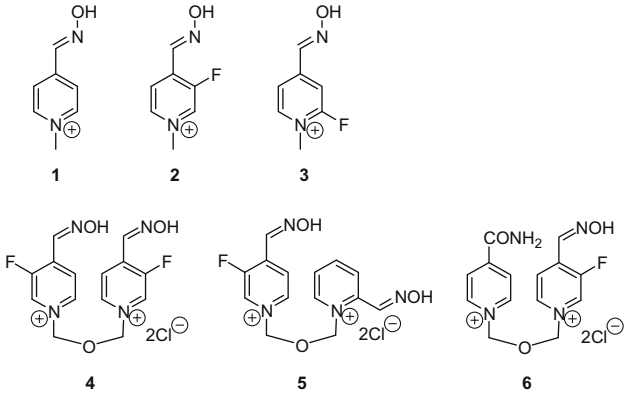


Figure 2. Structure of bis-pyridinium oximes.

The results of the theoretical treatments described above suggest that fluorinated oximes **2** and **3** should have higher lipophilicities and higher BBB permeabilities than non-fluorinated oxime **1**. As a result, we have synthesized oxime **2** along with the related bis-pyridinium oximes (**4–6**) that contain the oxime moiety found in **2** and compared their activities toward reactivation of organophosphorus compound inactivated AChE.

The first step in the preparation of the fluorinated mono-pyridinium oxime **2** involved treatment of 3-fluoro-4-pyridinecarboxaldehyde **6** with NH_2OH–HCl in EtOH containing pyridine. This process produces the aldoxime **7** in 96% yield (Scheme 1). The oxime **2** was then generated in 24% yield by N-methylation **8** with methyl iodide in $CHCl_3$.

Our interest also focused on fluorinated derivatives of bis-pyridinium oximes (**4–6**), obidoxime analogs, because the obidoxime is already clinically in use.

Methods for the preparation of the non-fluorinated oximes HI-6 and obidoxime, bearing a bis-chloromethyl-ether linker, have been published.[10] As a result, the fluorinated analogs (**4–6**) were prepared by using the established procedures. Accordingly, reaction of aldoxime **8** with 0.33 equiv of bis-chloromethyl-ether **9** at 45 °C in $CHCl_3$ yields the bis-pyridinium oxime **4** (15%). In contrast, reaction of aldoxime 10 with 5 equiv of bis-chloromethyl-ether 9 in $CHCl_3$ generates the mono-pyridinium oxime **11** (98%), which upon reaction with **8** provides the oxime **5** (40%). In the route for preparation of the bis-pyridinium oxime **6**, isonicotinamide **12** is reacted with 5 equiv of bis-chloromethyl-ether 9 in $CHCl_3$ to give intermediate **13** (26%), which reacts with aldoxime **8** in DMF to produce the oxime **6** (63%).

The pyridinium oximes and HI-6 were prepared by using known synthetic methods.[3] 2-PAM was purchased from Sigma–Aldrich. Diisopropyl fluorophosphates (DFP) and paraoxon are available from Fluka and Sigma–Aldrich, respectively. AChE, from bovine red blood cells (RBC), was purchased from Sigma–Aldrich. Known sequential protocols were used to monitor reactivation of AChE.[11]

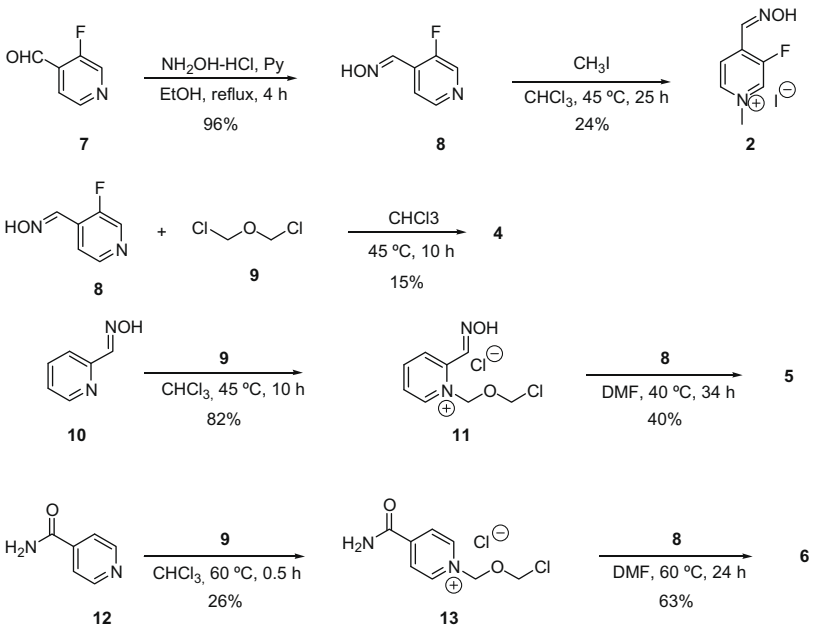
In order to assess the reactivation potencies of the oximes, paraoxon was used to inactivate AChE since this substance has structure that is similar to typical nerve gases. As shown in Table 2, oxime reactivations were carried out on paraoxon-inhibited HF and RBC AChEs. The potencies of the prepared oximes were compared with the three currently used AChE reactivators, 2-PAM, obidoxime and HI-6 (Fig. 3). The importance of fluorine substitution at pyridinium ring positions is demonstrated by comparison of data obtained using *N*-methyl-4-pyridinium oxime **1** and *N*-methyl-3-fluoro-4-pyridinium oxime **2** in paraoxon-inhibited HF and RBC AChEs.[12]

Because of structural similarities, the fluorinated oxime **4** should be compared with obidoxime, and the fluorinated oxime **6** can be compared with HI-6. The results of these measurements show that non-fluorinated oximes have higher reactivation potencies than their fluorinated analogs with paraoxon-inhibited RBC

Table 1
QM calculations of electron densities and hydrophilicity of compounds

Compound	Electron densities			Hydrophilicity index	Aqueous solubility*	BBB penetration*
	N1	N2	O			
1	6.576927	7.099565	7.703776	2.53	−0.767	−0.553
2	6.604342	7.113086	7.683838	−0.20	−1.152	−0.490
3	6.726558	7.101621	7.695776	−0.33	−1.484	−0.372

* These values are calculated using Accelrys/ADME prediction software. Lower solubility value represents less soluble in water. Greater BBB value represents more BBB penetration.



Scheme 1. Synthesis of new pyridinium oximes.

Table 2
Reactivation potency of oximes for paraoxon-inhibited AChEs

Compound	HF AChE		RBC AChE	
	Mean[a]	SE	Mean[a]	SE
1	7.5	2.47	20.5	2.38
2	19	2.85	46.3	3.36
4	50	8.98	40.9	3.96
5	2.2	0.85	26.9	2.87
6	22.1	3.17	29.5	2.99
2-PAM	48.4	11.63	62.6	3.55
Obidoxime	33	6.47	78.1	7.61
HI-6	4.6	1.3	48.2	3.94

[a] Mean is the average of % reactivation with 4 replications, and SE is the 'Standard Error'.

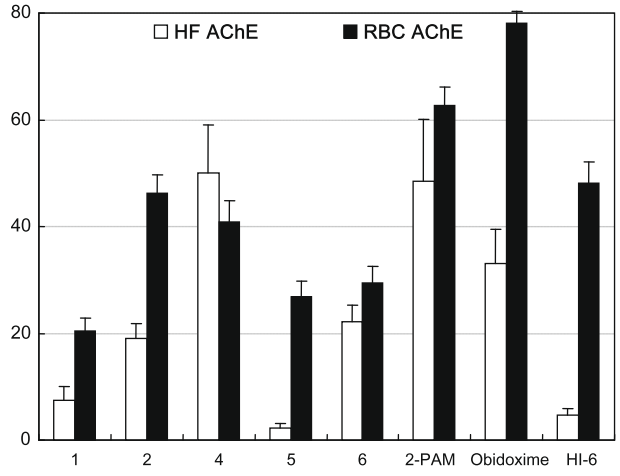


Figure 3. Reactivation potency of tested oximes for paraoxon-inhibited RBC AChEs.

AChE. The potency order was found to be obidoxime >**4**, and HI-6 >**6**. Among the oximes tested, obidoxime was observed to be a more potent reactivator than either 2-PAM or HI-6. In contrast, the trends outlined above are reversed in reactivation of paraoxon-inhibited HF AChE. In this case, fluorinated oximes have higher potencies than the corresponding non-fluorinated derivatives. This is demonstrated by the finding that **4** is a more potent reactivator than obidoxime, and **6** is also slightly more potent than HI-6. However **5** showed lower reactivation potencies compared with other oxime compounds. Thus, among the substances explored in this study, **4** has the highest reactivation potency in reactivation of paraoxon-inhibited HF AChE.

In summary, we have designed and synthesized several fluorinated oxime compounds in order to probe the effect of fluorine substitution on reactivation of inhibited AChE. The results of computational studies, indicating that fluorinated oxime **2** is more hydrophobic than the non-fluorinated oxime **1**, stimulated the design of new oximes with potentially increased BBB permeabilities. Among the substances explored in this study, **4** has the highest reactivation potency in reactivation of paraoxon-inhibited HF AChE. Further investigation will be carried out in our laboratory to probe the in vivo reactivation activities of the fluorinated oximes. It is hope that this effort leads to new guidelines for the design of promising oxime reactivators of inactivated AChE.

Acknowledgments

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11. *In vitro determination of AChE activity.* The enzyme activity was measured in a 96-well microplate using a microplate reader (Benchmark Microplate Reader, Bio-Rad) at 415 nm and 37 °C with acetylthiocholine (1 mM) as substrate and DTNB (1 mM) as chromogen in 0.05 M Tris–HCl buffer, pH 7.8. Total reaction volume was adjusted to 250 μL with slight modification from Ellman's AChE assay method. The enzyme concentrations of both AChEs in 250 μL of final reacting solutions were 0.05 mg/ml for HF AChE and 0.02 U/mL for RBC AChE, respectively. For RBC AChE, 1% of Triton X-100 (Sigma–Aldrich) was added in the Tris–HCl buffer to preserve enzyme activity. AChE activity was measured by using the change of optical density per minute (OD/min). The percentage of reactivation of AChE activity was calculated by comparison with the AChE activity without inhibitor showing 0.2 OD/min.
12. AChE inhibition and reactivation. DFP and paraoxon were used as organophosphorus inhibitors and the AChE reactivating capability of 2-PAM and HI-6 were examined against OP-inhibited HF and RBC AChE, respectively. AChE was inhibited with the minimum quantity of inhibitor necessary to inactivate 99% of activity for 10 min at room temperature. The concentration of DFP was 12.5 μM for HF AChE and 25 μM for RBC AChE, respectively, and 20 μM of paraoxon for both AChEs. To remove excess inhibitor after reaction with enzyme, the enzyme solution was mixed with 2-fold volume of hexane and vigorously shaken with a vortex mixer for 1 min. The aqueous phase was separated by centrifugation at 3000g for 10 min at 3 °C. The solution containing phosphorylated AChE was incubated with various concentration of 2-PAM or HI-6 for various reactivation periods. To remove small molecules such as reactivator and phosphorylated oxime, the reactivating mixture was filtered through a micro spin-column packed with Sephadex-G50 (Bio-Rad) in a centrifuge at 300g for 1 min at 3 °C. The AChE activity of the filtrate was measured in a 96-well microplate.*AChE reactivation with newly synthesized oxime compounds.* The reactivating abilities of the newly synthesized oximes were evaluated against DFP or paraoxon-inhibited HF or RBC AChE, respectively. The inhibited AChE was extracted with hexane, and then the aqueous phase was reacted with 5 mM of each oxime compound for 30 min for DFP-inhibited AChE and for 1 h for paraoxon-inhibited AChE, respectively.