Bioorganic & Medicinal Chemistry Letters 24 (2014) 5743–5748

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

New efficient imidazolium aldoxime reactivators for nerve agent-inhibited acetylcholinesterase

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ARTICLE INFO

Article history:
Received 28 July 2014
Revised 26 September 2014
Accepted 17 October 2014
Available online 22 October 2014

Keywords:
Human acetylcholinesterase
Reactivator
Nerve agent
Imidazolium aldoxime
Peripheral site ligand

ABSTRACT

Herein, we described a new class of uncharged non-pyridinium reactivators for nerve agent-inhibited acetylcholinesterase (AChE). Based on a dual site binding strategy, we conjugated the imidazolium aldoxime to different peripheral site ligands (PSLs) of AChE through alkyl chains. Compared with the known quaternary pyridinium reactivators, two of the resulting conjugates (**7g** and **7h**) were highlighted to be the first efficient non-pyridinium oxime conjugates exhibiting similar or superior ability to reactivate sarin-, VX- and tabun-inhibited AChE. Moreover, they were more broad-spectrum reactivators.

Organophosphate (OP) nerve agents (e.g., sarin, VX, tabun, and soman) are highly toxic compounds and pose potential threats to the military and the public (such as the terrorist attacks in Tokyo subway in 1995 and the recent Syria civil war).[1] Acetylcholinesterase (AChE) is a hydrolase which terminates cholinergic neurotransmission by hydrolyzing the neurotransmitter acetylcholine (ACh).[2] The acute toxic effect of OPs stems from irreversible inhibition of AChE by forming a covalent P–O bond in the active site (A-site) of the enzyme, and the resulting accumulation of unhydrolyzed ACh in synapse would lead to cholinergic crisis, respiratory distress, convulsive seizures and ultimately death.[3]

Current available drugs for OP-poisoning include an AChE reactivator, such as one of the standard pyridinium oximes (e.g., pralidoxime or 2-PAM, trimedoxime or TMB-4, obidoxime, HI-6, and MMB-4 Fig. 1),[4,5] a muscarinic receptor antagonist (e.g., atropine) and an anticonvulsant (i.e., diazepam).[6,7] It is generally believed that the highly nucleophilic oximes could break the P–O bond and restore the enzyme's activity.[8] Whereas one drawback for these quaternary reactivators is that they provide little or no protection against neurological effects of OP exposure in the central nervous system (CNS), because the permanent charges seriously limit their blood–brain barrier (BBB) penetration,[9] while the brain is a major target of nerve agents.[10] Another weakness is that there is no

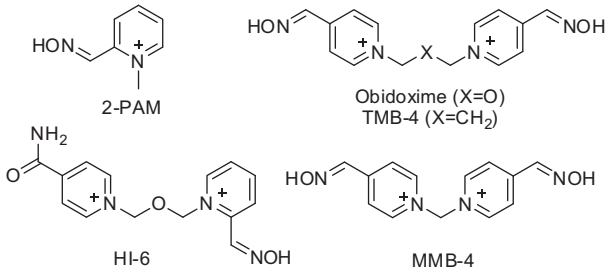

Figure 1. Current available pyridinium oximes in the treatment of OP poisoning.

universal broad-spectrum oxime suitable for the antidotal treatment of various OP-poisoning, especially in the cases of tabun and soman. For instance, HI-6, the best available reactivators to date, is inefficient in reactivating tabun-inhibited hAChE.[4,5] So it remains a challenge to develop effective antidotes for OP exposure.

Over the past several decades, a number of strategies have been developed to overcome the problems mentioned above.[11–13] It was proven that nonionic reactivators (e.g., monoisonitrosoacetone or MINA, amidine-oximes, Fig. 2) would facilitate the BBB penetration as a result of increased lipophilicity, and they showed obvious superiority to charged 2-PAM as antidotes for CNS poisoning.[14–17] Nevertheless, the absence of charge will cause decreasing reactivation potency because the uncharged reactivators don't properly bind

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http://dx.doi.org/10.1016/j.bmcl.2014.10.055
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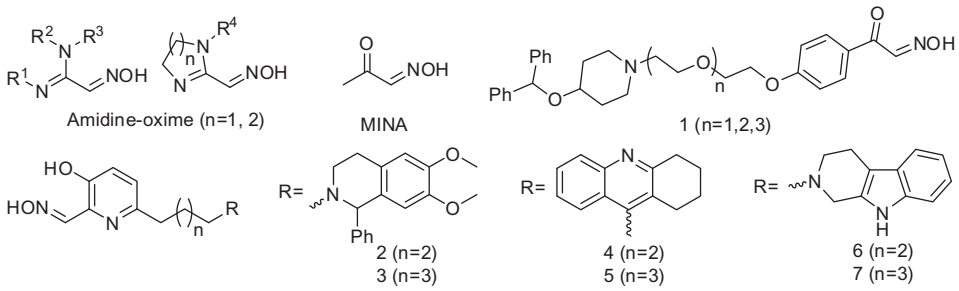


Figure 2. Chemical structures of some reported nonionic oximes.

to the aromatic moiety of the A-site,[18] which buries at the bottom of a deep active gorge into the AChE.[19] However, there is a peripheral site (P-site) at the entrance of the active gorge, serving as binding site for distinctive substrates.[20,21] Accordingly, a dual site binding strategy was proposed, a peripheral site ligand (PSL) was conjugated to the oxime group to contribute extra affinity for AChE at the P-site.[22,23] Based on this strategy, a series of promising reactivators were synthesized (conjugates **1**–**7**, Fig. 2), and among these compounds, conjugate **3** outperformed all others. But all these conjugates are pyridyl aldoximes and they are difficult to prepare due to a long synthetic route, moreover, their reactivation potencies against sarin-inhibited hAChE are still unknown.[24–27] Hence we sought to develop alternatives of the pyridyl aldoximes.

From the beginning, a kind of imidazolium aldoximes drew our attention, whose chemical structures are similar to the amidine oximes mentioned above.[16,17] Early in 1990s, quaternary imidazolium aldoximes had been intensively investigated as reactivators,[28–31] and recently, a series of nonquaternary imidazolium aldoximes (Fig. 3) were reported as antidotes for OP poisoning.[32–35] Nevertheless, the reactivation abilities of these oximes are still not satisfying and they need further modification for more efficient reactivators.

Therefore, enlightened by the dual site binding strategy, the PSL (phenyl-tetrahydroisoquinoline, PIQ) from conjugates **3** was connected to the imidazolium oxime through flexible alkyl chains, aiming at developing more efficient tertiary reactivators (Fig. 3). The designed conjugates were expected to possess enhanced BBB penetration due to dramatic improvement of calculated lipophilicity (a higher value of $S + \log P$ indicates higher lipophilicity; values are listed in Table 1). In this study, we prepared eight new tertiary imidazolium reactivators (Fig. 3), the in vitro screening essays demonstrated that conjugates **7g** and **7h** were efficient and relatively broad-spectrum reactivators.

Two different approaches were utilized for the synthesis of conjugates **7a**–**7h**. They are outlined in Schemes 1 and 2. In both approaches, the starting materials tetrahydroisoquinoline (TIQ) **1a** and PIQ **1b** were firstly synthesized. In path A, it was started with N-alkylation of **1a** and **1b** with two different halide **2a** and **2b** to give the intermediates **3a**, **3d**, **3e** and **3h**. They were treated with TsCl in pyridine to produce the tosylates **4a**, **4d**, **4e** and **4h.** Then N-alkylation of imidazole-2-carboxaldehyde **5a** with the tosylates provided compounds **6a**, **6d**, **6e** and **6h.** Finally, treatment of the latters with hydroxylammonium chloride afforded the target conjugates **7a**, **7d**, **7e** and **7h**, whose methylene length is either 3 or 6 units. Unfortunately, efforts to obtain conjugates with methylene length of 4 or 5 units using method A failed, because unexpected compounds were produced in the N-alkylation of **5a**. Thus, we turned to path B and synthesized the desired compounds successfully. The starting materials **2b** and **2c** were converted to tosylates **3b** and **3c** in a similar way as in path A. In comparison with halide moiety in **3b** and **3c**, the tosylate moiety was much easier to undergo an N-alkylation reaction with **5a** to obtain the intermediates **4b** and **4c**, and then they were readily converted to oximes **6b** and **6c** by treating with hydroxylammonium chloride. At last, N-alkylation was conducted again between **6b**, **6c** and **1a**, **1b** to give the desired compounds 7**b**, **7c**, **7f** and **7g**. In addition, compound **3** (Fig. 3), one of the most promising uncharged reactivators in the literature, was prepared in a similar way as described by Mercey et al.[26] In contrast to **7a**–**7h**, the synthetic route of pyridine-aldoxime **3** was long as ten steps from the starting material **1b**. Furthermore, the synthesis of **3** involved a Sonogashira coupling reaction and two protection and deprotection reactions, which were complex, costly and poor yield, while the synthesis of conjugates **7a**–**7h** was simple, economical and high-yield.

The in vitro experiments were conducted with fresh human whole blood serving as enzyme source. The enzyme activity was measured using a similar method of Ellman et al.[36] and the experimental detail was described in the section of supporting information. Two oxime concentrations (1 mM and 0.1 mM) were selected

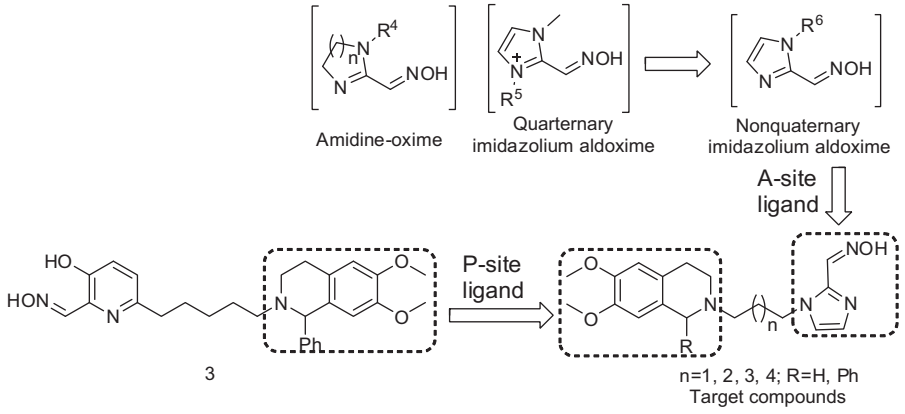
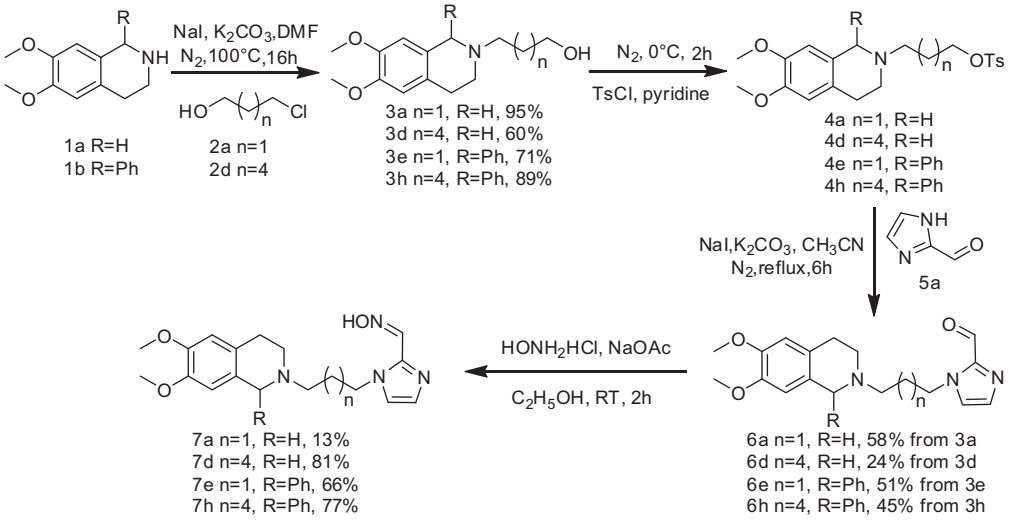


Figure 3. Chemical structures of the designed target compounds based on a dual site binding strategy.

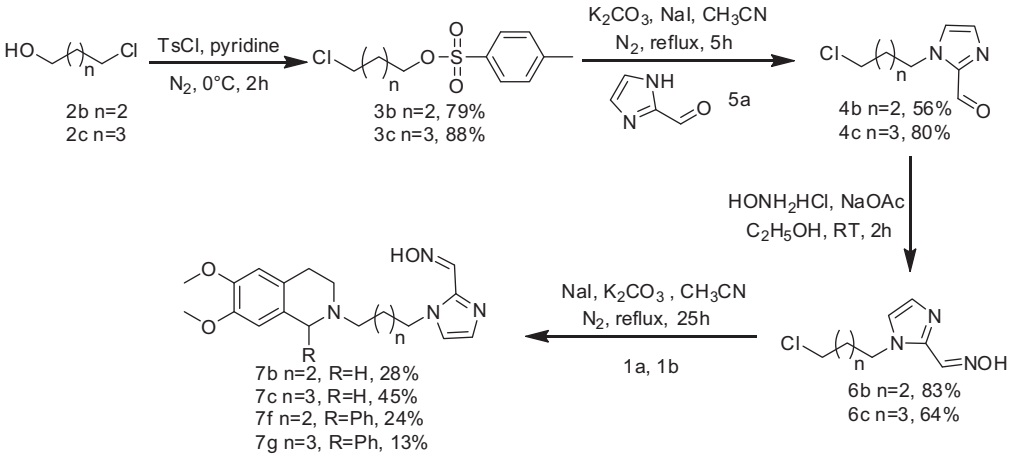
Table 1
Reactivation of nerve agent-inhibited hAChE by **3**, **5b**, **7a–7h** and the reference reactivators (1 mM and 0.1 mM)

Oxime	Reactivation rate (%)						$S + \log P$
	Sarin		VX		Tabun		
	1 mM	0.1 mM	1 mM	0.1 mM	1 mM	0.1 mM	
Obidoxime	35 ± 7	7 ± 1	84 ± 0	54 ± 2	84 ± 5	24 ± 1	−4.33
HI-6	84 ± 1	25 ± 0	83 ± 2	59 ± 0	10 ± 0	4 ± 0	−3.51
MMB-4	49 ± 3	45 ± 1	71 ± 3	23 ± 3	21 ± 0	5 ± 1	−3.65
TMB-4	18 ± 4	3 ± 0	80 ± 4	24 ± 4	91 ± 5	58 ± 5	−3.46
3	68 ± 1	64 ± 6	84 ± 4	68 ± 16	68 ± 7	54 ± 5	5.06
7a	−2 ± 1	−2 ± 2	31 ± 4	−1 ± 0	2 ± 1	−1 ± 1	2.34
7b	8 ± 1	0 ± 0	68 ± 1	28 ± 0	1 ± 0	1 ± 0	2.6
7c	14 ± 2	2 ± 0	89 ± 2	46 ± 4	7 ± 2	5 ± 4	2.99
7d	34 ± 0	6 ± 0	89 ± 1	54 ± 5	17 ± 2	32 ± 1	3.37
7e	2 ± 2	0 ± 0	13 ± 3	3 ± 0	1 ± 1	0 ± 0	3.73
7f	27 ± 1	4 ± 0	47 ± 14	6 ± 5	5 ± 2	3 ± 2	4.06
7g	69 ± 3	21 ± 1	92 ± 3	67 ± 5	47 ± 2	33 ± 13	4.45
7h	94 ± 7	50±7	80 ± 2	84 ± 0	53 ± 1	44 ± 3	4.84
5b	5 ± 2	3 ± 1	8 ± 0	7 ± 0	10 ± 0	3 ± 0	0.12

Experiments were performed in triplicate at 37 °C in phosphate buffer (0.10 M, pH = 7.4); AChE inhibition percentages ranging 85–98% were firstly achieved before the reactivation assays. Data shows the average and standard deviation. The values of $S + \log P$ (calculated by ADMET Predictor 5.5) were also given.



Scheme 1. Preparation of imidazolium aldoxime conjugates **7a**, **7d**, **7e**, **7h**, path A.



Scheme 2. Preparation of imidazolium aldoxime conjugates **7b**, **7c**, **7d**, **7e**, path B.

in the biological essays, because the current available oximes generally show obvious reactivation potency only in relatively high concentrations, especially in the cases of tabun (for examples, obidoxime exhibited high reactivation rate for tabun-inhibited hAChE at 1 mM concentration, but the reactivation rate greatly decreased at 0.1 mM concentration, data shown in Table 1).

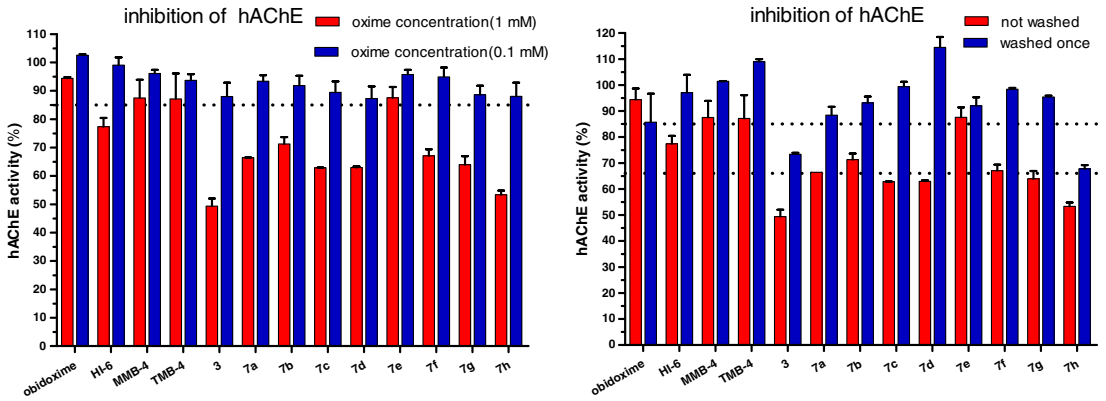


Figure 4. Inhibition of hAChE by conjugates **3**, **7a**–**7h** and the reference oximes at different concentrations (1 mM and 0.1 mM, left figure) and under different treatments (1 mM, right figure). Data depicted shows the average and standard deviation (calculated as a fraction of positive control samples without oximes) for AChE activity ($n = 3$ incubations per oxime) after inhibition. Unless otherwise indicated, the standard deviation bars were smaller than the data marker.

Considering the fact that the inhibition ability would help to provide an insight into the oximes binding affinity for hAChE, and a strong or irreversible inhibitor of AChE would lead to toxicity,[37,38] we firstly evaluated the AChE inhibition potency of these new reactivators (**7a**–**7h** and **3**).

Four quaternary reactivators (obidoxime, HI-6, MMB-4 and TMB-4, Fig. 1) were used as reference compounds in the inhibition experiments, the results are depicted in Figure 4. It is apparent that these new oximes, including **3**, were moderate or weak inhibitors of hAChE, while the reference reactivators just showed slight inhibitory abilities (Fig. 4). At 1 mM oxime concentration, enzyme inhibition percentages by **3**, **7g**, **7h** were 51%, 36% and 47% respectively, while the inhibition percentages by all other oximes were below 40%. In contrast, at 0.1 mM oxime concentration, the enzyme activity was almost completely released (>85%). We further determined the IC_{50} of **3**, **7g** and **7h** (data show in Table 2) and confirm that they were weak inhibitors of AChE. In order to determine whether the inhibition was reversible, the erythrocyte (AChE bound to it covalently) was washed once with PBS (0.1 M, pH = 7.4). For most tested oximes, the activities of the hAChE were greatly restored (>85%) after wash (Fig. 4). For examples, at 1 mM oxime concentration, hAChE activities increased from 53% to 68% for **7h**, 49% to 73% for **3** and 64% to 95% for **7g**, respectively. On the contrary, it was found that there was no change in the activity of the nerve agent-inhibited hAChE in an extra experiment. Therefore, we concluded that the synthesized oximes inhibited hAChE in a moderate and reversible way, this could not only provide suitable AChE affinity for reactivation of OP poisoning, but also avoid toxicology caused by strong inhibition of AChE. These results encouraged us to proceed to the reactivation experiments.

Sarin, VX and tabun were used to determine the reactivation potency of oximes **7a**–**7h**, **3** and the reference compounds mentioned above. The reactivation rates were calculated relatively to the poisoned control (85–98% AChE inhibition) and the results are shown in Table 1. In the case of sarin, the most promising uncharged reactivators were **7h**, **7g** and **3**. Their reactivation rates equaled and even exceeded those of MMB-4 and HI-6, while **7d** and **7f** showed relatively low reactivation potency. For VX-inhibited hAChE, it is inspiring that most of the novel oximes were promising reactivators; some of them even exhibited higher reactivation abilities than obidoxime and HI-6 (such as **7c**, **7d**, **7g**, **7h** and **3**). In terms of tabun-inhibited hAChE, to our best knowledge, oxime **3**, **7g** and **7h** were the most promising nonquaternary reactivators reported until now, while HI-6 and oximes **7a**–**7f** only slightly reactivated tabun-inhibited hAChE. For conjugate **3**, our results are in line with what Mercey et al. had reported, but they did not evaluate the reactivation ability against sarin-inhibited hAChE.[24,25] In contrast to those quaternary oximes, **7g**, **7h** and **3** emerged as more broad-spectrum for sarin-, VX- and tuban-inhibited hAChE, while obidoxime and TMB-4 were less efficient for sarin; HI-6 and MMB-4 were much less efficient for tabun poisoning. We also determined the reactivation potency of imidazolium-2-aldoxime (**5b**), the results demonstrated that most conjugates showed improved reactivation potency, especially in the cases of **7d**, **7g** and **7h**, which justified the concept of dual-site biding strategy.

In addition, in order to get a complete comprehension of the reactivation of conjugates **7g** and **7h**, we measured their reactivation rate constant K_r, dissociation constant K_D and second order reactivation rate constant K_{r2} for sarin-, VX- and tabun-inhibited hAChE,[39] the results were presented in Table 2. As a result of improved K_r or K_D, their reactivation efficiencies, or K_{r2} (K_r/K_D), equaled and even exceeded those of obidoxime, HI-6 and TMB-4 in similar conditions, which were in line with the conclusions we got above. For details, conjugate **7h** was more efficient than HI-6 and **3** for sarin poisoning due to a better affinity (lower K_D). For

Table 2
Reactivation rate constant (K_r), dissociation constant (K_D), second order reactivation rate constant (K_{r2}) and IC_{50} of obidoxime, HI-6, TMB-4, **3**, **7g** and **7h**

Oxime	K_r/min^{-1}			$K_D/\mu M$			$K_{r2}/mM^{-1}min^{-1}$			IC_{50} (mM)
	Sarin	VX	Tabun	Sarin	VX	Tabun	Sarin	VX	Tabun	
Obidoxime	0.011 ± 0.002	0.22 ± 0.02	0.028 ± 0.002	90 ± 39	32 ± 9	66 ± 16	0.12	6.95	0.43	2.21
HI-6	0.048 ± 0.003	0.25 ± 0.01	n.d.[a]	93 ± 19	27 ± 3	n.d.	0.52	9.30	n.d.	1.36
TMB-4	n.d.	n.d.	0.056 ± 0.004	n.d.	n.d.	79 ± 20	n.d.	n.d.	0.70	2.57
3	0.063 ± 0.022	0.22 ± 0.02	0.027 ± 0.003	88 ± 77	22 ± 7	18 ± 10	0.72	10.03	1.54	0.89
7g	0.040 ± 0.008	0.14 ± 0.02	0.009 ± 0.001	143 ± 78	24 ± 8	26 ± 15	0.28	5.75	0.36	0.93
7h	0.033 ± 0.004	0.21 ± 0.01	0.012 ± 0.001	41 ± 16	25 ± 4	14 ± 8	0.81	8.43	0.86	0.87

[a] not determined.

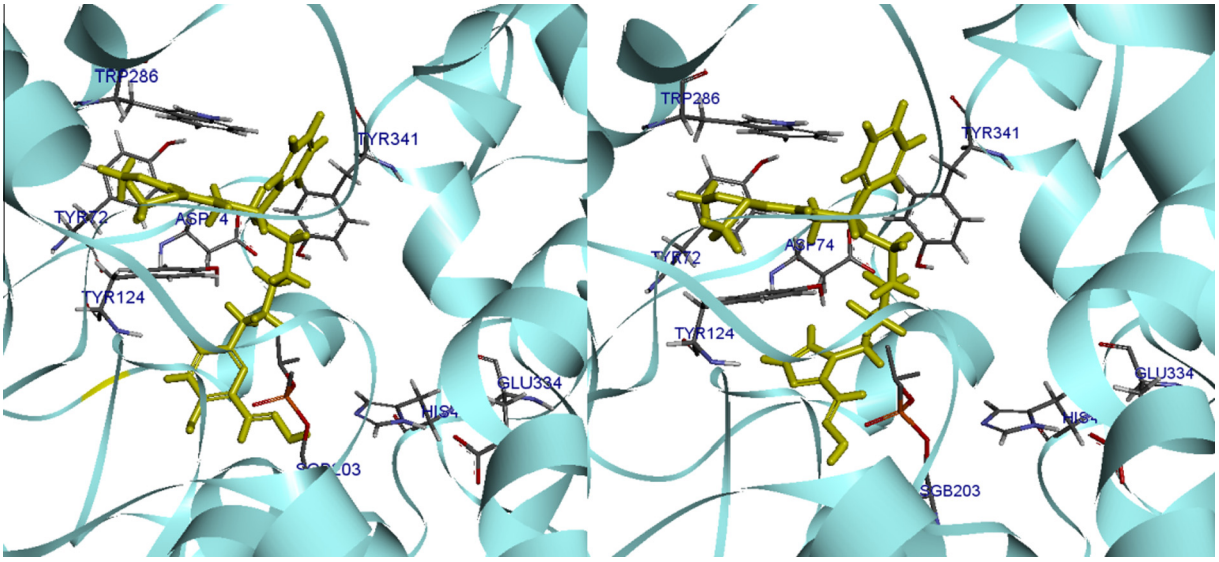


Figure 5. Docked conformation of molecules **3** (left) and **7h** (right) in the active site gorge of sarin^{nonaged}-mAChE (pdb code: 2WHP). The docked conformations of the reactivators are depicted as stick model in yellow and the key amino acid residues as stick model in grey.

VX-hAChE, **7h** was almost as efficient as HI-6, obidoxime and **3** due to similar K_r and K_D. For tabun poisoning, despite a lower reactivation rate K_r, conjugate **7h** was more efficient than obidoxime and TMB-4 for greatly improved affinity for tabun-hAChE (lower K_D), while conjugate **3** was the best reactivator for tabun due to relatively high K_r and low K_D.

In this study, TIQ, an analogue of PIQ, was also chosen as the PSL, which had been proven to be effective P-site binders of reactivators for OP poisoning,[40] but replacement of PIQ by TIQ decreased the reactivation efficacy. The failure of TIQ may be due to its low affinity for P-site of AChE, while PIQ has been confirmed as a moderate P-site binder.[41] However, a PSL possessing strong affinity towards the P-site should be avoided, because it would lead to toxicity.[37,38] Therefore, a proper PSL may play a critical role for an efficient reactivators.

Furthermore, in order to determine the influence of alkyl length between the A-site ligand and PSL on the reactivation potency, alkyl chains ranging from 3 to 6 methylene units were investigated. It is clear that, along with the increasing linker length (from compounds **7a** to **7d** and **7e** to **7h**), both inhibition and reactivation potency increased in most cases. The calculated K_D for **7g** and **7h** indicated that an increased linker length may provide better affinity for poisoned hAChE. Thus, we hypothesized that a relatively longer linker may provide more flexibility for the reactivators to have a better conformation to bind to AChE, and result in higher reactivation ability. To valid our hypothesis, preliminary molecular docking studies of the most promising reactivators **3** and **7h** into the active gorge of AChE were conducted and compared. The results demonstrated that the two conjugates interacted with AChE in a similar dual site binding mode (Fig. 5). The PSLs (PIQ) of both conjugates interact with the P-site in an optimized position (key amino acid residues include Tyr72, Tyr124, Trp286 and Tyr341and Asp74),[20,21] which results in a π–π sandwiching of PIQ moiety between Trp286 and Tyr124, and provides extra affinity for AChE; while the oxime groups were located at the A-site (key amino acid residues include Ser-sarin203 or SGB203, Glu334 and His447),[19] which were supposed to serve as reactivating groups.

In summary, based on a dual site binding strategy, we have designed and synthesized a new family of uncharged conjugates for reactivation of OP-inhibited hAChE. It was proven that conjugation of a proper PSL to the imidazolium aldoxime would lead to greatly enhanced reactivation potency and two conjugates (**7g** and **7h**) emerged as first efficient and more broad-spectrum non-pyridinium reactivators than those quaternary reactivators for sarin-, VX- and tuban-inhibited hAChE. For conjugates **3**, we determined its reactivation potency against sarin-inhibited hAChE for the first time, confirming its relatively wide scope efficiency. Considering the simple synthesis route in comparison to conjugates **3**, these novel imidazolium aldoximes show economical superiority and hold promise for further design of more efficient uncharged reactivators. Finally, it should be noted that PIQ was used as a racemic mixture in this study, the separation of the enantiomers is ongoing and it will be discussed in a future paper.

Acknowledgments

The financial support from the Major Program of Ministry of Science and Technology of China (No: 2013ZX09J109-02C) is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.10.055.

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