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Syntheses and *in vitro* evaluations of uncharged reactivators for human acetylcholinesterase inhibited by organophosphorus nerve agents

Julien Renou [a,b], Guillaume Mercey [a,b], Tristan Verdelet [a,b], Emilia Păunescu [a,b], Emilie Gillon [c], Mélanie Arboléas [c], Mélanie Loiodice [c], Maria Kliachyna [d], Rachid Baati [d], Florian Nachon [c], Ludovic Jean [a,b,*], Pierre-Yves Renard [a,b,*]

[a] *COBRA – CNRS UMR 6014 & FR 3038, Rue Lucien Tesnière, 76821 Mont Saint Aignan, France*
[b] *Université de Rouen, Place Emile Blondel, 76821 Mont Saint Aignan, France*
[c] *Cellule enzymologie, Département de Toxicologie, Institut de Recherche Biomédicale des Armées, 24, Avenue des Maquis du Grésivaudan, BP87, 38702 La Tronche, France*
[d] *Faculté de Pharmacie, Université de Strasbourg, CNRS UMR 7199, Laboratoire des Systèmes Chimiques Fonctionnels, 74 route du Rhin, BP 60024, 67401 Illkirch, France*

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ABSTRACT

Organophosphorus nerve agents (OPNAs) are highly toxic compounds that represent a threat to both military and civilian populations. They cause an irreversible inhibition of acetylcholinesterase (AChE), by the formation of a covalent P–O bond with the catalytic serine. Among the present treatment of nerve agents poisoning, pyridinium and bis-pyridinium aldoximes are used to reactivate this inhibited enzyme but these compounds do not readily cross the blood brain barrier (BBB) due to their permanent cationic charge and thus cannot efficiently reactivate cholinesterases in the central nervous system (CNS). In this study, a series of seven new uncharged oximes reactivators have been synthesized and their *in vitro* ability to reactivate VX and tabun-inhibited human acetylcholinesterase (*h*AChE) has been evaluated. The dissociation constant K_D of inhibited enzyme–oxime complex, the reactivity rate constant k_r and the second order reactivation rate constant k_{r2} have been determined and have been compared to reference oximes HI-6, Obidoxime and 2-Pralidoxime (2-PAM). Regarding the reactivation of VX-inhibited *h*AChE, all compounds show a better reactivation potency than those of 2-PAM, nevertheless they are less efficient than obidoxime and HI-6. Moreover, one of seven described compounds presents an ability to reactivate tabun-inhibited *h*AChE equivalent to those of 2-PAM.

1. Introduction

Organophosphorus (OP) compounds have been developed as chemical warfare agents (CWAs) or pesticides, and OP intoxications continue to be a threat to the general population during armed conflicts (e.g., Gulf Wars) and terrorist attacks (e.g., subway attacks in Japan in 1995) [1,2], while OP pesticide poisoning cause over 200,000 fatalities annually worldwide [3].

OP nerve agents (OPNAs) acts as irreversible inhibitors of acetylcholinesterase (AChE) via the formation of a covalent P–O bond at the serine hydroxyl group within AChE catalytic site. AChE plays a key role in neurotransmission and its inhibition prevents the hydrolysis of acetylcholine resulting in the accumulation of the neurotransmitter in the synaptic cleft. Once AChE is inhibited, the over-stimulation of cholinergic receptors, causing seizures, respiratory arrest and death. Nowadays, the therapy against OP intoxications includes the administration of an antimuscarinic agent (e.g., atropine), an anticonvulsant drug (e.g., diazepam) and an AChE reactivator such as pralidoxime (2-PAM), HI-6 or similar bis-quaternary structures (Fig. 1) [4,5].

These alpha-nucleophiles are able to attack the phosphorus atom of the phosphylated serine, yielding to the removal of the phosphyl group and thus restore AChE activity.

Over the last 60 years, many structural modifications have been realized on pyridinium and bis-pyridinium aldoximes, nevertheless all standard AChE reactivators are permanently charged and poorly cross the blood brain barrier (BBB) and limit their reactivation potency of inhibited AChE into the central nervous system (CNS) [6].

Moreover, the efficiency of these reactivators depends on the nerve agent used (for instance, HI-6 reactivates *in vitro* VX-inhibited AChE but has no effect on tabun-inhibited AChE) [7,8] and these molecules do not bind in an optimal orientation for the reactivation reaction, limiting their global efficiency. Another flaw of the pyridinium aldoxime is that the product of reactivation is a phosphyl oxime that can readily re-inhibit the enzyme [9,10].

Recently, several research groups have focused their efforts on the development of original reactivators to tackle one or several

* Corresponding authors at: COBRA – CNRS UMR 6014 & FR 3038, Rue Lucien Tesnière, 76821 Mont Saint Aignan, France. Fax: +33 2 35 52 29 71.
E-mail addresses: ludovic.jean@univ-rouen.fr (L. Jean), pierre-yves.renard@univ-rouen.fr (P.-Y. Renard).

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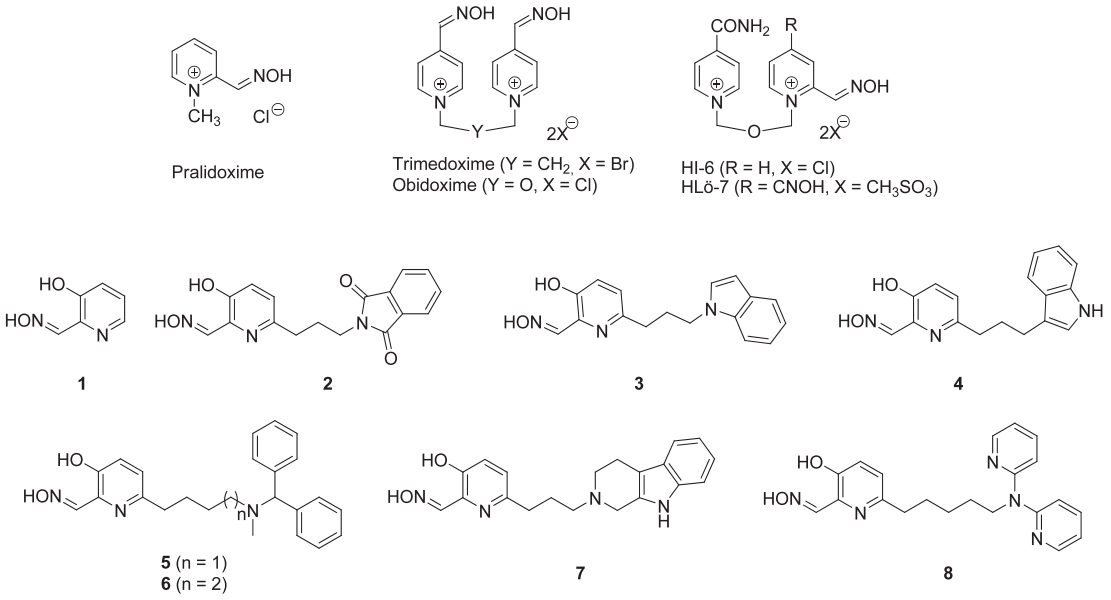

Fig. 1. Chemical structures of the main pyridinium aldoxime reactivators and uncharged AChE reactivators.

drawbacks cited above, especially to improve the reactivation of OP-inhibited AChE in the brain [11–19].

In this context, we described herein the syntheses and *in vitro* reactivation evaluation of seven new uncharged molecules that could be able to cross the BBB to reach OP-inhibited AChE in the CNS. The structures of these reactivators are composed of two moieties: the first moiety is an alpha-nucleophile, 3-hydroxy-2-pyridinealdoxime **1** (Fig. 1), recently reported, which has an ability to cleave the P–S bond of OPNAs and limits the recapture phenomenon [20,21], and the second moiety is a ligand that could interact with AChE peripheral site to increase reactivator's affinity toward the OP-inhibited AChE [22–25]. These two moieties are linked together through a carbon chain of various length.

2. Materials and methods

2.1. Synthesis procedures

Synthetic schemes, detailed procedures and full characterizations of oximes **2**–**8** and intermediate compounds are described in detail in the supporting information.

2.2. Inhibition of hAChE by OPNAs

Recombinant *h*AChE was produced and purified as previously described [26]. VX and tabun were from DGA maîtrise NRBC (Vert le Petit, France). HI-6 was from Pharmacie Centrale des Armées (Orléans, France). All other chemicals were from Sigma. Stock solution of VX and tabun were 5 mM in isopropanol. The inhibition of 120 μM *h*AChE is realized with a 5-fold excess of OPNAs and was performed in tris buffer (20 mM, pH 7.4, 0.1% BSA) at 25 °C. After a 20-min incubation, inhibited *h*AChE was desalted on PD-10 column (GE Healthcare).

2.3. Reactivation of hAChE inhibited by OPNAs

OPNA-inhibited *h*AChE was incubated at 37 °C with different concentrations of oxime in phosphate buffer (0.1 M, pH 7.4, 0.1% BSA, 5% methanol). Methanol was used for complete dissolution of the oximes. Every 1–10 min and for 1–5 h, depending on the reactivation rate, an aliquot of the reactivation mix was transferred to 1-mL cuvettes for measurement of *h*AChE activity using 1 mM acetylthiocholine in Ellman's buffer (phosphate 0.1 M, pH 7.4, 0.1% BSA, 0.5 mM DTNB, 25 °C) [27].

The enzyme activity in the control remained constant during the experiment. The percentage of reactivated enzyme ($\%E_{react}$) was calculated as the ratio of the recovered enzyme activity and activity in the control. The dissociation constant K_D of inhibited enzyme–oxime complex (E-POx) and the reactivity rate constant k_r were calculated by non-linear fit using the standard oxime concentration-dependent reactivation equation derived from the following scheme:

$$\text{E-P} + \text{Ox} \overset{K_D}{\leftrightarrows} \text{E-POx} \overset{k_r}{\rightarrow} \text{E} + \text{POx}$$

$$\%E_{react} = 100 \cdot (1 - e^{k_{obs} \cdot t}) \quad \text{and} \quad k_{obs} = \frac{k_r[\text{Ox}]}{K_D + [\text{Ox}]}$$

3. Results and discussion

The reactivation potencies of compounds **2**–**8** have been determined on the VX- and tabun-*h*AChE at pH 7.4 and 37 °C, close to physiological conditions. In order to evaluate the potential of these oximes, rate constants (k_r and k_{r2}) and the apparent dissociation constant of the reactivator/phosphyl-AChE complex (K_D) have been determined (Table 1). Concerning the results of *in vitro* reactivation of VX-*h*AChE, molecules **2**–**8** are 2–10-fold more efficient than 2-PAM, nevertheless they are 2.4–18-fold less efficient than obidoxime or HI-6.

These results could be explained by a better affinity (K_D) toward VX-inhibited AChE of our compounds **2** and **6** as compared to those one of 2-PAM (Table 1).

The results of *in vitro* reactivation test on tabun-inhibited human AChE show that, among the seven synthesized compounds **2**–**8**, only **7** exhibits an ability to reactivate the inhibited enzyme equivalent to those of 2-PAM (k_{r2} of 2-PAM and **7** are respectively 0.014 min^{-1} mM^{-1} and 0.015 ± 0.0008 min^{-1} mM^{-1}). After 340 h of incubation with 200 μM of **7** (maximal concentration tested, range was 10–200 μM), 43% of the enzyme was reactivated, though without reaching the reactivation plateau.