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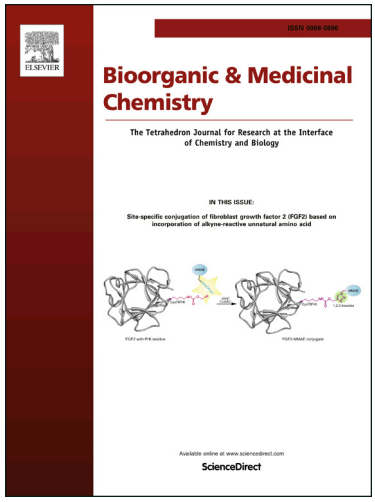

Conjugates of salicylaldoximes and peripheral site ligands: novel efficient non-quaternary reactivators for nerve agent-inhibited acetylcholinesterase

Zhao Wei, Yan-qin Liu, Sheng-zheng Wang, Lin Yao, Hui-fang Nie, Yong-an Wang, Xue-Ying Liu, Zhi-bing Zheng, Song Li

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Graphical Abstract

Conjugates of salicylaldoximes and peripheral site ligands: novel efficient nonquaternary reactivators for nerve agent-inhibited acetylcholinesterase

Based on a dual site binding strategy, the salicylaldoximes were conjugated to different peripheral site ligands through alkyl chains for construction of novel nonquaternary reactivators. Compared with the known quaternary pyridinium reactivators, some of the resulting compounds emerged as efficient and relatively broad-spectrum reactivators for nerve agent-inhibited human acetylcholinesterase.

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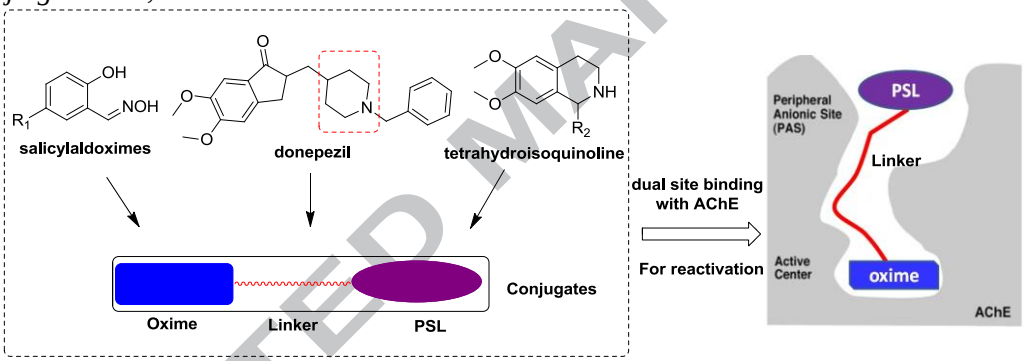

Construction of the novel dual site binding conjugates for reactivation of inhibited AChE

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ABSTRACT

A new family of nonquaternary reactivators for nerve agent-inhibited human acetylcholinesterase (hAChE) were designed, synthesized and tested in this paper. It was found that salicylaldoximes were able to quickly cleave the P-S bond of organophosphate and avoid the reinhibition phenomenon in the reactivation process, but they lacked reactivating ability due to poor affinity for AChE. Based on a dual site binding strategy, different peripheral site ligands of AChE were introduced to achieve extra affinity. The *in vitro* reactivation experiments demonstrated that some of the yielding conjugates exhibited similar or even superior ability to reactivate sarin-, VX- or tabun-inhibited hAChE in comparison with the mono- and bis-pyridinium aldoximes currently used. Moreover, due to greatly improved lipophilicity, these nonquaternary conjugates hold promise for the development of efficient centrally activating reactivators.

1. Introduction

Organophosphate (OP) nerve agents (such as sarin, tabun, soman and VX, Fig. 1) and pesticides (such as such as parathion, chlorpyrifos) are highly toxic compounds. The acute toxicity of OP stems from the phosphorylation of the active site serine residue in acetylcholinesterase (AChE), which terminates cholinergic neurotransmission by hydrolyzing the neurotransmitter acetylcholine (ACh) [1], the resulting irreversible inhibition of AChE causes accumulation of ACh at both peripheral and central cholinergic synapses, leading to cholinergic crisis, respiratory distress, convulsive seizures and ultimately death [2].

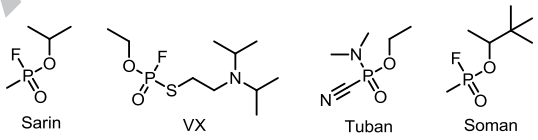


Figure 1. Chemical structures of some nerve agents

OPs represent potential threats to both military personnel and civilians [3]. To date, the nerve agents had been used in terrorist attacks (e.g., subway attack in Tokyo in 1995) [4], in murder (e.g., Kim Jong-nam's Killing in Kuala Lumpur in 2017) and in the armed conflicts (e.g., Gulf Wars) [5] as weapons of mass destruction. In addition, due to their relatively simple chemical synthesis, efforts to control the proliferation of the organophosphorus pesticides have been proved of limited success, resulting in a serious public health issue with about 3 000 000 acute intoxications and over 200 000 fatalities annually worldwide, mostly in developing countries [6]. Therefore, the development of more effective antidotes against OP poisoning remains a challenging issue.

Currently approved antidotal therapies for the treatment of OP poisoning in humans combine intramuscular injections of an AChE reactivator of the pyridinium aldoxime family (e.g., pralidoxime (2-PAM), trimedoxime (TMB-4), obidoxime, HI-6, Fig. 2) [7, 8], a muscarinic receptor antagonist (e.g., atropine), and an anticonvulsant (e.g., diazepam) [9, 10]. Pyridinium aldoxime therapy is directed toward nucleophilic reactivation of AChE covalently inhibited by OPs to restore the enzyme's activity [11].

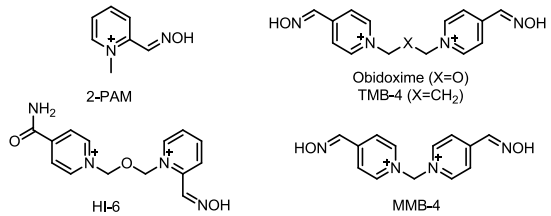


Figure 2. Current available pyridinium oximes in the treatment of OP poisoning

During the past several decades, researchers have studied various monopyridinium oximes and especially more efficient

bispyridinium oximes, but all known reactivators have several drawbacks: **(1)** there is no universal broad-spectrum oxime suitable for the antidotal treatment of various OP-poisoning. For instance, HI-6, the best available reactivators to date, rapidly reactivates sarin-, soman- and VX-inhibited AChE, but it's inefficient for tabun [7, 8]. **(2)** Phosphylated oximes (compound **c**, Fig. 4, left) formed during reactivation may act as a nerve agent and reinhibit AChE, which would reduce the antidotal efficacy of these pyridinium reactivators [12, 13]. **(3)** Most of all, due to their permanent positive, these quaternary reactivators are unable to cross the blood-brain barrier (BBB) and show poor reactivation efficiency in the central nervous system (CNS) [14], while the brain is a major target of nerve agents [15, 16]. Thereby, reactivators of broad-spectrum efficiency and especially of CNS antidotal efficiency are needed.

A number of strategies have been developed to address the problems mentioned above. Based on the use of prodrug, the charged pyridine ring of 2-PAM is replaced with a significantly less charged dihydropyridyl moiety, the resulting pro-2-PAM (Fig. 3) can penetrate the BBB and rapidly undergo oxidation in the brain to produce functionally active 2-PAM [17]. But the synthesis of prodrug is rather difficult and pro-2-PAM is unstable to autoxidation [18].

A recent spur of nonquaternary AChE reactivators has drawn our attention [19-22]. Due to increased lipophilicity, some nonquaternary reactivators (e.g. monoisonitrosoacetone (MINA), Fig. 3) [23, 24] readily penetrate the BBB and display superior *in vivo* reactivation activity to charged 2-PAM as antidotes for CNS poisoning. However, the absence of charge reduced the affinity to AChE and resulted in decreasing reactivation potency [25].

Kalisiak *et al* had designed and synthesized a class of amidine-oximes (Fig. 3), the basic amidine residue was expected to undergo protonation under physiological conditions to form a positively charged center similar to pyridinium oximes, increasing the binding affinity for OP-inhibited AChE. The *in vitro* reactivating ability for OP-inhibited AChE of amidine-oximes was improved in contrast to MINA, but still lower than 2-PAM [26, 27].

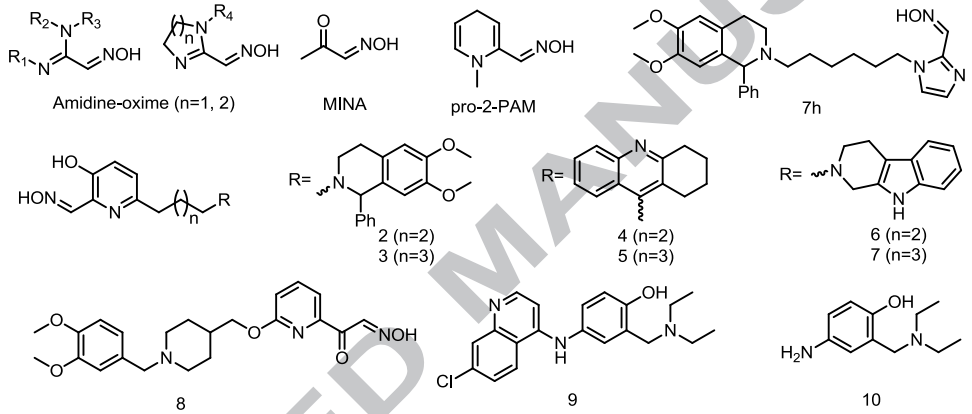


Figure 3. Chemical structures of some reported nonionic oximes

The bispyridinium oximes (e.g., HI-6 and obidoxime) exhibit superior reactivation properties to monopyridinium oximes due to the fact that the second cation interacts with the peripheral anionic site (P-site) of AChE and increases binding affinity [28]. Accordingly, De Koning and Mercey *et al* proposed to attach nonquaternary pyridine aldoxime to a ligand able to bind with the P-site, which lies at the entrance of the active gorge of AChE [29, 30]. It was found some of the resulting conjugates (Fig. 3, compounds **2-7**) exhibited dramatically enhanced reactivation potency as a result of greatly improved affinity for AChE [30-33]. These dual site binding nonquaternary reactivators hold promise for the development of more efficient centrally activating reactivators for nerve agent poisoning. However, the synthesis of these pyridine aldoxime conjugates is difficult and their reactivation potency against sarin-inhibited AChE is still unknown. In addition, McHardy *et al* reported a series of heteroaryl keto-oximes (Fig. 3, compounds **8**), which showed obvious reactivation potency for cyclosarin-inhibited hAChE [34]. Similarly, we used the dual site binding strategy to design and synthesize a series of imidazolium aldoxime conjugates, some of them (Fig. 3, compounds **7h**) exhibited similar or superior ability to reactivate sarin-, VX- and tabun-inhibited AChE in comparison to HI-6 and obidoxime [35]. Their synthesis is relatively easy and economical, but imidazolium aldoxime may encounter the problem of reinhibition (Fig. 4, left). Furthermore, it is noticeable that Katz *et al* had reported that some Mannich phenols (Fig. 3, compounds 9 and 10) were efficient reactivators for organophosphate inhibited AChE [36]. Coincidentally, we also designed and synthesized a series of Mannich phenols which showed obvious reactivating ability for nerve agent inhibited hAChE, and the corresponding research will be reported soon.

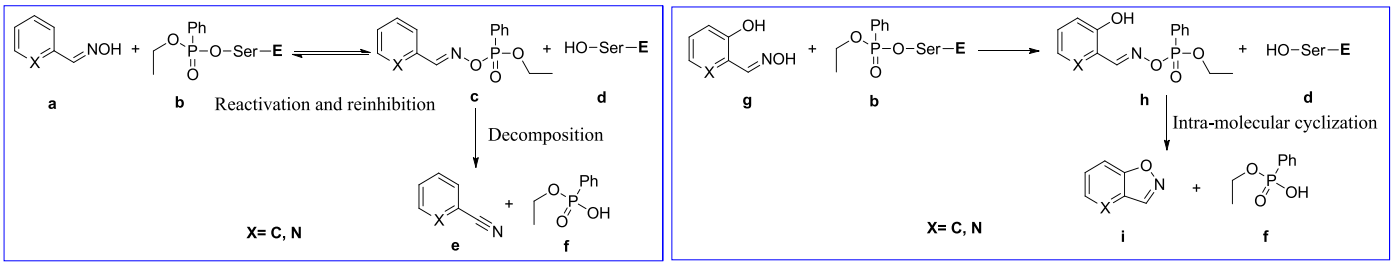


Figure 4. The mechanism of reactivation, reinhibition and intra-molecular cyclization of the phosphylated oximes

Recently, it was found that salicylaldoxime was able to cleave the P-O bond of OP, and then yielded corresponding isoxasole **i** and non-toxic phosphonic acids **f** through an intra-molecular attack of the phenol onto the nitrogen atom (Fig. 4, right) [37, 38]. The rapid formation of this isoxasole could thus avoid reinhibition by the active phosphylated oximes and allow the discovery of more efficient reactivators. Furthermore, enlightened by the structure of donepezil, a highly selective AChE inhibitor for Alzheimer's disease treatment, the piperidine ring was introduced as a linker between aldoximes and peripheral site ligands (PSLs) (Fig. 5), it was expected to interact with the AChE active gorge and contribute extra affinity for the enzyme [39], and it had been used by McHardy *et al* to design and synthesize novel reactivators (compound **8**) [34]. As a comparison, methylene was also used as linkage of aldoximes and PSLs. As a preliminary attempt, the moderate AChE P-site binder phenyltetrahydroisoquinoline (PIQ) and its analogue tetrahydroisoquinoline (TIQ) were chosen as PSLs (Fig. 5), which can avoid toxicology produced by heavy AChE inhibition [40].

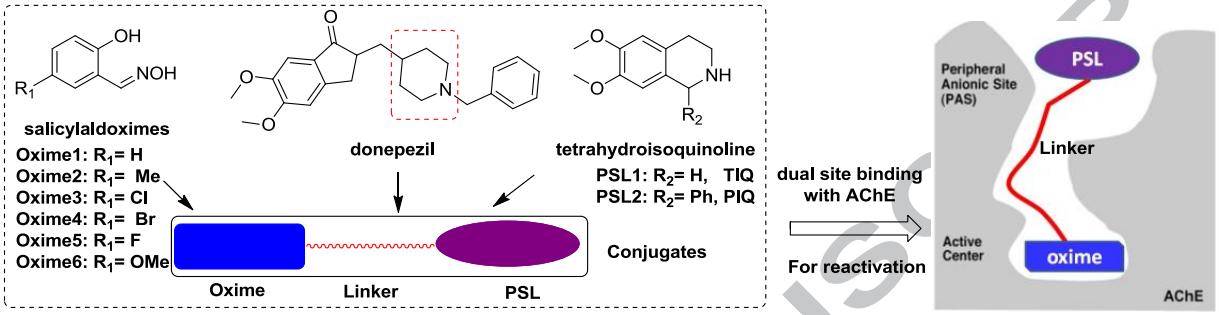


Figure 5. Construction of the novel dual site binding conjugates for reactivation of inhibited AChE

Consequently, a novel series of salicylaldoxime conjugates (Fig. 6) based on the dual site binding strategy were constructed. It was supposed that salicylaldoxime would lie at the bottom of AChE active gorge to reactivate phosphoryl serine, while the PSLs interacted with aromatic group in the P-site and compensate the salicylaldoxime's affinity for AChE (Fig. 5). The designed conjugates were expected to possess enhanced BBB penetration due to dramatic improvement of calculated lipophilicity (higher value of S+logP indicates higher lipophilicity, values are listed in table 1).

In this paper, we prepared 28 new tertiary salicylaldoxime reactivators (Fig. 6), the *in vitro* screening essays demonstrated that conjugates **3c, 4c** and **5c** were efficient and relatively broad spectrum reactivators. They have advantageous properties including **(1)** improved chemical stability (compared to pro-2-PAM) and synthesis feasibility (compared to pyridine aldoxime conjugates **2-7**); **(2)** greatly improved lipophilicity (compared to quaternary oximes); **(3)** and relatively increased *in vitro* reactivation efficacy of nerve agents-inhibited AChE (compared to quaternary oximes).

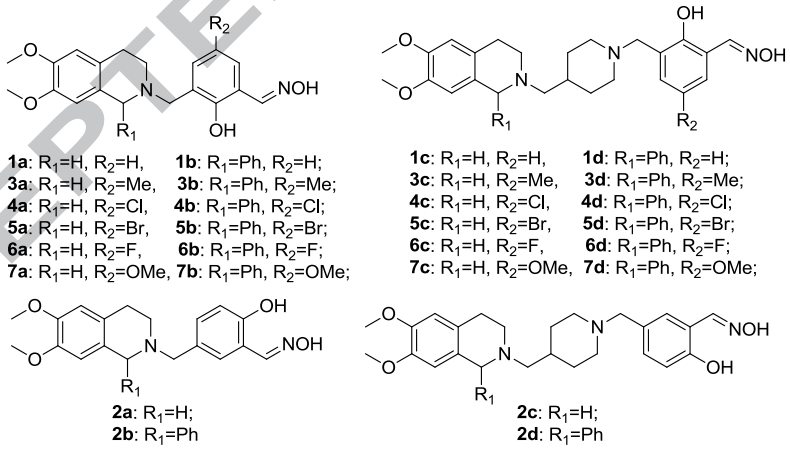


Figure 6. Novel designed and synthesized compounds in this paper

2. Materials and methods

2.1. Chemicals

All reagents and solvents were used as received from commercial sources. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz on a JNM-ECA-400 instrument in in $CDCl_3$ or DMSO-d_6, respectively. Proton and carbon chemical shifts are expressed in parts per million (ppm) relative to internal tetramethylsilane (TMS) and coupling constants (J) are expressed in Hertz (Hz). The splitting pattern abbreviations are as follows: multiplicity (s: singlet, d: doublet, dd: double doublet, dm: double multiplet, ds: double single, dt: double triplet, t: triplet, td triple doublet, tm, triple multiplet, q: quartet, quint: quintuplet, m: multiplet, br: broad). Low-resolution mass spectra were obtained using an API 3000 LC/MS with an ESI source or an Agilent 620B TOF LC/MS with an ESI source. Thin-layer chromatography (TLC) was carried out on alumina sheets precoated with silica gel 10-40 um (pH 6.2-6.8) and the chromatography were performed on silica gel (200-300 mesh), compounds were visualized under UV light at 254 nm, R_f values were given for guidance. 2-PAM was purchased from Sigma-Aldrich. HI-6 and obidoxime were synthesized ourselves [41-43]. The synthetic procedures are described in detail in the supporting information.

2.2. General in vitro hAChE screening information

Human acetylcholinesterase (hAChE), bovine serum albumin (BSA), acetylthiocholine (ATCh), 5, 5'-dithiodis-2-nitrobenzoic acid (DTNB) and 2-PAM were purchased from Sigma-Aldrich. Sarin, VX, tabun and soman were from Anti chemical command and Engineering Institute of the Chinese people's Liberation Army. (Caution! Nerve agents used in our research are highly toxic and must be handled with extreme care by well-trained personnel. Use of these materials has been approved by Anti chemical command and Engineering Institute of the Chinese people's Liberation Army. After reactivation studies, biochemical samples were neutralized by stirring with 2 M NaOH for 12 h, the remaining solutions were brought back to pH ~7 and disposed in chemical waste.) Centrifugation was conducted at 4 °C in a 3-18K instrument from Sigma, the absorption was measured on a Bio Rad Microplate Reader Model 550 (Parts).

The *in vitro* experiments were conducted with hAChE serving as enzyme source. At the beginning of each experiments, the stock solution of hAChE (dissolved in 20 mM HEPES, pH 8.0, contain 0.1% TRITON X-100) were diluted by PBS (0.1 M, pH=7.4, 0.1% BSA) and stored at 0-4°C. 10 mM oxime concentration solutions were prepared in water contain 2.5% acetic acid and 10% PEG-400 and they were further diluted by PBS (0.1 M, pH=7.4) to the required concentrations. It was found that there was no effect of CH3COOH and PEG-400 on hAChE by a control experiment. All the biological evaluation experiment were conducted triplicate in 96-well plate, the enzyme activity was measured by the time-dependent hydrolysis of ATCh in which the product thiocholine was detected by reaction with the Ellman's reagent DTNB and absorbance at 415 nm [44].

2.3. hAChE inhibition experiments

Initially 10 μL of hAChE (20 U/L in 0.1 M PBS, pH 7.4, 0.1% BSA) was incubated with 5 μL of each oxime (final concentrations: 0.2, 2, 100, 500 and 1000 μM) for 30 min at 25 °C. A blank experiment (positive control) was run in parallel in which oxime was replaced by PBS. For each sample above in the 96-well plate, 30 μl of ATCh (3.0 mM in 0.1 M PBS, pH 8.0, 0.1% BSA) along with 150 μl DTNB (0.75 mM in 0.1 M PBS, pH 7.0) and 10 μl HCl (0.1 M) was added in each well. Reaction product was monitored every 5 minutes, up to 20 minutes by testing the absorption value at 415 nm (0<abs<2.5). Enzyme activity was calculated by using the formula: %Activity= 100*S/P. (S=absorption value of test substance; P = absorption value of positive control (100% activity)). IC_{50} was determined by non-linear fitting using the standard IC_{50} equation: %Activity = $100*IC_{50}/(IC_{50}+[Ox])$.

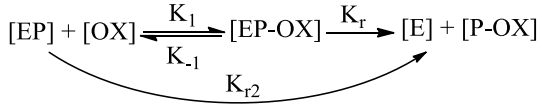
2.4. hAChE reactivation experiments

A stock solution of hAChE (20 U/mL, from sigma) was diluted 50-fold with PBS (0.1 M, pH 7.4, 0.1% BSA). Different nerve agents was diluted to proper concentrations (VX, $3*10^{-6}$M; sarin, $2.75*10^{-5}$M; tabun, $1*10^{-6}$M; soman, $5*10^{-8}$M) with PBS (0.1 M, pH 7.4, 0.1% BSA), which had been determined by a pre-experiment. The diluted hAChE (175 μL) was incubated with different nerve agents (325 μL) at 37 °C for 30 min (at 4 °C for soman to delay rapid aging) and an excess of nerve agents was removed from inhibited enzyme immediately by filtration through a 10 kDa MWCO filter with a modified PES membrane (Amicon Ultra-0.5, Millipore Corporation, Billerica, MA) at 4 °C, followed by two washes prior to the final resuspension in PBS/BLG (pH 7.4). The remaining solution in the filter (50 μL) was diluted to 3.5 mL with PBS (0.1 M, pH 7.4, 0.1% BSA). Under these conditions, 95% or greater inhibition of enzyme was gotten and different nerve agents were diluted more than 7000-fold, indicating that most of the nerve agent was removed and inhibition of hAChE had been terminated. Then the inhibited enzyme (20 U/L, 80 μL) was incubated with oximes (40 μL, 0.3 mM) at 37 °C for 30 min (final concentration of oximes was 0.1 mM). At different time intervals, 15 μL of the incubation mixture was taken and enzyme activity was measured by using same method described above.

Blank samples were run in parallel and consisted of: **(a)** A positive control (P): uninhibited enzyme (80 μL) was used instead of the inhibited enzyme; **(b)** a negative control (N): PBS (40 μL, 0.1 M, pH 7.4, 0.1% BSA) was used instead of oximes. Reactivation was calculated using the formula: %Reactivation 100*(S-N)/(P-N) [29, 45].

2.5. Determination of reactivation kinetics

In order to determine the reactivation rate constant (K_r), dissociation constant (K_D) and second order reactivation rate constant (K_{r2}) of the selected reactivators, the reactivation rate at different time intervals and at different concentrations were measured in the same way as described above. The observed first-order rate constant K_{obs} for each oxime concentration, the dissociation constant K_D of inhibited enzyme-oxime complex (EP-OX) and the reactivation rate constant K_r were calculated by non-linear fitting using the standard oxime concentration dependent reactivation equation derived from the following scheme [46, 47]:



$$\%\text{Reactivation} = 100*(1-e^{-K_{obs}*t})$$

$$K_{obs} = K_r[OX]/(K_D+[OX])$$

In this scheme, EP is the phosphylated enzyme, [EP-OX] is the reversible Michaelis-type complex between EP and the oxime [OX], E is the active enzyme and P-OX the phosphylated oxime. K_D is equal to the ratio $(K_{-1}+K_r)/K_1$, and it typically approximates the dissociation constant of the [EP-OX] complex, where from it follows that: $K_{r2} = K_r/K_D$.

Experimental details to determine the concentration dependence of the apparent reactivation rate Kobs for the reactivation of VX-, sarin- and tabun-inhibited hAChE are described in the supporting information.

2.6. ADMET Predictions

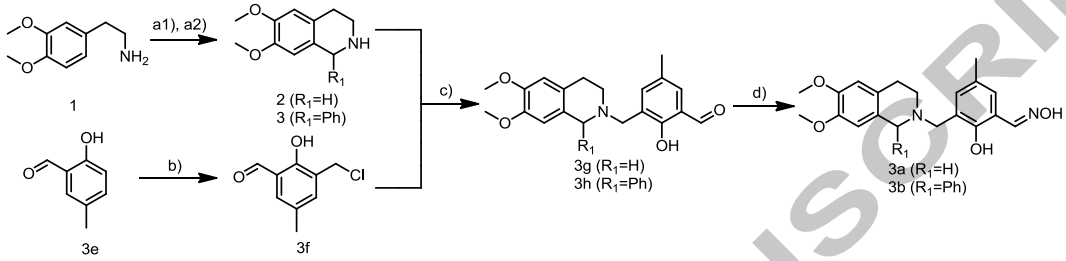
All reagents and solvents were used as received from commercial sources. Initially structures of the compounds were saved in the 'sdf' format using ChemBio3D Ultra software (Cambridge Soft Corporation, 2013). The 'sdf' file of molecular structures of these compounds was uploaded into the ADMET predictor software, pH value was set at 7.4, pKa was limited between 1.0 to 14.0 and the 'activate-one-out in all associative models' option was selected. The program was executed to calculate various parameters such as S+logP, $S+S_w$, S+Peff, S+logBBB, $S+V_d$ and so on. The predicted data could be saved as output file in 'xls' format.

3. Results and discussion

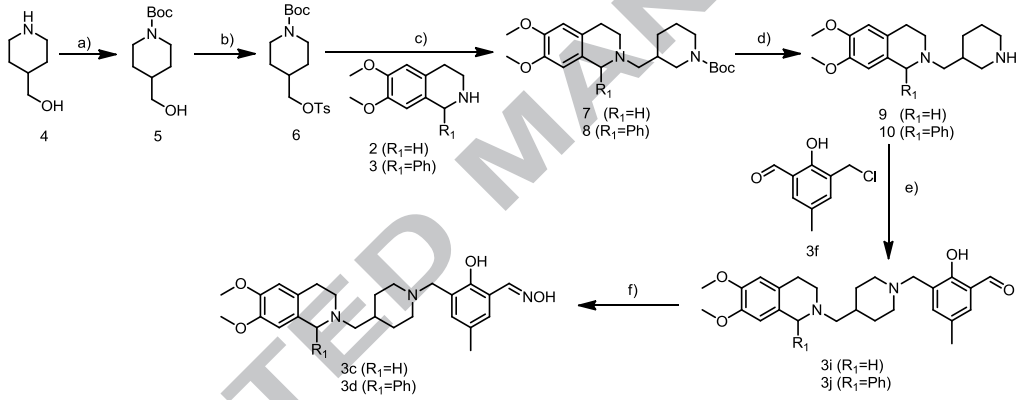
3.1. Synthesis

A representative synthesis route highlighted in Scheme 1 was used to prepare the conjugate templates **3a** and **3b** as shown in Fig. 6. It was started with a ring close reaction of dimethoxyphenylethylamine **1** to give TIQ **2** and PIQ **3**. At the same time, salicylaldehyde derivatives such as **3e** underwent a chloromethylation reaction with aldehyde and concentrated hydrochloric to provide the chloromethyl derivatives **3f** in excellent yields. Then condensation of **2, 3** and **3f** afforded the intermediates **3g** and **3h**. Finally, they were readily converted to oximes **3a** and **3b** by treating with hydroxylammonium chloride. The rest conjugates from **1a, 1b** to **7a, 7b** were obtained in a similar way to **3a** and **3b**.

The synthetic route outlined in Scheme 2 was used to prepare the conjugates **1c, 1d** to **7c, 7d** and the details for oximes **3c** and **3d** were presented. Firstly, piperidinemethanol **4** were protected by Boc to produce the intermediate **5**, it was converted to tosylate **6** by treating with tosyl chloride (TsCl) in pyridine. Then N-alkylation between the tosylate **6** and TIQ **2** or PIQ **3** provided the intermediates **7** or **8**, and a subsequent deprotection of Boc was followed to give the tertiary amines **9** or **10**. Finally, condensation between **9, 10** and **3f** was conducted and the resulting compounds **3i** and **3j** were treated with hydroxylammonium chloride to furnish the desired oximes **3c** and **3d**.



Scheme 1. Preparation of conjugates **3a** and **3b**. Conditions and reagents: **a1**, R=H) HCOOH(98%), 50 °C, 75%; **a2**, R = Ph) benzaldehyde, TFA, reflux, 76%; **b)** con. HCl, 70 °C, 87%; **c)** DCM, NEt_3, r.t. 78%; **d)** $HONH_2HCl$, Na_2CO_3, EtOH/DCM, 79%.



Scheme 2. Preparation of conjugates **3c** and **3d**. Conditions and reagents: **a)** Boc_2O, DCM, r.t. 76%; **b)** DMAP, pyridine, 0 °C, 87%; **c)** NaI, K_2CO_3, CH_3CN, reflux, 69%; **d)** TFA, DCM, r.t. 91%; **e)** DCM, NEt_3, r.t. 82%; **f)** $HONH_2HCl$, NaOAc, EtOH/DCM, 91%.

3.2. ADMET Predictions

Lipophilicity and BBB penetration potency of all the compounds were evaluated through *in silico* predictions by using ADMET Predictor software version 7.0 (Pharmogo Co., Limited.), which was recommended as an accurate, quick and useful tool to predict physicochemical and biological properties of drug-like chemicals [48, 49]. S+logP is predicted octanol-water partition coefficient and S+logBBB is predicted $\log(C_{brain}/C_{blood})$, where C_{brain} stands for the concentration of compound in the brain and C_{blood} stands for the concentration of compound in the blood. A higher S+LogP value indicates higher lipophilicity and a higher S+logBBB value indicates higher BBB penetration ability. The calculated results are listed in table 1.

Sakurada *et al* reported that the concentration of 2-PAM in the corpus striatum is about 10% of the plasma level in rat *in vivo* [50]. Falb and Erdmann *et al* reported that central nervous system concentrations of obidoxime amounting to 3-5% of plasma levels in both rats and mice *in vivo* [51]. It can be found that the predicted S+logBBB values of 2-PAM (-0.69) and obidoxime (-1.26) (table 1) are almost in accordance with these experimental results. Accordingly, these new synthesized compounds were expected to penetrate BBB much more easily than 2-PAM and HI-6 as a result of their greatly tremendous S+logBBB values, especially for compounds (such as **6b, 1c**-**6c** and **4d**) with higher predicted S+LogBBB values.

3.3. Inhibition evaluation

A proper affinity to AChE is essential for a good reactivator, while strong inhibition of AChE should avoided because it would result in heavy toxicity [29, 45, 52]. Thus it is necessary to evaluate the AChE inhibition abilities of these oximes firstly. Quaternary oximes 2-PAM, HI-6 and obidoxime were used as reference compounds. IC_{50} was determined by using multiple concentrations of the oximes, and the results are displayed in table 1. It can be found that these new synthesized oximes were moderate or weak inhibitors of hAChE with IC_{50} greater than 100 μM, which might allow a proper affinity to hAChE for reactivation of the poisoned enzyme. The results and the previous studies in this field [26, 27, 45] confirmed us that an oxime concentration of 100 μM is suitable for the *in vitro* reactivation experiments.

3.4. in vitro reactivation evaluation

During the course of the *in vitro* reactivation experiment, four most common nerve agents (VX, sarin, tabun and soman) were used for the inhibition of hAChE, and reactivation studies of these new synthesized oximes were evaluated together with three reference quaternary oximes (*i.e.*, 2-PAM, HI-6 and obidoxime). Firstly, the diluted hAChE was incubated with sufficient amounts of the nerve agent to get a 95% or greater inhibition of the enzyme and the excess of nerve agents were removed by filtration immediately. Then the inhibited AChE was incubated with different oximes (0.1 mM) for reactivation and the enzyme activity was measured. The percentage of reactivated enzyme (%Reactivation) was calculated as the ratio of the recovered enzyme activity and activity in the control and the results are listed in table 1.

The reactivation study shows that: (1) For VX-inhibited hAChE, depart from **1a, 3a** and **7b**, methylene linked conjugates **1a-7a** and **1b-7b** were poor reactivators, while piperidine linked conjugates **1c-7c** and **1d-7d** exhibited superior reactivation ability, some of them even exhibited higher reactivation potency as compared to obidoxime and HI-6 (such as **1c, 4c, 6c, 3d, 6d** and **7d**). It was supposed that a methylene linker was not long and flexible enough for the reactivators to have an optimal orientation in regard to the reactivation reaction. Furthermore, compounds whose linker in the *ortho* position to the phenol function seemed to be better reactivators than compounds whose linker in the *para* position (such as **1a** *vs* **2a**, **1c** *vs* **2c** and **1d** *vs* **2d**), it was also noteworthy that the phenol *para* linked conjugates were heavier inhibitor of hAChE than the corresponding *ortho* linked conjugates.

Table 1. a. Reactivation of sarin-, VX-, tabun- and soman-inhibited hAChE by new synthesized and the reference oximes (0.1 mM). **b.** IC_{50}, calculated S+LogP and S+LogBBB of the reference and new synthesized oximes.

Oximes	Reactivation (%)				IC_{50}	S+LogP	S+LogBBB
	VX	sarin	tabun	soman			
2-PAM	68.4±5.9	31.1±3.5	13.4±0.3	7.8±2.2	995.7±107	-2.81	-0.69
HI-6	76.8±1.9	52.9±6.1	2.2±0.1	36.1±2.6	667.8±60.8	-5.03	-1.39
obidoxime	86.0±2.8	34.2±3.7	36.7±1.7	9.0±0.5	2169±234	-4.61	-1.26
1a	45.0±3.4	3.4±0.2	3.2±0.4	1.6±0.3	1265±227	3.50	-0.44
2a	18.9±1.9	5.6±1.2	7.7±1.2	1.1±0.2	559.3±85.2	3.24	-0.42
3a	69.2±1.4	10.0±1.6	6.7±0.3	1.9±0.8	1058±109	3.89	-0.49
4a	2.0±0.2	0.9±0.2	2.5±0.6	1.6±0.9	1567±173	4.17	-0.24
5a	6.1±1.1	1.2±0.1	1.2±0.5	3.6±1.2	956.7±79.2	4.17	-0.37
6a	3.3±0.4	1.2±0.1	1.3±0.1	3.3±0.5	692.5±107	3.73	-0.18
7a	15.3±4.7	0.2±0.2	3.6±1.2	2.0±0.2	1382±67.0	3.32	-0.51
1b	0.4±0.3	1.0±0.1	0.6±0.2	2.1±0.6	907.8±168	4.69	-0.32
2b	2.9±0.8	0.3±0.4	2.8±1.3	2.5±0.4	625.6±119	4.54	-0.38
3b	0.1±0.0	1.3±0.1	1.0±0.7	1.8±1.1	1124±158	5.07	-0.39
4b	1.2±0.2	0.9±0.0	0.1±0.1	2.4±0.8	1207±144	5.22	-0.14
5b	1.1±0.1	1.2±0.2	1.6±0.3	2.6±0.8	895.8±123	5.24	-0.27
6b	29.2±4.6	1.2±0.2	0.3±0.3	1.4±0.6	754.7±107	4.87	-0.06
7b	38.6±1.3	1.0±0.1	0.6±0.1	1.4±0.3	911.3±87.9	4.50	-0.45
1c	88.5±0.9	27.8±2.5	16.7±0.1	8.0±3.8	802.4±63.0	4.20	-0.05
2c	55.3±0.5	14.8±5.4	0.3±0.4	4.7±0.1	393.3±26.0	3.97	-0.06
3c	76.7±0.9	48.4±12.5	38.6±1.9	6.7±0.8	417.5±42.6	4.64	-0.09
4c	84.8±0.9	51.5±7.4	26.6±0.5	7.1±0.2	636.7±33.6	4.85	0.16
5c	81.2±5.4	51.4±9.5	25.0±1.0	6.5±0.8	568.0±46.3	4.86	0.03
6c	83.1±3.2	34.2±7.0	20.1±1.4	6.3±0.8	658.1±55.6	4.48	0.17
7c	80.9±1.7	33.4±7.4	19.7±1.3	7.2±0.3	847.9±78.7	4.10	-0.17
1d	77.5±3.2	4.5±0.3	3.1±0.3	1.1±0.2	740.4±67.1	5.65	-0.19
2d	33.8±2.3	7.0±0.1	1.8±0.1	1.4±0.3	233.7±13.5	5.31	-0.10
3d	82.9±1.9	19.9±0.3	17.5±1.3	1.5±0.1	524.7±77.7	6.03	-0.25
4d	61.4±2.9	22.9±12.0	9.5±0.8	1.1±0.2	940.8±173	6.14	-0.03
5d	72.8±5.1	15.6±10.3	8.9±1.3	4.5±0.4	977.4±158	6.09	-0.17
6d	79.2±0.3	4.0±0.7	6.2±1.7	1.1±0.6	684.5±104	5.57	-0.21
7d	82.6±7.0	7.8±0.9	13.1±1.1	2.1±0.9	543.4±81.6	5.50	-0.33

Experiments were performed in duplicate at 37 °C in phosphate buffer (0.10 M, pH 7.4), data shows the average and standard deviation, the values of S+logP and S+logBBB were calculated by ADMET Predictor 7

(2) In the case of sarin, a similar trend was observed, piperidine linked conjugates and whose linker in the *ortho* position to the phenol function outperformed the other oximes tested, such as **1c, 3c-7c** and **4d**. It was also noticeable that conjugates containing a TIQ as PSL were more potent reactivators than those containing PIQ. Additionally, methyl or halogen substitution in the *para* position of salicylaldoxime created more efficient reactivators such as **3c-6c**, some of them (**3c, 4c** and **5c**) were even more potent reactivators for sarin poisoning in comparison with 2-PAM and obidoxime.

(3) Tabun-inhibited hAChE is an OP complex known to be reluctant to reactivation due to its weak electrophilicity and to the steric hindrance imposed on the phosphorus atom in the tabun-hAChE adduct. All of the new synthesized and reference oximes displayed relatively lower reactivating ability for tabun-inhibited hAChE than that for VX and sarin, but same reactivation trend was concluded. Conjugates with TIQ and

linked to the *ortho* position to the phenol function of salicylaldoxime though piperidine, such as **3c-7c**, were still efficiency reactivators superior to 2-PAM, whereas not good enough than obidoxime.

(4) It was a pity that no satisfying results were obtained for soman-inhibited hAChE, which is notoriously difficult to reactivate, except that HI-6 showed obvious reactivation ability.

To further confirm the efficiency of the dual site binding strategy, **PSL1** and **oxime2-oxime4** (Fig. 5) were also tested in the reactivation experiment together with **3c-5c**, the results (Fig. 7) showed that **PSL1** and o**xime2-oxime4** exhibited slight or no reactivating ability towards VX-, sarin- and tabun-inhibited hAChE. However, conjugation of the salicylaldoximes and PSLs through a flexible linker would dramatically improve the reactivation potency, such as reactivators **3c-5c**. Thereby, we believed that a proper PSL was essential for improvement of reactivating ability of these salicylaldoximes. In general, based on the reactivation results, TIQ and piperidine were considered as proper PSL and linker, and piperidine should link to the *ortho* position to the phenol function of salicylaldoxime. Moreover, methyl and halogen substitution in the *para* position of salicylaldoxime would achieve improvement of reactivating ability.

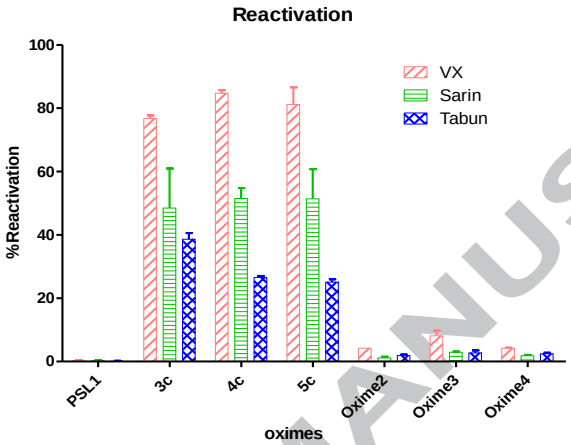


Figure 7. Reactivation of **PSL1, 3c-5c,** and **oxime2-oxime4** for VX-, sarin- and tabun-inhibited hAChE

3.5. Determination of reactivation kinetics

In order to get a deeper comprehension of the reactivation mechanism of selected conjugates **3c-7c**, 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. Reactivation kinetics were compared to those of known reactivators, including HI-6 and obidoxime. OP-inhibited hAChE was incubated at 37 °C with at least 5 concentrations of oxime in phosphate buffer (0.1 M, pH 7.4, 0.1%BSA). At time intervals ranging from 5 to 180 min depending on the reactivation rate, the reactivation rate at different concentrations of different oximes were measured. The corresponding reactivation kinetics were calculated by non-linear fit using the standard oxime concentration dependent reactivation equation and the details were described in the experimental section. The results were presented in table 2.

It can be found that all of the tested conjugates were efficient reactivators for VX-inhibited hAChE, especially the halogen substitution **4c-7c**, even exceeded that of HI-6, and while the methyl substituted **3c** just remained a little less efficient than HI-6. In terms of sarin, conjugates **3c-5c** exhibited higher reactivation ability respectively. Compared with halogen substituted **4c** and **5c**, despite the reactivation rate constant K_r of **3c** is quite lower, its second order reactivation rate constant K_{r2} was greatly improved due to increased affinity towards the enzyme (lower dissociation constant K_D). Although none of these oximes do not surpassed the reactivating efficiency of HI-6 for sarin poisoning, they represented an interesting new starting new starting point for further improvement. Quite remarkably, conjugates **3c-5c** displayed even higher reactivation efficiency for the obstinate tabun-inhibited hAChE. Despite the reactivation rate constant (K_r) was lower compared to obidoxime, compound

Table 2. Reactivation rate constant (K_r), dissociation constant (K_D), second order reactivation rate constant (K_{r2}) of obidoxime, HI-6, and **3c-7c.**

oxime	$K_r/10^{-3}min^{-1}$			$K_D/\mu M$			$K_{r2}/mM^{-1}min^{-1}$		
	VX	sarin	Tabun	VX	sarin	Tabun	VX	sarin	Tabun
HI-6	48±0.6	344±156	n.d.	15±0.5	234±142	n.d.	3.09	1.47	n.d.
Obidoxime	n.d.	n.d.	30.0±1.4	n.d.	n.d.	91±9.2	n.d.	n.d.	0.325
3c	49±2.8	19±1.5	5.6±0.6	23±2.7	38±9	7.6±4.0	2.10	0.502	0.739
4c	-	127±1.7	3.4±0.2	-	262±55	8.9±2.9	7.19±0.68	0.485	0.382
5c	-	107±2.1	2.9±0.2	-	204±66	7.2±2.7	5.20±0.50	0.521	0.412
6c	-	41±4.4	5.2±0.2	-	268±44	53±4.3	6.18±0.48	0.153	0.098
7c	-	91±5.7	4.9±0.1	-	512±42	51±3.0	3.10±0.19	0.177	0.097

Experiments were performed in duplicate at 37 °C in phosphate buffer (0.10 M, pH 7.4), data shows the nonlinear fitting results and standard deviation.

3c was 2-fold more efficient than obidoxime as a result of its greatly increased affinity. However, compounds **6c** and **7c** exhibited poor reactivation of tabun-inhibited hAChE due to decreased affinity towards the enzyme. Interestingly, it can be found that the dissociation constants (K_D), indicating the binding affinity for the inhibited hAChE, were always in consistent with IC_{50} of these reactivators, but they were not proportional to each other. Thus we proposed that a relatively moderate inhibition of reactivators for hAChE might achieve improvement in reactivating efficiency as a result of increasing affinity for the target enzyme

4. Conclusion

In summary, based on a dual site binding strategy, we have described the design, synthesis and the *in vitro* biological evaluation of a series of novel, nonquaternary conjugates for reactivation of OP-inhibited hAChE in this paper. The *in vitro* experiment demonstrated that a combination of TIQ, piperidine and salicylaldoxime would result relatively broad-spectrum and efficient reactivators, such as conjugates **1c** and **3c-7c**, while the short methylene linked conjugates **1a-7a** and **1b-7b** exhibited poor reactivation ability. Moreover, piperidine linked to the *ortho* position and methyl or halogen substitution of salicylaldoxime contributed to the improvement of reactivation ability, the resulting conjugates **3c-5c** were highlighted as the most efficient reactivators, some of them even surpassed the currently approved bis-pyridinium oximes HI-6 and obidoxime for VX- and tabun-inhibited hAChE. It has been proven that introduction of a proper PSL is essential for improvement in reactivation potency and the dual site binding strategy hold promise for further design of more efficient nonquaternary reactivators. Meanwhile, all the new synthesized compounds were moderate or weak inhibitors of hAChE and we found that inhibition of hAChE may be related with increasing affinity for the enzyme. Compared with the reported pyridine aldoxime conjugates **2-7** (Fig. 3), these novel salicylaldoxime conjugates show economical superiority due to their synthesis feasibility. Finally, it must be stressed that these novel reactivators were expected to penetrate the BBB readily and show efficiency in CNS reactivation as a consequence of their strikingly improved lipophilicity. The *in vivo* assays are in process and maybe further modification is required for development of centrally activating reactivators.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Abbreviations

OP, organophosphate; hAChE, human acetylcholinesterase; ACh, acetylcholine; 2-PAM, pralidoxime; MINA, monoisonitrosoacetone; BBB, blood-brain barrier; CNS, central nervous system; P-site, peripheral anionic site; PSL, peripheral site ligand; TIQ, tetrahydroisoquinoline; PIQ, phenyltetrahydroisoquinoline; TsCl, tosyl chloride; TFA, trifluoracetic acid; DMAP, 4-dimethylaminopyridine; DCM, dichloromethane; PE, petroleum ester; EA, ethylamine; TLC, thin-layer chromatography; TMS, tetramethylsilane; ppm, parts per million; r.t., room temperature; n.d., not determined; ATCh, acetylthiocholine; DTNB, 5, 5'-dithiodis-2-nitrobenzoic acid; BSA, albumin from bovine serum; BLG, β-lactoglobulin; PBS, phosphate buffered saline.

References and notes

1. Silman, I.; Sussman, J. L. Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. *Curr. Opin. Pharmacol.* **2005,** *5*, 293-302. DOI: 10.1016/j.coph.2005.01.014.
2. Marrs, T. C. Organophosphate poisoning. *Pharmacol. Ther.* **1993,** *58*, 51-66.
3. Jeyaratnam, J. Acute pesticide poisoning: a major global health problem. *World Health Stat.* Q. **1990,** *43*, 139-144.
4. Tu, A. T. Toxicological and chemical aspects of sarin terrorism in Japan in 1994 and 1995. *Toxin. Rev.* **2007,** *26*, 231-274.
5. Ember, L. Lab tests confirm sarin in Iraqi shell. *Chem. Eng. News* **2004**, *82*, 15.
6. Eddleston, M.; Buckley, N. A.; Eyer, P.; Dawson, A. H. Management of acute organophosphorus pesticide poisoning. *Lancet* **2008,** *371*, 597-607. DOI: 10.1016/S0140-6736(07)61202-1.
7. Jokanović, M.; Stojiljković, M. P. Current understanding of the application of pyridinium oximes as cholinesterase reactivators in treatment of organophosphate poisoning. *Eur. J. Pharmacol.* **2006,** *553*, 10-17. DOI: 10.1016/j.ejphar.2006.09.054.
8. Jokanović, M; Prostran, M. Pyridinium oximes as cholinesterase reactivators. Structure-activity relationship and efficacy in the treatment of poisoning with organophosphorus compounds. *Curr. Med. Chem.* **2009,** *16*, 2177-2188. DOI: 10.2174/092986709788612729.
9. Jokanović, M. Medical treatment of acute poisoning with organophosphorus and carbamate pesticides. *Toxicol. Lett.* **2009,** *190*, 107-115. DOI: 10.1016/j.toxlet.2009.07.025.
10. Bajgar, J. Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. *Adv. Clin. Chem.* **2004,** *38*, 151-216.
11. Kassa, J. Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. *J. Toxicol. Clin. Toxicol.* **2002,** *40*, 803-816.
12. Luo, C.; Saxena, A.; Smith, M.; Garcia, G.; Radic, Z.; Taylor, P.; Doctor, B. P. Phosphoryl oxime inhibition of acetylcholinesterase during oxime reactivation is prevented by edrophonium. *Biochemistry* **1999,** *38*, 9937-9947. DOI: 10.1021/bi9905720.
13. Ashani, Y.; Bhattacharjee, A.K.; Leader, H.; Saxena, A.; Doctor, B.P. Inhibition of cholinesterases with cationic phosphonyl oximes highlights distinctive properties of the charged pyridine groups of quaternary oxime reactivators. *Biochem Pharmacol.* **2003,** *66*,191-202.
14. Shih, T. M.; Skovira, J. W.; O'Donnell, J. C.; McDonough, J. H. *In vivo* reactivation by oximes of inhibited blood, brain and peripheral tissue cholinesterase activity following exposure to nerve agents in guinea pigs. *Chem. Biol. Interact.* **2010,** *187*, 207-214. DOI: 10.1016/j.cbi.2010.03.006.
15. Lorke, D. E.; Kalasz, H.; Petroianu, G. A.; Tekes, K. Entry of oximes into the brain: a review. *Curr. Med. Chem.* **2008,** *15*, 743-753. DOI: 10.2174/092986708783955563.
16. Little, P. J.; Scimeca, J. A.; and Martin, B. R. Distribution of [3H]diisopropylfluorophosphate, [3H]soman, [3H]sarin, and their metabolites in mouse brain. *Drug Metab. Dispos.***1988,** *16*, 515-520.
17. Demar, J. C.; Clarkson, E. D.; Ratcli ffe, R. H.; Campbell, A. J.; Thangavelu, S. G.; Herdman, C. A.; Leader, H.; Schulz, S. M.; Marek, E.;Medynets, M. A.; Ku, T. C.; Evans, S. A.; Khan, F. A.; Owens, R. R.; Nambiar, M. P.; Gordon, R. K. Pro-2-PAM therapy for central and peripheral cholinesterases. *Chem. Biol. Interact.* **2010,** *187*, 191-198. DOI: 10.1016/j.cbi.2010.02.015.
18. Bodor, N.; Shek, E.; Higuchi, T. Improved delivery through biological membranes. 1. Synthesis and properties of 1-methyl-1,6-dihydropyridine-2-carbaldoxime, a pro-drug of N-methylpyridinium-2- carbaldoxime chloride. *J. Med. Chem.* **1976,** *19*, 102-107.
19. Korabecny, J.; Soukup, O.; Dolezal, R.; Spilovska, K.; Nepovimova, E.; Andrs, M.; Nguyen, T. D.; Jun, D.; Musilek, K.; Kucerova-Chlupacova, M.; Kuca, K. From pyridinium-based to centrally active acetylcholinesterase reactivators. *Mini-Rev. Med. Chem.* **2014,** *14*, 215-221. DOI: 10.2174/1389557514666140219103138.

20. Mercey, G.; Verdelet, T.; Renou, J.; Kliachyna, M.; Baati, R.; Nachon, F.; Jean, L.; Renard, P. Y. Reactivators of acetylcholinesterase inhibited by organophosphorus nerve agents. *Acc. Chem. Res.* **2012,** *45*, 756-766. DOI: 10.1021/ar2002864.
21. Wei, Z.; Zheng, Z. B.; Li, S. Progress in acetylcholinesterase reactivators aiming to cross blood-brain barrier. *Chin. J. Pharmacol. Toxicol.* **2013,** *27*, 731-738.
22. McHardy, S.F.; Wang, H.L.; McCowen, S.V.; Valdez, M.C. Recent advances in acetylcholinesterase Inhibitors and Reactivators: an update on the patent literature (2012-2015). *Expert. Opin. Ther. Pat.* **2017**, *27*, 455-476. doi: 10.1080/13543776.2017.1272571.
23. Rutland, J. P. The effect of some oximes in sarin poisoning. *Br. J. Pharmacol. Chemother.* **1958,** *13*, 399-403. DOI: 10.1111/j.1476-5381.1958.tb00228.x.
24. Shih, T.M.; Skovira, J. W.; O'Donnell, J. C.; McDonough, J. H. Treatment with tertiary oximes prevents seizures and improves survival following sarin intoxication. *J. Mol. Neurosci.* **2010,** *40*, 63-69. DOI: 10.1007/s12031-009-9259-7.
25. Kuca, K.; Jun, D.; Musilek, K. Structural requirements of acetylcholinesterase reactivators. *Mini-Rev. Med. Chem.* **2006,** *6*, 269-277. DOI: 10.2174/138955706776073510.
26. Kalisiak, J.; Ralph, E. C.; Zhang, J.; Cashman, J. R. Amidine-oximes: reactivators for organophosphate exposure. *J. Med. Chem.* **2011,** *54*, 3319-3330. DOI: 10.1021/jm200054r.
27. Kalisiak, J.; Ralph, E. C.; Cashman, J. R. Nonquaternary reactivators for organophosphate-inhibited cholinesterases. *J. Med. Chem.* **2012,** *55*, 465-474. DOI: 10.1021/jm201364d.
28. Kuca, K.; Bartosova, L.; Jun, D.; Patocka, J.; Cabal, J.; Kassa, J.; Kunesova, G. New quaternary pyridine aldoximes as casual antidotes against nerve agents intoxications. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech. Repub.* **2005,** *149*, 75-82.
29. De Koning, M. C.; Joosen, M. J.; Noort, D.; Van Zuylen, A.; Tromp, M. C. Peripheral site ligand-oxime conjugates: A novel concept towards reactivation of nerve agent-inhibited human acetylcholinesterase. *Bioorg. Med. Chem.* **2011,** *19*, 588-594. DOI: 10.1016/j.bmc.2010.10.059.
30. Mercey, G.; Verdelet, T.; Saint-André, G.;, Gillon, E.; Wagner, A.; Baati, R.; Jean, L.; Nachon, F.; Renard, P. Y. First efficient uncharged reactivators for the dephosphylation of poisoned human acetylcholinesterase. *Chem. Commun.* **2011,** *47*, 5295-5297. DOI: 10.1039/c1cc10787a.
31. Mercey, G.; Renou, J.; Verdelet, T.; Kliachyna, M.; Baati, R.; Gillon, E.; Arboléas, M.; Loiodice, M.; Nachon, F.; Jean, L.; Renard, P. Y. Phenyltetrahydroisoquinoline-pyridinaldoxime conjugates as efficient uncharged reactivators for the dephosphylation of inhibited human acetylcholinesterase. *J. Med. Chem.* **2012,** *55*, 10791-10795. DOI: 10.1021/jm3015519.
32. Renou, J.; Loiodice, M.; Arboléas, M.; Baati, R.; Jean, L.; Nachon, F.; Renard, P. Y. Tryptoline-3-hydroxyl- pyridinaldoxime conjugates as efficient reactivators of phosphylated human acetyl and butyrylcholinesterases. *Chem Commun.* **2014**, 50, 3947-3950. DOI: 10.1039/c4cc00561a.
33. Kliachyna, M.; Santoni, G.; Nussbaum, V.; Renou, J.; Sanson, B.; Colletier J. P.; Arboléas M.; Loiodice, M.; Weik, M.; Jean, L.; Renard, P. Y.; Nachon, F.; Baati, R. Design, synthesis and biological evaluation of novel tetrahydroacridine pyridine aldoxime and amidoxime hybrids as efficient uncharged reactivators of nerve agent-inhibited human acetylcholinesterase. Eur. *J. Med. Chem.* **2014**, *78*, 455-467. DOI: 10.1016/j.ejmech.2014.03.044.
34. McHardy, S.F.; Bohmann, J.A.; Corbett, M.R.; Campos, B.; Tidwell, M.W.; Thompson, P.M.; Bemben, C.J.; Menchaca, T.A.; Reeves, T.E.; Cantrell, W.R. Jr.; Bauta, W.E.; Lopez, A.; Maxwell, D.M.; Brecht, K.M.; Sweeney, R.E.; McDonough, J. Design, synthesis, and characterization of novel, nonquaternary reactivators of GF-inhibited human acetylcholinesterase. *Bioorg. Med. Chem. Lett.* **2014,** *24*,1711-1714. doi: 10.1016/j.bmcl.2014.02.049.
35. Wei, Z.; Liu, Y. Q.; Zhou, X. B.; Luo, Y.; Huang, C. Q.; Wang, Y. A.; Zheng, Z. B.; Li, S. New efficient imidazolium aldoxime reactivators for nerve agent-inhibited acetylcholinesterase. *Bioorg. Med. Chem. Lett.* **2014,** *24*, 5743-5748. DOI: 10.1016/j.bmcl.2014.10.055.
36. Katz, F.S.; Pecic, S.; Tran, T.H.; Trakht, I.; Schneider, L.; Zhu, Z .; Ton-That, L.; Luzac, M.; Zlatanic, V.; Damera, S.; Macdonald, J.; Landry, D.W.; Tong, L.; Stojanovic, M.N. Discovery of New Classes of Compounds that Reactivate Acetylcholinesterase Inhibited by Organophosphates. *Chembiochem.* **2015,** *16*, 2205-2215. doi: 10.1002/cbic.201500348.
37. Louise-Leriche, L.; Paunescu, E.; Saint-André, G.; Baati, R.; Romieu, A.; Wagner, A.; Renard, P. Y. A HTS assay for the detection of organophosphorus nerve agent scavengers. *Chem. Eur. J.* **2010,** *16*, 3510-3523. DOI: 10.1002/chem.200902986.
38. Dale, T. J.; Rebek, J. Jr. Hydroxy oximes as organophosphorus nerve agent sensors. *Angew. Chem. Int. Ed. Engl.* **2009,** *48*, 7850-7852. DOI: 10.1002/anie.200900488.
39. Cheung, J.; Rudolph, M. J.; Burshteyn, F.; Cassidy, M. S.; Gary, E. N.; Love, J.; Franklin, M. C.; Height, J. J. Structures of human acetylcholinesterase in complex with pharmacologically important ligands. *J. Med. Chem.* **2012,** *55*, 10282-10286. DOI: 10.1021/jm300871x.
40. Krasiński, A.; Radić, Z.; Manetsch, R.; Raushel, J.; Taylor, P.; Sharpless, K. B.; Kolb, H. C. In situ selection of lead compounds by click chemistry: target-guided optimization of acetylcholinesterase inhibitors. *J. Am. Chem. Soc.* **2005,** *127*, 6686-6692. DOI: 10.1021/ja043031t.
41. Edward, J. P.; Brennie, E. H. Jr.; George, M. S. Pyridinium Aldoximes. *J. Org. Chem.* **1958,** *23*, 714-717. DOI: 10.1021/jo01099a019.
42. Hsiao, L. Y. Y.; Getzville, N. Y.; Musallam, H. A.; Damascus, Md. Bis-methylene ether pyridinium compound preparation. U.S. Patent 5,130,438, 1992.
43. Eddolls, J.; Mccormack, P.; Hodgson, A. Process for the manufacture of HI-6 dimethanesulfonate. H.K. Patent 1, 137, 422, 2013.
44. Ellman, G. L.; Courtney, K. D.; Andres, V. Jr.; Feather-stone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961,** *7*, 88-95.
45. De Koning, M. C.; Van Grol, M.; Noort, D. Peripheral site ligand conjugation to a non-quaternary oxime enhances reactivation of nerve agent-inhibited human acetylcholinesterase. *Toxicol. Lett.* **2011,** *206*, 54-59. DOI: 10.1016/j.toxlet.2011.04.004
46. Worek, F.; Thiermann, H.; Szinicz, L.; Eyer, P. Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. *Biochem. Pharmacol.* **2004**, *68*, 2237-2248. DOI: 10.1016/j.bcp.2004.07.038.
47. Kovarik, Z.; Radic, Z.; Berman, H. A.; Simeon-Rudolf, V.; Reiner, E.; Taylor, P. Mutant cholinesterases possessing enhanced capacity for reactivation of their phosphonylated conjugates. *Biochemistry* **2004**, *43*, 3222-3229. DOI: 10.1021/bi036191a.
48. Lin, A.; Cai, Z.; Hu, G.; Li, Q. Identification of ALK5 inhibitor via structure-based virtual screening and ADMET prediction. *J. Recept. Signal. Transduct. Res.* **2015,** *35*, 559-564. DOI: 10.3109/10799893.2015.1024852.
49. Hassan, S. F.; Rashid, U.; Ansari, F. L.; Ul-Haq, Z. Bioisosteric approach in designing new monastrol derivatives: an investigation on their ADMET prediction using in silico derived parameters. *J. Mol. Graph. Model.* **2013,** *45*, 202-210. DOI: 10.1016/j.jmgm.2013.09.002.
50. Sakurada, K.; Matsubara, K.; Shimizu, K.; Shiono, H.; Seto, Y.; Tsuge, K.; Yoshino, M.; Sakai, I.; Mukoyama, H.; Takatori, T. Pralidoxime iodide [2-pAM] penetrates across the blood-brain barrier. *Neurochem. Res.* **2003,** *28*, 1401-1407.
51. Falb, A.; Erdmann, WD. Penetration of ^{14}C-obidoxime through the so-called blood-brain barrier of mice and rats. *Arch. Toxikol.* **1969,** *24*, 123-132.
52. Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radić, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. Click chemistry in situ: acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from an array of building blocks. *Angew. Chem. Int. Ed.* **2002,** *41*, 1053-1507. DOI: 10.1002/1521-3773(20020315)41:6<1053::AID-ANIE1053>3.0.CO;2-4 .

Supporting information

The supporting information includes details of the synthesis procedures and analytical data, details of hAChE inhibition and reactivation experiments with oximes, oxime concentrations (μM) for determination of the observed first-order rate constant kobs and non-liner fitting plot of kobs vs obidoxime, HI-6 and 3c-7c. These materials is available via the Internet at

Highlights:

1. Novel nonquaternary reactivators for nerve agent-inhibited AChE were constructed.
2. Some exhibited superior *in vitro* reactivation efficiency in comparison to 2-PAM.
3. Introduction of peripheral site ligand dramatically enhanced reactivation ability.
4. These conjugates may develop as efficient centrally activating reactivators.