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First efficient uncharged reactivators for the dephosphylation of poisoned human acetylcholinesterase†

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Nerve agents are highly toxic organophosphorus compounds with strong inhibition potency against acetylcholinesterase (AChE). Herein, we describe two first extremely promising uncharged reactivators for poisoned human AChE with a superior or similar *in vitro* ability to reactivate the enzyme as compared to that of HI-6, obidoxime, TMB-4 and HLö-7.

Organophosphorus-nerve agents (OPNA) act as irreversible acetylcholinesterase inhibitors. They are used either as pest control agents (such as parathion, chlorpyrifos and diazinon) or as warfare agents (for highly toxic agents such as sarin, tabun, soman and VX) (Fig. 1).[1,2]

OPNA inhibit acetylcholinesterase (AChE, EC 3.1.1.7) *via* the formation of a covalent P–O bond at the serine hydroxyl group within the enzyme's active site. Inhibited enzyme is not able to hydrolyze acetylcholine anymore and thus cannot fulfill its essential role in neurotransmission. The resulting accumulation of the neurotransmitter in the synaptic cleft leads to the over-stimulation of cholinergic receptors, causing seizures, respiratory arrest and death. Pest control agents have been accounted for over 200 000 deadly chronic intoxication yearly,[3] thus it is of prior importance to find an efficient way to reactivate the phosphylated enzyme, and not only for the treatment of acute intoxications which could be caused by a terrorist attack. Presently, a combination of an antimuscarinic agent (*e.g.* atropine), AChE reactivator such as one of the standard pyridinium oximes (pralidoxime, trimedoxime or TMB-4, obidoxime, HI-6, HLö-7)[4] and anticonvulsant drug (*e.g.* diazepam) has been used for the treatment of organophosphate poisoning in humans (Fig. 2).[2] Those highly nucleophilic compounds add to the phosphorus atom of the phosphylated serine, yielding to the removal of the phosphonate from the enzyme, which thus induces the recovery of the enzyme catalytic activity .

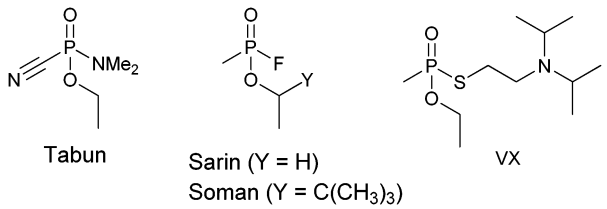

Fig. 1 Structure of some warfare agents.

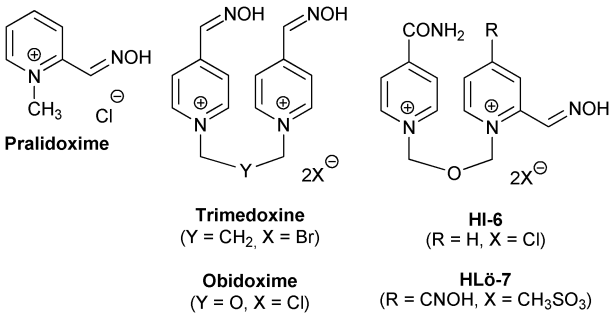

Fig. 2 Structure of AChE reactivators.

Structural modifications of pyridinium oximes have been widely described in the literature,[5,6] but all existing reactivators are permanently charged cationic compounds that poorly cross the blood–brain barrier (BBB) and thus cannot efficiently reactivate cholinesterases in the central nervous system (CNS).[7] For instance, HI-6 does not readily penetrate into the CNS[7b] and the mean BBB penetration ratio of pralidoxime was approximately 10%.[7c] Moreover, those reactivators do not bind optimally to the AChE catalytic site,[8] limiting their global efficiency, and their activity depends on the nerve agent used (for instance, HI-6 reactivates *in vitro* VX-poisoned AChE, it has no effect onto Tabun-poisoned AChE). Another drawback for these reactivators is that the resulting phosphylated oxime can act as a nerve agent since the recovered

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† Electronic supplementary information (ESI) available: Experimental procedures, copies of spectral data of **2** and **3** and reactivation kinetics. See DOI: 10.1039/c1cc10787a

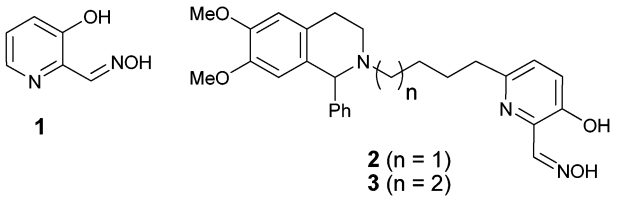


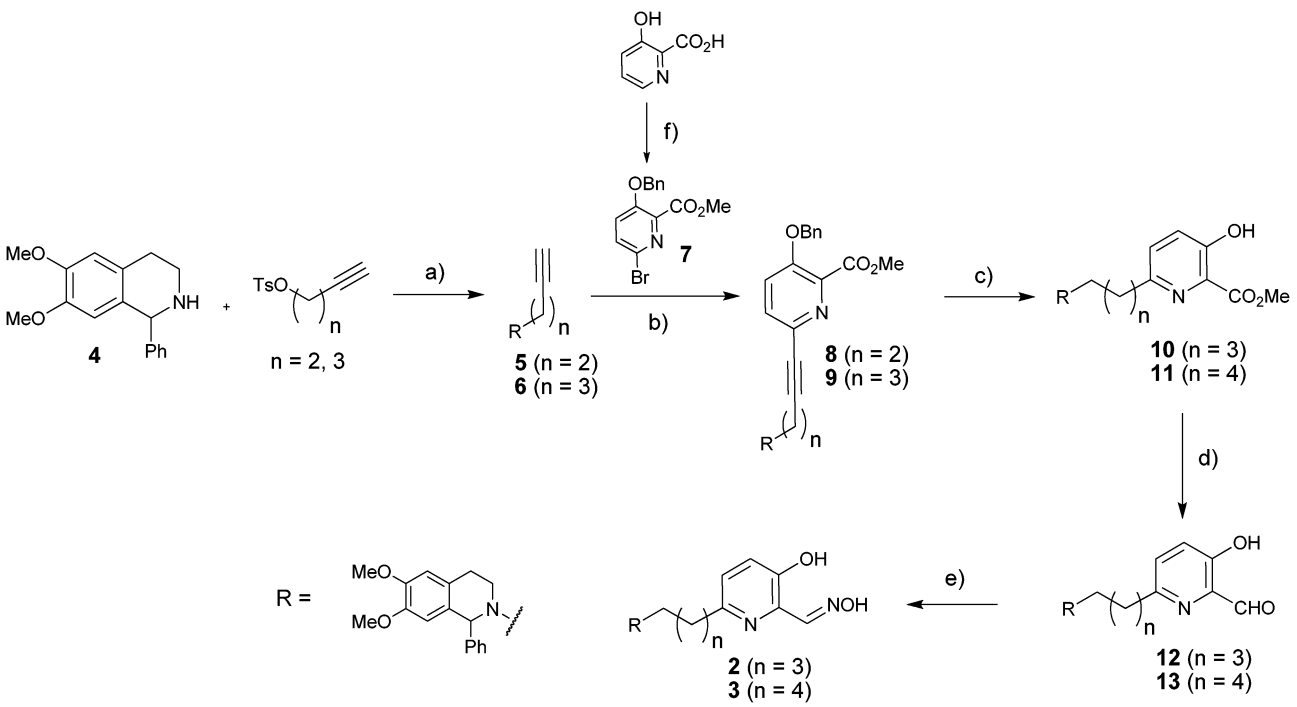
Fig. 3 Structure of non-quaternary AChE reactivators.

catalytically active serine can further attack this phosphonate (recapture phenomenon). Aiming at the development of a non-ionic reactivator, which can more easily penetrate the BBB than all existing reactivators by removing the permanent positive charge, and thus increasing their lipophilicity, bind efficiently to the AChE active site, and does not suffer from the recapture phenomenon, we report in this article the synthesis and evaluation of the first wide scope and uncharged AChE reactivators with a similar or superior *in vitro* ability to reactivate human AChE than HI-6, obidoxime, trimedoxime and Hlö-7.

Recently, the ability of 3-hydroxy-2-pyridinealdoxime **1** to cleave the P–S bond of OPNA was reported yielding the corresponding isoxasole and non-toxic phosphonic acid.[9,10] The rapid formation of this isoxazole through an intramolecular attack of the phenol onto the nitrogen atom could thus prevent the recapture of the activated phosphonate by the catalytically active serine. As expected from its reasonably low pK_a (8.2 ± 0.1), non-quaternary oxime **1** showed a noticeable ability to reactivate VX-poisoned human AChE (h-AChE) (k_r = 0.5 ± 0.1 min^{-1}, pH 7.0, 25 °C) yet with a very low affinity towards inhibited enzyme (K_D = 32 ± 11 mM) (Fig. 3).

In order to increase its affinity, we proposed to attach the oxime **1** to a ligand able to bind with the peripheral site of the enzyme. The latter lies at the entrance to the AChE gorge at the bottom of which is placed the active site of the enzyme. Oxime and ligand are linked through an alkyl or heteroalkyl chain fitting in AChE gorge. A tightly balanced compromise for AChE binding encouraged us to choose a moderate AChE peripheral site binder such as phenyl-tetrahydroisoquinoline **4**[11] in order to limit reversible AChE inhibition by the resulting isoxazole. Moreover, molecular docking simulations suggested that the best candidates for reactivation seem to be compounds whose linker is in the meta position to the oxime function.

The synthesis of these reactivators started with the N-alkylation of phenyltetrahydroisoquinoline **4**[12] with two different tosylates to give compounds **5** and **6**. Then, a Sonogashira coupling reaction between alkynes and bromopyridine **7** prepared in three steps from 3-hydroxypicolinic acid[13] (esterification, followed by electrophilic bromination and protection of the hydroxyl group into benzyl ether) afforded efficiently the desired compounds **8** and **9** in 66% and 75% yield, respectively. Reduction of alkyne and deprotection of the hydroxyl group then furnished compounds **10** and **11** in excellent yield. For the introduction of the required aldehyde moiety, best results were obtained using a sequence comprising the protection of the phenol group as TBS ether, the reduction of methyl ester into corresponding aldehyde by using DIBAL-H, and a subsequent deprotection



Scheme 1 Synthesis of reactivators **2** and **3**. *Conditions and reagents*: (a) K_2CO_3, CH_3CN, reflux, 15 h, 46% (*n* = 2), 71% (*n* = 3); (b) **7**, $Pd(PPh_3)_4$, CuI, NEt_3, THF, RT, 15 h, 66% (*n* = 2), 75% (*n* = 3); (c) H_2 (1 atm), $Pd(OH)_2$, EtOAc, 15 h, RT, 92% (*n* = 3), 92% (*n* = 4); (d) 1. TBSCl, imidazole, DMF, RT, 2 h; 2. DIBAL-H, CH_2Cl_2, −78 °C, 10 min; 3. TBAF, THF, 0 °C, 30 min (over three steps) 53% (*n* = 3), 53% (*n* = 4); (e) NH_2OH, HCl, NaOAc, EtOH, RT, 1 h, 88% (*n* = 3), 71% (*n* = 4); (f) 1. MeOH, H_2SO_4 cat., reflux, 24 h, 80%; 2. Br_2, 0 °C, H_2O, 2 h, 53%; 3. BnBr, K_2CO_3, acetone, reflux, 15 h, 93%.

Table 1 Reactivation rate constant (k_r), dissociation constant (K_D) and bimolecular reactivation rate constant (k_{r2}) of HI-6, obidoxime, HLö-7, trimedoxime, **2** and **3**

	k_r/min^{-1}	K_D/μM	k_{r2}/mM^{-1} min^{-1}
VX-hAChE			
Obidoxime	0.60 ± 0.05	54 ± 12	11
	0.893[a]	27.4[a]	32[a]
HLö-7	0.49[a]	7.8[a]	63[a]
Trimedoxime	—	—	0.50 ± 0.02
HI-6	0.44 ± 0.15	50 ± 26	9
2	0.82 ± 0.16	47 ± 20	17
3	0.35 ± 0.05	6 ± 2	61
Tabun-hAChE			
Obidoxime	0.040 ± 0.006	250 ± 110	0.16
	0.040 ± 0.001[a]	97 ± 11[a]	0.4[a]
HLö-7	0.020 ± 0.0007[a]	106 ± 15[a]	0.2[a]
Trimedoxime	0.085 ± 0.005	145 ± 25	0.7
HI-6	∅[b]	∅[b]	∅[b]
2	0.042 ± 0.006	25 ± 9	1.7
3	0.015 ± 0.002	5 ± 2	3.4

[a] From ref. 14. [b] No reactivation up to 5 mM HI-6.

gave the compounds **12** and **13** in 53% yield. A temporary protection of the phenol group as TBS ether proved necessary for an efficient reduction of the ester into aldehyde. Finally, treatment of aldehyde with hydroxylamine afforded quantitatively oximes **2** and **3** (Scheme 1).

Oximes **2** and **3** are weak inhibitors of native hAChE with IC_{50} about 100 μM (respectively, 50% inhibition at 100 μM and 30% at 50 μM).

The determination of reactivation rate constants of VX- and tabun-poisoned hAChE by oximes **2** and **3** under physiological conditions showed that this new family of non-quaternary reactivators is extremely promising. Their reactivation efficiencies, or k_{r2} (k_r/K_D), equal and even exceed those of HI-6, obidoxime and HLö-7 in similar conditions, due to improved k_r and/or K_D (Table 1). For details, oxime **2** is 2-fold more efficient than HI-6 at reactivating VX-hAChE due to higher k_r. Oxime **3** is about 6-fold more efficient than HI-6 and obidoxime due to a better affinity and at least as efficient as HLö-7. The most dramatic improvement is achieved for the reactivation of tabun-poisoned AChE. Oxime **2** is 10-fold more efficient than obidoxime due solely to higher affinity. Oxime **3**, despite a 3-fold lower k_r, is remarkably 20-fold more efficient than obidoxime because of K_D improving over 50-fold, and with a better affinity, oxime **3** is much more efficient than HLö-7. Despite a lower reactivation rate constant (2- and 6-fold lower), oximes **2** and **3** are 2-fold and 5-fold more efficient than trimedoxime due to drastically improved affinity towards tabun-poisoned hAChE.

The increase in linker length by one methylene unit has a similar effect for the reactivation of both VX- and tabun-poisoned hAChE: reactivity decreases whereas affinity increases. Thus, there is a trade-off between affinity and reactivity. This trade-off suggests that the longer linker gives more flexibility to the reactivator, allowing better binding to the inhibited enzyme, but in a less optimal orientation in regard to the reactivation reaction.

In conclusion, we have described two non-quaternary pyridine aldoximes as a new extremely promising family of AChE reactivators. They are as or more efficient than all existing oximes to reactivate VX- and tabun-inhibited AChE. Now, other analogues are in preparation to determine the optimized length and position of the linker to the oxime function. In this communication, **4** was used as a racemic mixture, but we are aware of the plausible enzyme kinetic effects of each enantiomers and it will be discussed in a future paper. Moreover, in order to evaluate their ability to cross the BBB, *in vivo* assays will be realized and the corresponding results will be reported in due time.

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