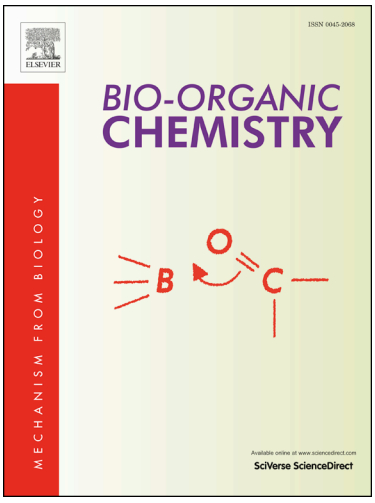

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ABSTRACT

A new series of nonquaternary conjugates for reactivation of both nerve agents and pesticides inhibited hAChE were described in this paper. It was found that substituted salicylaldehydes conjugated to aminobenzamide through piperidine would produce efficient reactivators for sarin, VX and tabun inhibited hAChE, such as **L6M1R3, L6M1R5** to **L6M1R7**, **L4M1R3** and **L4M1R5** to **L4M1R7**. The *in vitro* reactivation experiment for pesticides inhibited hAChE of these new synthesized oximes were conducted for the first time. Despite they were less efficient than obidoxime, some of them were highlighted as equal or more efficient reactivators in comparison to 2-PAM. It was found that introduction of peripheral site ligands could increase oximes' binding affinity for inhibited hAChE in most cases, which resulted in greater reactivation ability.

1. Introduction

Organophosphates (OP) including nerve agents (e.g., sarin, VX, tabun and soman, Fig. 1) and pesticides (e.g., paraoxon, parathion, phorate, dichlorvos and chlorophos, Fig. 1) are highly toxic compounds [1]. Nerve agents can be used as weapons of mass destruction (Iran-Iraq War) [2] or be used for terrorist attacks (e.g., subway attack in Tokyo in 1995) [3]. The broad use of the pesticides in agriculture leads to a serious public health issue with about 3 000 000 acute intoxications and over 200 000 fatalities annually worldwide, mostly in developing world [4]. Thus there is a well-recognized need for development of more effective antidotes against OP poisoning [5].

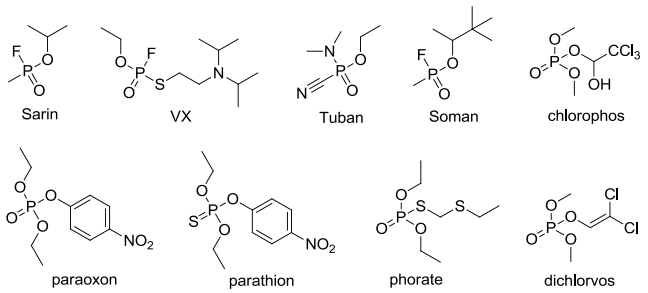


Figure 1. Chemical structures of some nerve agents and pesticides

Acetylcholine (ACh) is an important neurotransmitter at postsynaptic membranes and neuromuscular junctions, acetylcholinesterase (AChE) acts as a key enzyme in regulation of neurotransmission; it hydrolyzes ACh and terminates the action of this neurotransmitter [6]. OPs readily phosphorylate the serine hydroxyl group at the active site (A-site) of the AChE, resulting accumulation of unhydrolyzed ACh at neuronal synapses and overstimulation of receptors in synapses, which would lead to cholinergic crisis, respiratory distress, convulsive seizures and ultimately death [7].

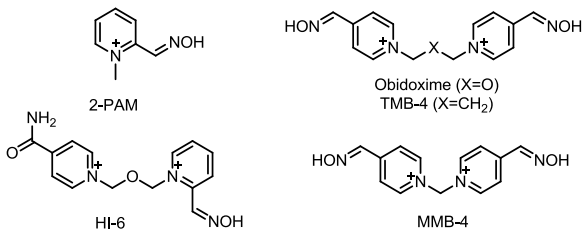


Figure 2. Current available pyridinium oximes in the treatment of OP poisoning

Currently, the approved treatment for OP intoxication includes administration of an AChE reactivator of the pyridinium aldoxime family (e.g., pralidoxime (2-PAM), trimedoxime (TMB-4), obidoxime, HI-6, Fig. 2) [8, 9] a muscarinic receptor antagonist (e.g., atropine), and an anticonvulsant (e.g., diazepam) [10, 11]. Pyridinium aldoxime is considered to reactivate phosphonylated AChE via a direct nucleophilic attack of the oximate anion on the phosphorus group and restore the enzyme's activity [12]. However, due to their permanent positive charge, these quaternary reactivators are unable to cross the blood-brain barrier (BBB) to reactivate inhibited AChE in the central nervous system (CNS) [13], while the brain is a major target of nerve agents [14, 15]. As a result, various nonquaternary AChE reactivators were designed and synthesized [16-18].

As the first tertiary oxime, monoisonitrosoacetone (MINA, Fig. 3) [19, 20] readily penetrate the BBB and reactivate inhibited AChE in the brain, but the absence of charge reduced the affinity to AChE as compared to 2-PAM and resulted in decreasing reactivation potency [21]. Amidine-oximes described by Kalisiak *et al* (Fig. 3) were expected to undergo protonation at physiological pH to form a positively charged center and compensate binding affinity for OP-inhibited AChE. Although their *in vitro* reactivating ability for OP-inhibited AChE was improved in contrast to MINA, but they were still less efficient than 2-PAM [22, 23].

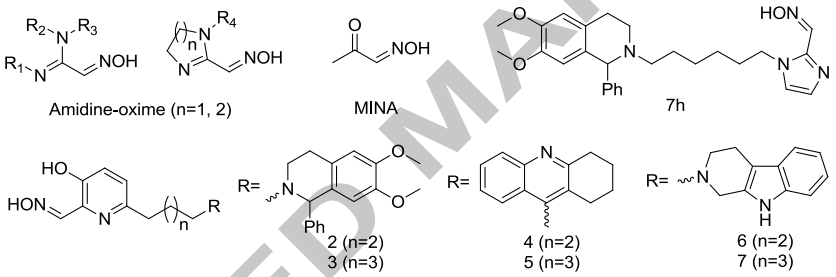
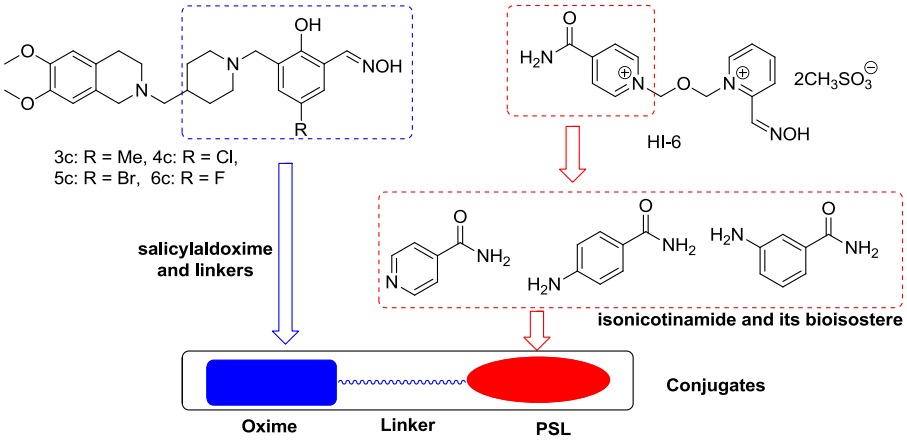


Figure 3. Chemical structures of some reported nonquaternary oximes

Previously, we found that some conjugates of tetrahydroisoquinoline and salicylaldoximes showed reactivation potency against hAChE inhibited by sarin, VX and tabun, some of them even surpassed the currently approved bis-pyridinium oximes HI-6 and obidoxime such as compounds **3c-6c** [24]. Tetrahydroisoquinoline was introduced as a peripheral site ligand (PSL) of AChE, which was considered to interact with the peripheral site of AChE and increase the affinity for the enzyme, while salicylaldoximes was expected to function in the AChE active gorge. This dual site binding strategy had been proved successful and produced some efficient nonquaternary oxime reactivators, such as compounds **7h** [25] and **2-7** [26-29] showing in **figure 3**. HI-6 was considered as one of the best quaternary reactivators for nerve agents poisoning so far [8], but its permanent charge block the BBB penetration for CNS reactivation. In this paper, a similar dual site binding strategy was used to construct a novel series of nonquaternary reactivator for nerve agent poisoning (Fig. 4). The ligand of isonicotinamide in HI-6 and its bioisostere 4-aminobenzamide and 3-aminobenzamide were introduced as PSLs, while the salicylaldoxime in **3c-6c** was chosen as the reactivating part of the conjugates (Fig. 4).



Firure 4: Construction of nonquaternary reactivators for OP poisoning

To valid our hypothesis, preliminary molecular docking studies of some new designed compounds were conducted. Docked conformations of moleculars **L6M1R1** and **L6M1R7** were depicted in **fig. 5**. It was demonstrated that the designed conjugates interacted with AChE in a dual site binding mode as predicted. The PSLs of 4-aminobenzamide interacted with the P-site in a π-π sandwiching way, which provided extra affinity for AChE; while the oxime groups were located at the A-site, which were supposed to serve as reactivating groups. Similar docking results were observed for designed compounds bearing 3-aminobenzamide as PSLs. Comparing the docking conformations of **L6M1R1** and **L6M1R7,** it could be observed that different substituents of the 3-hydroxy benzyloxime block the position of the oxime in the catalytic site of the enzyme and might result different reactivation abilities. In order to screening more efficient reactivators, benzyloxime bearing various substituents (such as methyl, bromide, chloride, fluorine or methoxy) were chosen as the reactivating part of the target conjugates. In our previous study, introduction of these selected substituents produced more efficient nonquaternary reactivators, such as compounds **3c** to **6c** showed in figure 4 [24].

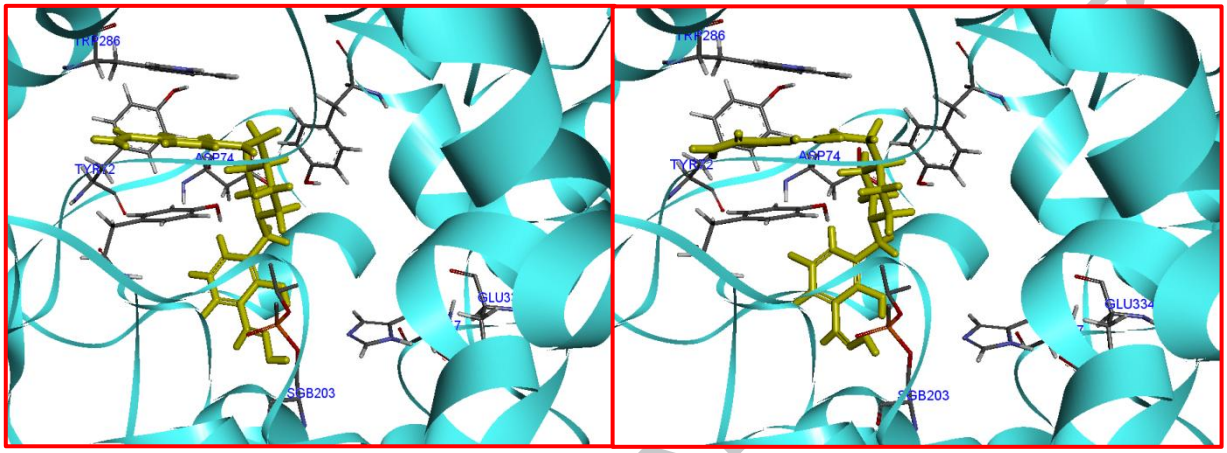


Fig. 5 Docked conformations of molecules **L6M1R1** (left) and **L6M1R7** (right) in the A-site gorge of sarin^{nonaged}-mAChE (pdb code: 2WHP). The key amino acid residues in the P-site include Tyr72, Tyr124, Trp286, Tyr341 and Asp74 [30, 31]; in the A-site include Ser-sarin203 or SGB203, Glu334 and His447 [32]. The docked conformations of the reactivators are depicted as stick model in yellow and the key amino acid residues as stick model in grey.

17 new tertiary salicylaldoxime reactivators were prepared in this paper (Fig. 6), conjugates **L6M1R3, L6M1R5** to **L6M1R7**, **L4M1R3** and **L4M1R5** to **L4M1R7** emerged as relatively efficient reactivators. In comparison to quaternary reactivators, they have advantageous of greatly improved lipophilicity and increased *in vitro* reactivation efficacy of nerve OP-inhibited AChE.

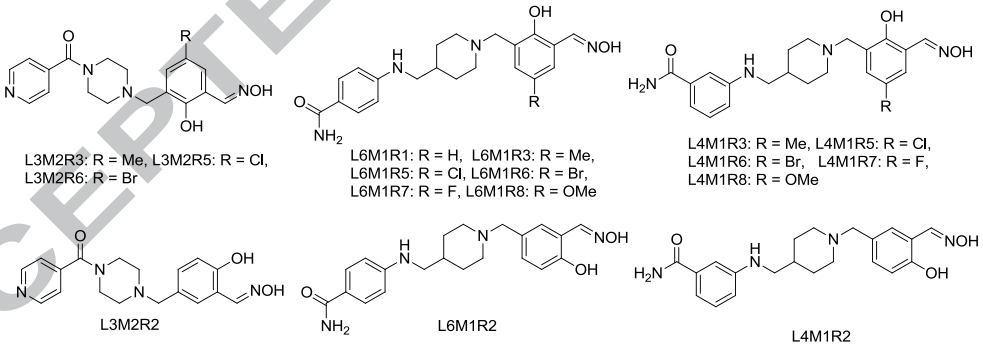


Figure 6: The structures of novel designed nonquaternary reactivators

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. 2-PAM was purchased from Sigma-Aldrich. HI-6 and obidoxime were synthesized ourselves [33-35].

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. Paraoxon, parathion, phorate and dichlorvos were from commercial sources. 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).

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. A solution of oxime (10 mM) were prepared in water containing 2.5% acetic acid and 10% PEG-400 and it was further diluted by PBS (0.1 M, pH=7.4) to the required concentrations. It was found that there was no effect of CH_3COOH 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 412 nm [36].

2.3. hAChE inhibition experiments

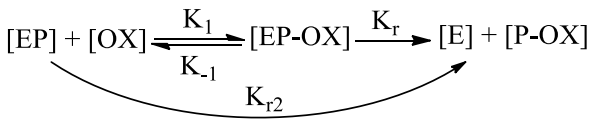
Initially a stock solution of hAChE (20 U/mL, from sigma) was diluted 2000-fold with PBS (0.1 M, pH 7.4, 0.1% BSA). 20 μL of diluted hAChE was incubated with 10 μL of each oxime (final concentrations: 10, 50, 200, 500 and 1000 μM) for 15min at 25 °C. A blank experiment (positive control) was run in parallel 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. Then the resulting mixture was centrifuged at 4 °C for 1 min to remove bubbles, the reaction product was monitored immediately by testing the absorption value at 412 nm (0<abs<2). 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 2000-fold with PBS (0.1 M, pH 7.4, 0.1% BSA). The concentrations of different nerve agents and pesticides were determined by a pre-experiment similar to the inhibition experiment to attain an inhibition plateau between 80% to 95%, the final concentration were as followings: VX, $3*10^{-8}$M; sarin, $6*10^{-7}$M; tabun, $6*10^{-8}$M; soman, $3*10^{-8}$M, paraoxon, $4.5*10^{-8}$M, parathion, $6*10^{-5}$M, phorate, $8*10^{-3}$M, dichlorvos, $6*10^{-6}$M. The diluted hAChE (20 μL) was incubated with different nerve agents and pesticides (10 μL) at 25 °C for 15 min (at 4 °C for soman to delay rapid aging). Then the inhibited enzyme was incubated with oximes (15 μL, 0.3 mM) at 25 °C for 30 min (final concentration of oximes was 0.1 mM). At last, activity of the enzyme in the incubation mixture was measured by using same method described in the section of inhibition experiment. Blank samples were run in parallel and consisted of: **(a)** A positive control (P): uninhibited enzyme (20 μL) was used instead of the inhibited enzyme; **(b)** a negative control (N): PBS (25 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) [37].

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. Initially the diluted hAChE (20 μL) was incubated with different nerve agents and pesticides (10 μL) at 25 °C for 15 min. Then the inhibited enzyme was incubated with oximes in various concentrations, and 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 immediately. The reaction product was monitored every 5 minutes, up to 2 hours by testing the absorption value at 412 nm (0<abs<2). Same blank samples were run in parallel as describing in the section of reactivation section. 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 [38, 39]:



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

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

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 K_{obs} for the reactivation of VX-, sarin- and tabun-inhibited hAChE are described in the supporting information.

2.6. ADMET Predictions

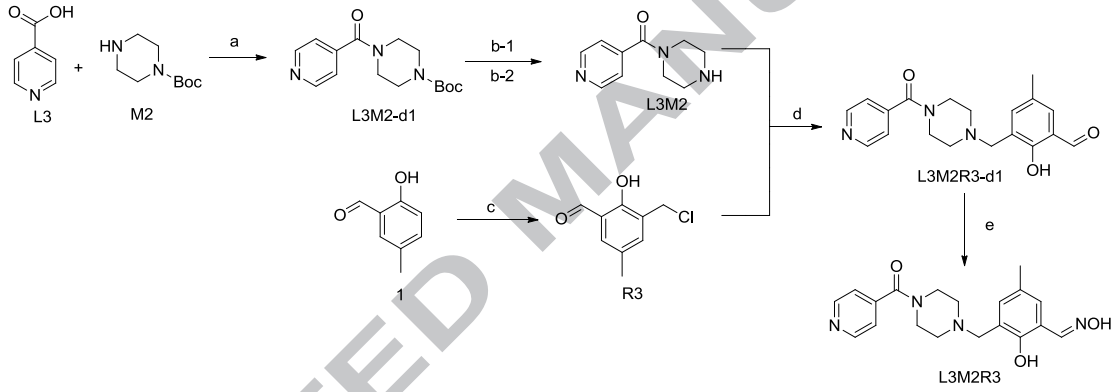
The lipophilicity and BBB penetration potency of all the compounds was predicted through in *silico* predictions by using ADMET Predictor software version 7.0 (Pharmogo Co., Limited.), which had been used by Lin *et al* and Hassan *et al* and was considered as an accurate, quick and useful tool to predict physicochemical and biological properties of drug-like chemicals.[30,31][40, 41]

Firstly, 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.

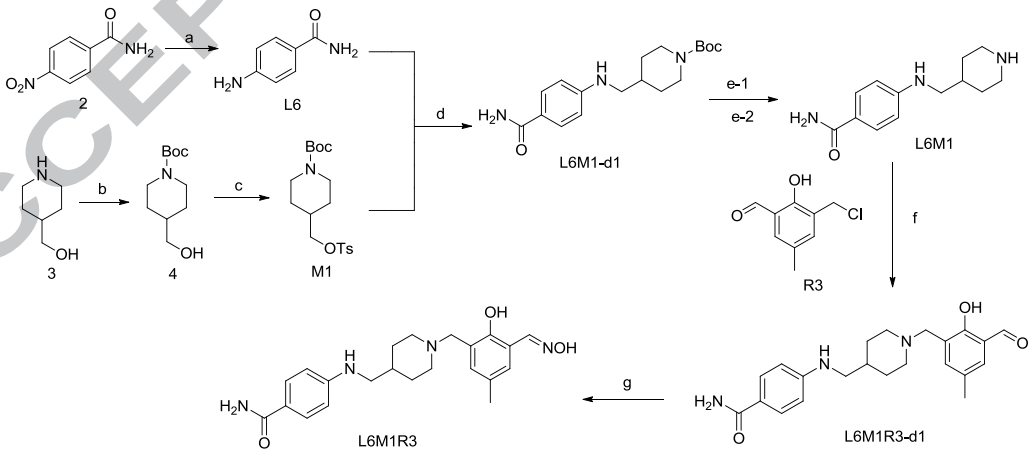
3. Results and discussion

3.1 Synthesis

The synthetic route to prepare conjugate **L3M2R3** (Fig. 5) was outlined in Scheme 1. Firstly amide **L3M2-d1** was prepared through condensation of isonicotinic acid and amine **M2** by using 1-hydroxybenzotriazole (HOBt) and N'-(ethylcarbonimidoyl)-N,N-dimethylpropane-1,3-diamine monohydrochloride (EDCI) in THF. Deprotection of Boc in **L3M2-d1** was followed to give the tertiary amine **L3M2**. 5-Methylsalicylaldehyde **1** underwent a chloromethylation reaction with paraformaldehyde and concentrated hydrochloric to provide the chloromethyl derivatives **R3** in excellent yields. Then N-alkylation between **L3M2** and **R3** provided the intermediate **L3M2R3-d1**. Finally, **L3M2R3-d1** was readily converted to oximes **L3M2R3** by treating with hydroxylammonium chloride. Conjugates **L3M2R3, L3M2R5** and **L3M2R6** (Fig. 5) were obtained in a similar way to **L3M2R3**.



Scheme 1. Preparation of conjugate **L3M2R3**. Conditions and reagents: a) HOBt, EDCI, THF, r.t. 96%; b-1) $SOCl_2$ /MeOH/DCM; b-2) MeOH/NaOH; c) con. HCl, $(CH_2O)_n$, 70 °C, 87%; d) DCM, NEt_3, r.t. 67%; e) $HONH_2HCl$, NaOAc, EtOH/DCM, r.t. 85%.



Scheme 2. Preparation of conjugates **L6M1R3**. Conditions and reagents: **a)** H_2NNH_2, $FeCl_3$/C, EtOH, 96%; **b)** Boc_2O, DCM, r.t. 76%; **c)** DMAP, pyridine, 0 °C, 87%; **d)** NaI, K_2CO_3, DMF, 100℃; TFA, DCM, r.t. 91%; e-1) $SOCl_2$ /MeOH/DCM; e-2) MeOH/NaOH; f) DCM, NEt_3, r.t. 71%; g) $HONH_2HCl$, NaOAc, EtOH/DCM, 78%.

Synthesis route highlighted in Scheme 2 was used to prepare the conjugate **L6M1R3** as shown in **figure 5**. Firstly, 4-nitrobenzamide **3** was reduced to 4-aminobenzamide **L6** by using hydrazine, active carbon and ferric chloride in ethanol. Piperidinemethanol **3** was protected by Boc to produce the intermediate **4** and then was converted to tosylate **M1** by treating with tosyl chloride (TsCl) in pyridine. Then N-alkylation between the tosylate **M1** and **L6** provided the intermediates **L6M1-d1** and a subsequent deprotection of Boc was followed to give the tertiary amines **L6M1**. Finally, condensation between **L6M1** and **R3** was conducted and the resulting compounds

L6M1R3-d1 were treated with hydroxylammonium chloride to furnish the desired oxime L6M1R3. Oximes **L6M1R1, L6M1R2, L6M1R5 to L6M1R8, L4M1R2, L4M1R3** and **L4M1R5** to **L4M1R7** (Fig. 5) were obtained in a similar way to **L6M1R3**.

3.2. ADMET Predictions

The predicted S+logP and S+logBBB values were listed in **Table 1**. S+logP means predicted octanol-water partition coefficient and S+logBBB means predicted $\log(C_{brain}/C_{blood})$, where C_{brain} stands for compounds' the concentration of compound in the brain and C_{blood} stands for 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. According to the predicted values, these new synthesized compounds were expected to penetrate BBB much more easily than obidoxime and HI-6 as a result of their greatly tremendous S+logP values, especially for compounds (such as **L3M2R5, L6M1R5** and **L6M1R7**) with higher predicted S+LogBBB values. Due to their bulky structure or relatively lower lipophilicity, the predicted S+LogBBB values of some compounds (such as **L6M1R3**, **L6M1R6**, **L6M1R8**, **L4M1R3**, **L4M1R6** and **L4M1R8**) is lower than that of 2-PAM, but still much higher than that of HI-6 and obidoxime. They still hold promise to cross the BBB because their predicted lipophilicity is much higher than these quaternary reactivators. The *in vivo* experiment of selected reactivators showing reactivation efficiency would be conducted to confirm their real BBB penetration in our further study.

3.3. Inhibition evaluation

It is necessary to evaluate the AChE inhibition abilities of these oximes firstly because a proper affinity to AChE is essential for a good reactivator, while strong inhibition of AChE would result in heavy toxicity [37]. IC_{50} was determined by using multiple concentrations of the oximes, and the results are displayed in **table 1**. The results show that most of these new synthesized oximes were weak inhibitors of hAChE with IC_{50} greater than 200 μM, which might allow a proper affinity to hAChE for reactivation of the poisoned enzyme [26, 27]. Oximes **L4M1R2** exhibit moderate inhibition potency against hAChE, a similar phenomenon was observed in our previous study [24], the phenol *para* linked conjugates showed heavier inhibition ability 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
L3M2R2	8.0±2.0	3.0±0.1	3.0±0.1	3.5±0.3	1426±157	1.21	-0.48
L3M2R3	58.3±13.5	4.1±0.5	1.6±0.2	2.0±0.6	3084±683	1.70	-0.51
L3M2R5	47.6±0.8	5.2±0.9	1.9±0.4	3.1±1.1	2600±610	2.11	-0.24
L3M2R6	51.3±7.5	7.6±0.2	1.6±0.1	2.4±0.3	1252±306	2.17	-0.36
L6M1R1	90.7±4.9	23.1±7.4	12.6±1.3	6.1±3.7	797.0±82.1	2.73	-0.73
L6M1R2	54.5±0.6	10.2±5.8	1.1±0.1	7.8±4.9	345.9±36.0	2.60	-0.66
L6M1R3	69.1±2.2	58.1±6.8	31.2±0.2	4.2±0.2	245.3±27.3	3.17	-0.76
L6M1R5	95.5±11.0	64.1±8.0	46.6±1.7	4.6±0.5	510.1±37.5	3.25	-0.61
L6M1R6	84.0±0.5	83.3±14.9	51.2±0.2	5.1±0.8	559.1±94.8	3.32	-0.74
L6M1R7	86.6±12.2	45.9±8.5	23.8±0.1	4.3±0.2	819.3±55.3	2.92	-0.57
L6M1R8	87.1±0.8	53.8±6.0	28.1±1.0	7.3±0.3	810.0±53.1	2.65	-0.82
L4M1R2	3.7±0.2	4.2±2.2	0.4±0.8	6.4±2.2	140.4±17.9	2.75	-0.72
L4M1R3	75.4±4.1	16.8±14.2	35.6±3.7	8.7±5.8	535.1±58.4	3.24	-0.84
L4M1R5	102.7±7.6	23.6±20.0	44.3±4.7	9.2±2.0	1055±126	3.36	-0.69
L4M1R6	96.5±13.0	21.1±11.9	42.5±7.0	16.1±5.9	983±136	3.41	-0.81
L4M1R7	84.8±3.1	19.3±12.2	32.6±19.4	8.4±5.7	1104±189	3.02	-0.65
L4M1R8	78.3±1.3	27.4±11.0	38.3±9.6	6.6±1.2	1022±150	2.74	-0.90

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.

3.3 in vitro reactivation evaluation

Four most common nerve agents (VX, sarin, tabun and soman) and four pesticides were used in the *in vitro* reactivation experiment, Quaternary oximes 2-PAM, HI-6 and obidoxime were used as reference compounds. Initially the diluted hAChE was incubated with sufficient amounts of OPs to get an inhibition of the enzyme ranging from 80% to 95%. Then different oximes (0.1 mM) were added 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. The reactivation results of nerve agents and pesticides inhibited hAChE are listed separately in **table 1** and **table 2**.

Table 2. Reactivation of pesticides inhibited hAChE by new synthesized and the reference oximes (0.1 mM).

Oximes	Reactivation (%)			
	paraoxon	**parathion**	**dichlorvos**	**phorate**
2-PAM	25.28±0.17	50.64±0.24	17.95±1.94	22.12±3.66
HI-6	2.15±0.31	28.80±0.43	2.04±0.04	6.67±0.66
obidoxime	41.7±0.42	61.41±1.40	28.15±4.07	37.12±4.03
L3M2R2	2.96±0.01	34.08±1.41	0.81±0.52	9.01±0.29
L3M2R3	36.17±0.42	2.51±0.17	9.05±0.25	18.78±1.69
L3M2R5	16.34±0.40	29.24±1.22	5.85±0.61	4.85±3.25
L3M2R6	9.92±0.71	10.38±1.39	4.96±0.91	0.02±3.21
L6M1R2	0.08±0.05	2.41±0.44	5.43±0.65	9.91±0.93
L6M1R3	16.02±1.09	2.98±1.73	8.16±0.40	7.00±1.33
L6M1R5	32.23±0.61	18.52±3.93	12.71±0.99	16.13±3.24
L6M1R6	32.27±0.98	24.41±0.10	28.13±1.08	24.76±0.36
L6M1R7	37.40±0.13	19.68±0.14	7.15±0.03	16.91±0.13
L6M1R8	16.27±1.63	6.64±1.38	12.06±0.72	1.50±1.10
L4M1R3	25.69±3.84	9.63±4.55	11.71±1.80	6.67±6.15
L4M1R5	45.33±4.20	31.98±2.73	31.91±2.49	26.42±2.63
L4M1R6	38.36±1.54	28.82±2.78	26.23±1.87	18.14±3.03
L4M1R7	47.24±5.33	34.08±1.41	13.81±1.08	25.35±4.11

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 reactivation study of nerve agents shows that: 1) Most of the new synthesized oximes reactivated VX inhibited hAChE efficiently except for **L3M2R2** and **L4M1R2**, some of them even exceeded that of HI-6 and obidoxime, such as **L6M1R1**, **L6M1R3** to **L6M1R8** and **L4M1R3** to **L4M1R8**, conjugates bearing **R2** as the oxime part appeared to be relatively poor reactivators for VX poisoning. **2)** For soman inhibited hAChE, only **L4M1R6** exhibited weak reactivating ability, but it was still much lower than that of HI-6, the others were all poor reactivators. **3)** For sarin poisoning, conjugates containing 4-aminobenzamide as a PSL exhibited promising reactivation potency, such as oximes **L6M1R3, L6M1R5 to L6M1R8**, and they were also efficient reactivators for tabun poisoning. It's interesting that the corresponding conjugates containing 3-aminobenzamide also showed reactivation efficiency for tabun inhibited hAChE. **4)** It could be concluded that conjugates with 4-aminobenzamide or 3-aminobenzamide linked to the *ortho* position to the phenol function of salicylaldoxime though piperidine were efficiency reactivators. In addition, introduction of methyl or halogen substitution in the *para* position of salicylaldoxime can increase reactivation ability, such as **L6M1R3, L4M1R3, L6M1R5 to L6M1R8 and L6M1R5 to L6M1R8**, some of them were even more potent reactivators for sarin and tabun poisoning in comparison with 2-PAM, HI-6 and obidoxime. **5)** It was a pity that no satisfying results were obtained for sarin and tabun inhibited hAChE in the case of conjugates containing isonicotinamide.

Some selected new conjugates were also tested for hAChE inhibited by pesticides including paraoxon, parathion, dichlorvos and phorate, the results were listed in **table 2**. **It can be concluded that: 1)** Obidoxime performed best among the three tested quaternary oximes, while HI-6 almost showed no reactivation efficiency for hAChE inhibited by paraoxon, dichlorvos and phorate. **2)** For the conjugates containing isonicotinamide as PSL, only **L3M2R3** showed some efficiency for paraoxon and phorate poisoning, the others were poor reactivators. **3)** For the conjugates containing 3-aminobenzamide or 4-aminobenzamide as PSL, a similar structure-activity relationship was observed, **L6M1R6, L4M1R5** to **L4M1R7** emerged as best nonquaternary reactivators, some were as efficient as 2-APM, but still lower than obidoxime. **4)** Although their toxicity is not higher than paraoxon and parathion [42], dichlorvos and phorate seems more difficult to be reactivated. A similar phenomenon was observed for the reactivation of nerve agents poisoning, although the toxicity of VX is highest [42], it was proven that VX inhibited hAChE could be reactivated much more easily in contrast to sarin, tabun and soman.

3.4. Determination of reactivation kinetics

In order to get a deeper comprehension of the reactivation mechanism of these new nonquaternary oximes, efficient compounds were selected and their reactivation rate constant K_r, dissociation constant K_D and second order reactivation rate constant K_{r2} for sarin, VX, tabun paraoxon, parathion and dichlorvos inhibited hAChE. 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 3**.

Table 3. Reactivation rate constant (K_r), dissociation constant (K_D), second order reactivation rate constant (K_{r2}) of obidoxime, HI-6, and selected new nonquaternary oximes for nerve agent inhibited hAChE.

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
L6M1R3	-	17±1.3	6.3±0.7	-	49±14	15±7.0	1.45±0.007	0.337	0.411
L6M1R5	-	57±2.7	8.9±1.1	-	341±23	36±13	1.34±0.033	0.167	0.245
L6M1R6	-	-	15.8±1.4	-	-	43±11	1.45±0.007	0.256±0.004	0.37
L6M1R7	-	15±3.5	3.8±0.3	-	196±75	27±5.9	1.677±0.045	0.076	0.142
L6M1R8	723±219	19±1.6	4.6±0.3	791±249	173±26	33±5.9	0.917	0.111	0.139
L4M1R3	-	19±2.3	8.4±0.7	-	109±26	26±6.7	1.547±0.041	0.178	0.325
L4M1R5	-	-	13.1±1.3	-	-	66±16	1.593±0.102	0.153±0.001	0.197
L4M1R6	-	-	14.7±0.4	-	-	59±4.0	1.974±0.167	0.245±0.006	0.248
L4M1R7	-	47±13	7.3±0.7	-	699±237	89±17	2.435±0.222	0.067	0.083
L4M1R8	-	41±16	13.9±1.5	-	650±318	104±24	0.541±0.011	0.063	0.134

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.

For VX inhibited hAChE, the calculated results showed that K_{obs} was proportional to the concentration of oximes (data showed in the supporting information), so no K_r or K_D could be calculated, K_{r2} was calculated directly. The results showed that **L4M1R7** was the most efficient reactivators, its K_{r2} value was just a little lower than HI-6's, the other halogen and methyl substituted compounds also exhibited good reactivation efficiency. However, all of the selected conjugates were poor reactivators for sarin poisoning in comparison to HI-6 due to their lower reactivation rate constant K_r, some of them even showed poor binding affinity towards sarin-hAChE (indicated as higher K_D), such as **L6M1R5, L4M1R7** and **L4M1R8**. It was remarkable that compounds **L6M1R3, L6M1R6** and **L4M1R3** were proven as more or equal efficient reactivators for the obstinate tabun-inhibited hAChE. Although their reactivation rate constant (K_r) was lower than obidoxime's, their final reactivation efficiency (K_{r2}) was greatly improved due to higher binding affinity for hAChE (indicated as lower K_D). Similar results were observed in our last paper [24], so we can come to the conclusion that introduction of PSL such as 3-aminobenzamide or 4-aminobenzamide to the oximes would contribute to binding affinity to OP inhibited hAChE and produce more efficient nonquaternary reactivators.

In the case of reactivation for paraoxon and dichlorvos inhibited hAChE, although the selected conjugates (**L6M1R6, L4M1R3** and **L4M1R5**) displayed poor reactivation efficiency in contrast to obidoxime, their binding affinity for hAChE was much higher than obidoxime's. The low reactivation rate constant (K_r) accounted for their disabled reactivation efficiency. However, their binding affinity for parathion inhibited hAChE were lower than obidoxime's, maybe hAChE poisoned by parathion present a different conformation which did not interact with these compounds properly.

Table 4. Reactivation rate constant (K_r), dissociation constant (K_D), second order reactivation rate constant (K_{r2}) of obidoxime and selected new nonquaternary oximes for hAChE inhibited by pesticides.

oxime	$K_r/10^{-3}min^{-1}$			$K_D/\mu M$			$K_{r2}/mM^{-1}min^{-1}$		
	paraoxon	parathion	dichlorvos	paraoxon	parathion	dichlorvos	paraoxon	parathion	dichlorvos
Obidoxime	435±77	739±46	856±183	35.3±24	16.3±44	120±51	12.32	45.25	7.13
L6M1R6	29.6±0.3	32.3±3.8	34.3±2.1	12.4±6.3	28.0±11	33.4±6.4	2.39	1.18	1.03
L4M1R3	19.1±0.4	18.1±5.0	12.8±1.9	11.4±9.9	23.7±23	15.5±9.2	1.67	0.76	0.83
L4M1R5	46.7±0.5	54.2±8.7	43.9±0.6	30.5±11	58.6±25	57.6±2.3	1.54	0.92	0.76

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.

4. Conclusion

On the base of our previous study, we have described the design, synthesis and the *in vitro* biological evaluation of a new series of nonquaternary conjugates for reactivation of both nerve agents and pesticides inhibited hAChE in this paper. Some salicylaldehydes bearing 3-aminobenzamide or 4-aminobenzamide as PSLs produced efficient reactivators for sarin, VX and tabun inhibited hAChE. Conjugates with piperidine linked to the *ortho* position and methyl or halogen substituted in the *para* position of salicylaldoxime emerged as more efficient reactivators, some of them even surpassed the currently approved bis-pyridinium oximes HI-6 and obidoxime, such as **L6M1R3, L6M1R5** to **L6M1R7**, **L4M1R3** and **L4M1R5** to **L4M1R7**. While those conjugates containing isonicotinamide as PSL were proved to be poor reactivators for most OP inhibited hAChE, such as **L3M2R2, L3M2R3, L3M2R5** and **L3M2R6**. For the first time, we conducted *in vitro* reactivation experiment for pesticides inhibited hAChE of these new synthesized oximes. Despite they were less efficient than obidoxime, some of them were highlighted as equal or more efficient reactivators than

2-PAM, while HI-6 only exhibited slight reactivation ability. It has been proven that introduction of PSLs could increase oximes' binding affinity for inhibited hAChE in most cases, which contribute to the reactivation efficiency. Finally, due to their strikingly improved lipophilicity, these novel reactivators hold promise for BBB penetration and reactivate inhibited hAChE efficiently in CNS, the *in vivo* experiment would be conducted in a future study. Further structural transformation and modification of these nonquaternary oximes are in process with the expectation for finding of efficient 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; TsCl, tosyl chloride; TFA, trifluoracetic acid; 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.

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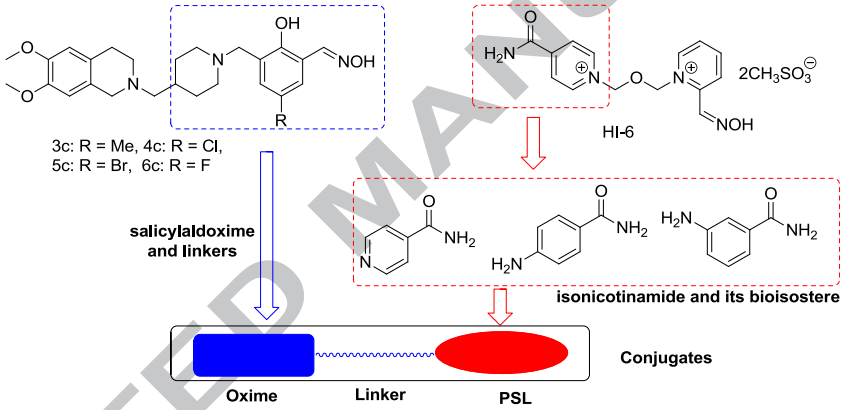
Graphical abstract

Design, synthesis and evaluation of new classes of nonquaternary reactivators for acetylcholinesterase inhibited by organophosphates

It was found that substituted salicylaldehydes conjugated to aminobenzamide through piperidine would produce efficient reactivators for sarin, VX or tabun inhibited hAChE. Meanwhile, some of them emerged as equal or more efficient reactivators for pesticides inhibited hAChE in comparison to 2-PAM.



Construction of nonquaternary reactivators for OP poisoning

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

Highlights:

1. Novel nonquaternary reactivators for both nerve agents and pesticides 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.