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Novel nonquaternary reactivators showing reactivation efficiency for soman-inhibited human acetylcholinesterase

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Highlights:

- We designed and synthesized a series of new nonquaternary oximes aiming for reactivation of OP-poisoned hAChE.
- Some of these new oximes showed obvious *in vitro* reactivation potency of soman-inhibited hAChE.
- Greater affinity of oximes **7b-9b** to hAChE might partially account for their higher reactivation efficiency for soman-inhibited hAChE.

ABSTRACT: Soman is a highly toxic nerve agent with strong inhibition of acetylcholinesterase (AChE), but of the few reactivators showing antidotal efficiency for soman-inhibited AChE presently are all permanently charged cationic oximes with poor penetration of the blood-brain barrier. To overcome this problem, uncharged reactivators have been designed and synthesized, but few of them were efficient for treating soman poisoning. Herein, we used a dual site biding strategy to develop more efficient uncharged reactivators. The ortho-hydroxylbenzaldoximes were chosen as reactivation ligands of AChE to prevent the secondary poisoning of AChE, and simple aromatic groups were used as peripheral site ligands of AChE, which were linked to the oximes in a similar way as that found in the reactivator HI-6. The *in vitro* experiment demonstrated that some of the resulting conjugates have robust activity against soman-inhibited AChE, and oxime **8b** was highlighted as the most efficient one. Although not good as HI-6 *in vitro*, these new compounds hold promise for development of more efficient centrally acting reactivators for soman poisoning due to their novel nonquaternary structures, which are predicted to be able to cross the blood-brain barrier.

Abbreviations: AChE, Acetylcholinesterase; ACh, Acetylcholine; A-site, Active site; P-site, Peripheral site; PSL, Peripheral site ligand; CNS, Central nervous system; BBB, Blood-brain barrier; TLC, Thin-layer chromatography; BSA, Bovine serum albumin; ATCh, acetylthiocholine; DTNB, 5, 5'-dithiodis-2-nitrobenzoic acid.

Keywords: Soman, Acetylcholinesterase, Nonquaternary reactivators, Peripheral site ligand, Dual-site binding, Reactivation

1. Introduction

Acetylcholinesterase (AChE) is a hydrolase, it terminates the action of the neurotransmitter acetylcholine (ACh) at postsynaptic membranes and neuromuscular junctions by hydrolyzing ACh.[1] Nerve agents (e.g., sarin, VX, tabun, and soman) exert their acute toxicity through inhibition of AChE by forming a covalent P-O bond with the serine hydroxyl group at the active site (A-site) of the enzyme. The subsequent accumulation of unhydrolyzed ACh at neuronal synapses and neuromuscular junctions results in cholinergic crisis, respiratory distress, convulsive seizures and ultimately death.[2] Considering their potential threats to the military and the public (such as the terrorist attacks in Tokyo subway in 1995 and the recent Syria civil war),[3] it is of great importance to find an efficient way to reactivate the nerve agent-inhibited enzyme.

Phosphylated AChE can be reactivated by nucleophilic agents such as oximes (e.g., pralidoxime or 2-PAM, trimedoxime or TMB-4, obidoxime, HI-6, and MMB-4, **Fig.1**).[4,5] Unfortunately, all the current available reactivators are quaternary oximes. They provide little or no protection against neurological effects of organophosphate (OP) exposure in the central nervous system (CNS) due to their poor blood-brain barrier (BBB) penetration due to their permanent charges,[6,7] while the nerve agents can readily diffuse through the BBB and inhibited the central AChE quickly.[8-10] To overcome this problem, a number of nonquaternary reactivators was designed and synthesized (e.g. monoisonitrosoacetone or MINA, amidine-oximes, pro-2-PAM and RS194B **Fig. 2**).[11-14] Their BBB penetration was facilitated as a result of increased lipophilicity. They showed obvious superiority to charged 2-PAM as antidotes for CNS poisoning. Presently, few uncharged reactivators were found to show antidotal efficiency for soman-inhibited AChE.[15,16]

HI-6 was the first quaternary oxime that demonstrated reactivation efficiency for soman-inhibited AChE.[17] Nevertheless, HI-6 encountered the same problem of poor BBB penetration owing to its permanent charges.[18,19] Moreover, the resulting phosphylated oximes (such as intermediate **c**, **Fig. 3**) still has high reactivation activity, and they can act as a nerve agent which may lead to secondary poisoning of

AChE (also known as recapture phenomenon, **Fig. 3**, left).[20] Thus it is of primary importance to develop uncharged reactivators with little or no recapture activity for soman poisoning.

Recently, a series of ortho-hydroxylbenzaldoximes were found showing ability to efficiently and selectively cleave the P-S bond of organophosphorus nerve agents. Moreover, the yielding phosphylated intermediate **f** can rapidly form isoxazole **g** and non-toxic phosphonic acid **h** through an intramolecular attack of the phenol onto the nitrogen atom (**Fig. 3**, right).[21,22] It prevent the recapture phenomenon, allowing the discovery of a more efficient uncharged structural scaffold for centrally acting reactivators of phosphylated AChE. However, these nucleophiles exhibited poor affinity to the inhibited enzyme due to the loss of permanent charges, which lead to high dissociation constants (K_D). It was believed that the second quaternary pyridyl group of HI-6 would interact with the peripheral site (P-site) of AChE and increase its affinity. Although its reactivation rate constant (K_r) is lower than 2-PAM (0.44±0.15 min^{-1} vs 0.73±0.09 min^{-1}), the bimolecular reactivation rate constant (K_{r2}) of HI-6 (K_{r2}=9.0 $mM^{-1}min^{-1}$) was greatly improved in comparison to 2-PAM (K_{r2}=2.3 $mM^{-1}min^{-1}$) due to its higher affinity for VX-hAChE (K_D=50±26 μM^{-1} for HI-6 vs K_D=300±140μM^{-1} for 2-PAM).[20,23] Moreover, it was found that the mono-oximes' affinity to AChE and their reactivation potency would be greatly enhanced after a peripheral site ligand (PSL) of AChE was linked, such as reactivator **7h** (**Fig. 2**).[24]

Accordingly, we used a dual site binding strategy to attach the ortho-hydroxylbenzaldoximes to some PSLs in a similar way as HI-6. As a preliminary attempt, simple aromatic groups were used as PSLs, which was expected to interact with the aromatic group in the P-site and compensate the ortho-hydroxylbenzaldoximes's affinity to AChE. Their lipophilicity (S+logP) and BBB penetration (S+logBBB) was estimated (data shown in **Table 1**). They were expected to show higher BBB penetration ability due to improved lipophilicity. Moreover, these oximes were expected to be effective reactivators against soman-inhibited AChE due to their structure similarity to HI-6. Additionally, molecular docking simulations demonstrated that the resulting conjugates interacted

with AChE in a dual site binding mode as predicted. The results suggested that the phenol group may act as a proper PSL. Docked conformations of moleculars **8a** and **8b** were depicted in **Fig. 4** and it can be observed that the PSLs interact with the AChE P-site in a π-π sandwiching way, while the oxime groups were located at the A-site, which were supposed to act as reactivation groups.

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 $CDCl_3$ 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). Low-resolution mass spectra were obtained using an API 3000 LC/MS with an ESI source and high-resolution mass spectra were obtained using an Agilent G6230A 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 was performed on silica gel (200-300 mesh), compounds were visualized under UV light at 254 nm, R_f values were given for guidance. Analytical HPLC was performed on a Agilent 1260 instrument equipped with a DAD detector under the following conditions: Innoval C18 column (5 μm, 100 Å, 4.6×250 mm) with MeOH and 0.1% aq. trifluoroacetic acid (TFA) as eluents [0.1% aq. TFA/MeOH (90/10) 5 min, followed by linear gradient from 10% to 80% of MeOH 40 (min)] at a flow rate of 1.0 mL/min and UV detection at 270 nm. The details of synthetic procedures can be found in the Supplementary data.

2.2. General *in vitro* hAChE screening information

Human acetylcholinesterase (hAChE), bovine serum albumin (BSA), acetylthiocholine (ATCh), 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) and 2-PAM were purchased from Sigma-Aldrich. HI-6 were synthesized ourselves according to the literature protocols.[25, 26] Soman was obtained from Anti chemical command and Engineering Institute of the Chinese people's Liberation Army. 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, from Sigma) were diluted by PBS (0.1 M, pH 7.4, 0.1% BSA) and stored at 0-4 °C. Soman was diluted in distilled water to $5*10^{-8}$ M concentration. 10 mM oxime concentration solutions were prepared in water contain 2.5% acetic acid and 10% PEG-400. They were further diluted by PBS (0.1 M, pH 7.4) to 3 mM concentrations. It was found that there was no effect of CH_3COOH or PEG-400 on hAChE by a control experiment.

2.3.hAChE inhibition experiments with oximes

Each oxime was tested for its ability to inhibit the enzyme activity of hAChE in 96-well plate as measured by the time-dependent hydrolysis of acetylthiocholine (ATCh) in which the product (thiocholine) was detected by reaction with the Ellman's reagent, 5, 5′-dithiodis-2-nitrobenzoic acid (DTNB) and absorbance at 415 nm. Initially 10 µL of hAChE (20 U/L in 0.1 M PBS, pH 8.0, 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 ($0<abs<2.5$).[27] 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: $\%Activity = 100*IC_{50}/(IC_{50}+[Ox])$.[28]

2.4.hAChE reactivation experiments with oximes

A stock solution of hAChE (20 U/mL, from sigma) was diluted 50-fold with PBS (0.1 M, pH 8.0, 0.1% BSA). The diluted hAChE (175 µL) was incubated with soman ($5*10^{-8}$M, 325 µL) and an excess of soman 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 soman was diluted more than 7000-fold, indicating that most of soman was removed and inhibition of hAChE had been terminated. Then the inhibited enzyme (20 U/L, 80 μL) was incubated with oximes (40 μL, 3 mM) at 37 °C for 10 min to 24 h (final concentration of oximes was 1 mM). At different time intervals, 15 μL of the incubation mixture was taken and enzyme activity was measured by using same method described in **section 2.3.**

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]

2.5.ADMET Predictions

In this study, we evaluated lipophilicity and BBB penetration potency of all the compounds 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 the most accurate, quick and useful tool to predict physicochemical and biological properties of drug-like chemicals.[30,31]

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, pK_a was limited to 1-14 and the 'activate-one-out in all associative models' option was selected. The program is executed to calculate the various parameters such as S+logP, S+S_w, S+Peff, S+logBBB, S+Vd and so on. The predicted data can be saved as output file in 'xls' format. 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.

3. Results and discussion

3.1. Synthesis

The synthetic routes of these reactivators were highlighted in **scheme 1**. It was started with chloromethylation of different salicylaldehydes **1, 2, 3** to give the intermediates **4, 5, 6** in excellent yields. At the same time, different benzaldehydes **10a, 10b** and **10c** were treated under reductive amination conditions with ethylamine and sodium triacetoxyborohydride to provide the tertiary amines **11a, 11b** and **11c** in 54% to 69% yields. Then condensation between the benzyl chlorides **4, 5, 6** and the amines **11a, 11b** and **11c** produced the desired aldehyde derivatives **4a-6a, 4b-6b** and **4c-6c** in moderate to good yields. Finally, treatment of the latters with hydroxyl-ammonium chloride afforded the target oximes **7a-9a, 7b-9b** and **7c-9c** in 51% to 93% yields.

3.2. 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.[32] Thus it is necessary to evaluate the AChE inhibition abilities of these oximes firstly. HI-6 and an efficient nonquaternary reactivator **7h**[24] were used as reference compounds. IC_{50} was determined and displayed in **Fig. 5**. It can be found that these new synthesized oximes were moderate or weak inhibitors of hAChE with IC_{50} greater than 100 μM. It allowed a proper affinity to hAChE for reactivation of the poisoned enzyme. The results encouraged us to proceed to the reactivation experiments.

3.3.*In vitro* reactivation evaluation

The search for antidotes and reactivators of OP-inhibited AChE started more than 60 years ago and the theory and methodology for evaluation of reactivation kinetic of OP-inhibited AChE had been established.[33] However, soman-inhibited hAChE undergo an extremely rapid aging process (the aging half-time being in the range of 2-3 min at 37 ˚C), and aged hAChE was quite difficult to be reactivated.[34-37] Thus the concentration of non-aged soman-hAChE decreased quickly and

complicated the evaluation of reactivation kinetics of soman-inhibited hAChE. In order to get an effective assessment of these new synthesized reactivators, %Reacrivation at different time intervals was determined, which can directly reflect the reactivation ability of these tested oximes.

The results of the reactivation experiment under physiological conditions are listed in **Table 1**. It was apparent that this new series of reactivators demonstrated obvious reactivation potency against soman-inhibited hAChE, while 2-APM and the reference uncharged oxime **7h** exhibited no reactivation ability. Oximes **7b-9b** were more efficient reactivators than the others. Oxime **8b** was highlighted as the most efficient one, albeit, it still lagged behind the efficient *in vitro* reactivator, HI-6. As the reactivation time prolonged, reactivation potency of HI-6 show no obvious change during the first half hour and it tended to remain steady after 2 h. The reactivation potency of these new tertiary oximes was also fairly stable up to 24 h. It may be due to rapid aging of soman-inhibited hAChE.

Experiments were performed (oxime final concentration 1mM) in triplicate at 37 $^{\circ}$C in phosphate buffer (0.10 M, pH=7.4); hAChE inhibition percentages were greater than 95%; the reactivation potency was calculated relatively to the poisoned control. S+logP and S+logBBB was predicted by ADMET Predictor software version 7.0, a higher S+LogP value indicates higher lipophilicity and a higher S+logBBB value indicates higher BBB penetration ability.

It is obvious that these reactivators bearing pyridinealdoximes show higher reactivating potency against soman-inhibited hAChE in contrast to **7h**, which contains an imidazolealdoxime. In another hand, a shorter linker with a hetero atom between the P-site ligands and the A-site ligands may be beneficial to reactivating soman-inhibited hAChE, while the linker of **7h** is too long and has no hetero atom. However, loss of permanent charges decreases the nucleophilic ability of oxime groups in **7a-9c**, which may account for their lower reactivating potency to soman-inhibited hAChE than HI-6. Therefore, pyridinealdoxime and shorter linker with hetero atom, especially a linker similar to that of HI-6, maybe key factors to develop efficient reactivators for soman poisoning.

Apart from these, it can be concluded that compounds (**7b-9b**) with a 4-hydroxybenzyl P-site ligand may produce higher reactivation activity against soman-inhibited hAChE. At the same time, it was noticeable that reactivators **7b-9b** were relatively heavier inhibitors of hAChE (**Fig. 5**), which indicated a higher affinity for hAChE. Consequently, we summarized that the greater affinity of **7b-9b** to hAChE may partially account for their higher reactivation efficiency. We believed that it was necessary to improve hAChE affinity for much better reactivators. However, strong inhibition of hAChE should be avoided because it would result in toxicity.[25]

3.4.ADMET Predictions

As we have mentioned previously, S+logBBB is the predicted result of $\log(C_{brain}/C_{blood})$, so a higher S+logBBB value indicates higher BBB penetration ability. Sakurada et al. found that the concentration of 2-PAM in the corpus striatum is about 10% of the plasma level in rat *in vivo*.[38] Falb and Erdmann observed that central nervous system concentrations of obidoxime amounting to 3-5% of plasma levels in both rats and mice *in vivo*.[39] It can be found that the predicted S+logBBB values of 2-PAM (-0.69) and obidoxime(-1.26, not listed in **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.

4. Conclusion

In conclusion, based on a dual site binding strategy and intended to avoid the recapture phenomenon of the reactivated AChE from the very beginning, a new series of nonquaternary ortho-hydroxylbenzaldoximes with totally novel structures were designed, synthesized and tested in this paper. To our best knowledge, they were the first family of uncharged reactivators showing obvious *in vitro* reactivation efficacy against soman-inhibited hAChE until now. Although not as efficient as HI-6, they were expected to exhibit higher BBB penetration and demonstrate promising *in vivo* reactivation ability as a result of their nonquaternary structures. The *in vivo* assays are in process and the corresponding results will be discussed in a future paper.

Furthermore, these new structural scaffolds also lay the foundation for development of more efficient centrally acting reactivators for soman poisoning.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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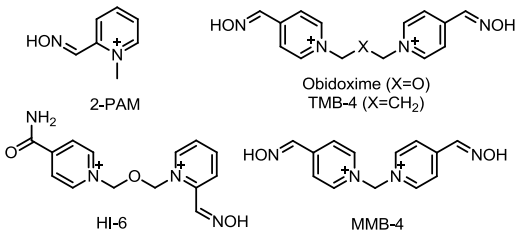


Fig. 1 Structures of current available quaternary AChE reactivators.

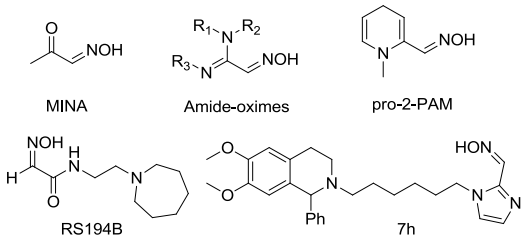


Fig. 2 Structures of uncharged AChE reactivators.

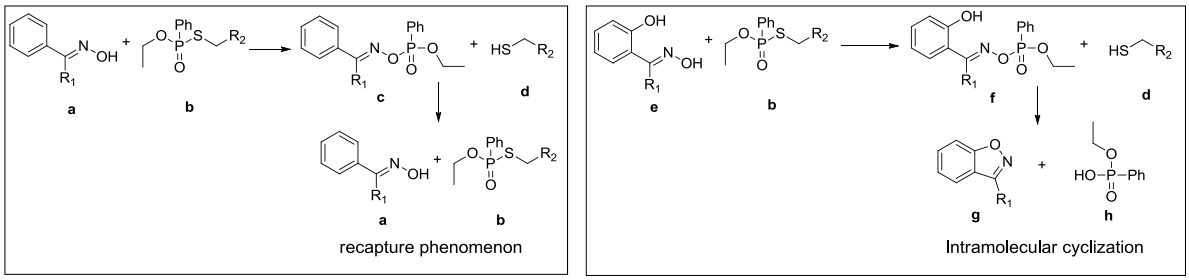


Fig. 3 The mechanism of recapture phenomenon and intramolecular cyclization of the phosphylated oximes

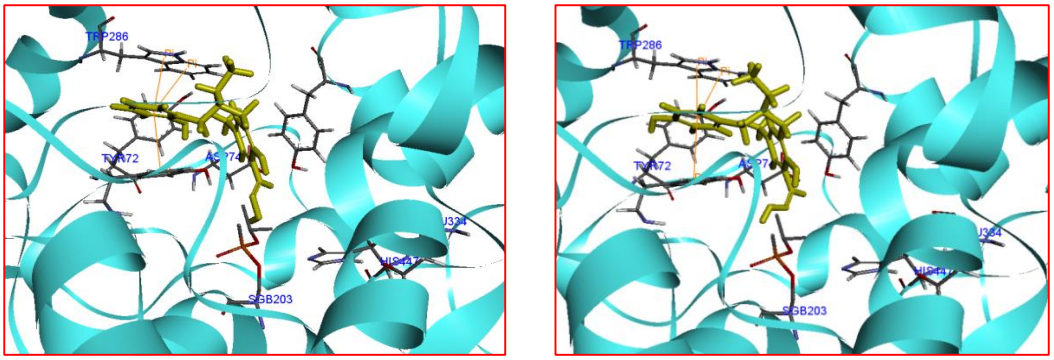


Fig. 4 Docked conformations of molecules **8a** (left) and **8b** (right) in the A-site gorge of sarin^{nonaged}-mAChE (pdb code: 2WHP). The key amino acid residues in the P-site include Tyr72, Tyr124, Trp286, Tyr341 and Asp74; in the A-site include Ser-sarin203 or SGB203, Glu334 and His447. The docked conformations of the reactivators are depicted as stick model in yellow and the key amino acid residues as stick model in grey.

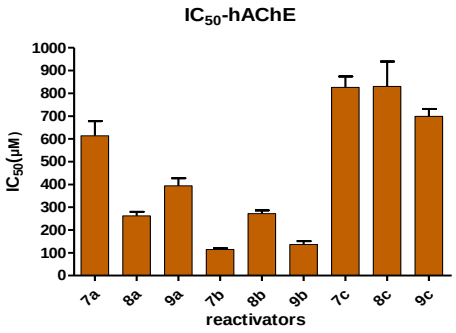
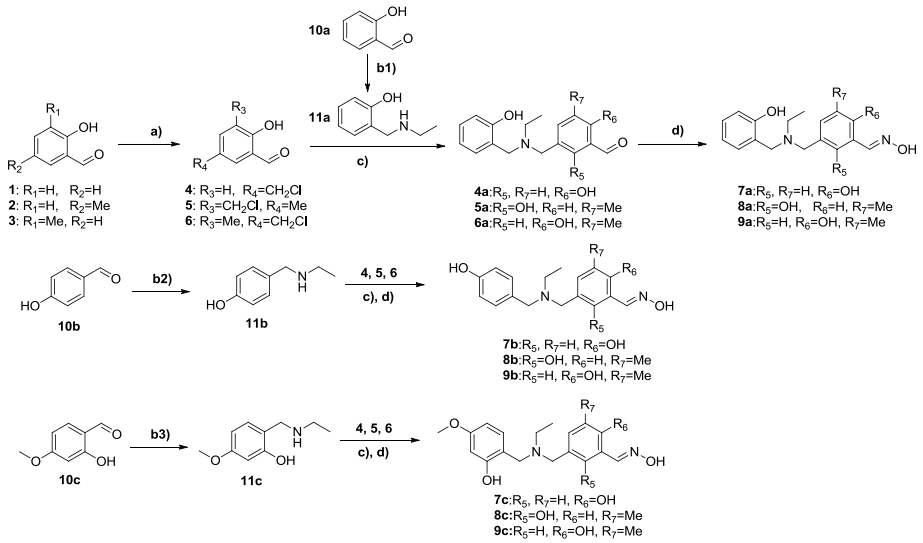


Fig. 5 IC_{50} of uncharged reactivators **7a-9a**, **7b-9b** and **7c-9c**.



Scheme 1 Synthesis of reactivators 7a-7c, 8a-8c and 9a-9c. Conditions and reagents: (a) con. HCl, $(CH_2O)_n$, 50-60 °C, 2-3 h, 77%-97%; (b1) 1. aq. $C_2H_5NH_2$ (65%-70%), 50 °C，3 h；2. $NaBH(CO_2CH_3)_3$, C_2H_5OH, 50-60 °C, 1 h (over two steps) 69%; (b2) 1. aq. $C_2H_5NH_2$ (65%-70%), CaO, C_2H_5OH, r.t., 2.5 h; 2. $NaBH(CO_2CH_3)_3$, C_2H_5OH, 3h (over two steps) 55%; (b3) 1. $C_2H_5NH_2$·HCl, C_2H_5OH, r.t., 0.5 h; 2. $NaBH_4$, C_2H_5OH, 0 °C, 3 h (over two steps) 54%; (c) DIEPA, CH_2Cl_2, r.t. 1h, 54%-98%; (d) NH_2OH, HCl, C_2H_5OH/ CH_2Cl_2, r.t., 1h, 51%-93%.

Table 1 Reactivation of soman-inhibited hAChE by **7a-9a, 7b-9b, 7c-9c** and the reference oximes at different time intervals and predicted logP and logBBB values of **7a-9a, 7b-9b, 7c-9c.**

oximes	Reactivation (%)				S+logP	S+LogBBB
	10 min	30 min	2 h	24 h		
HI-6	28.0±1.0	28.2±1.8	37.5±6.6	36.9±3.7	-5.03	-1.39
2-PAM	1.9±0.5	2.1±0.7	1.3±0.2	1.8±0.6	-2.81	-0.69
7h	-0.9±0.1	-1.0±0.2	-0.2±0.0	-1.0±0.5	4.89	-0.47
7a	9.7±0.5	9.5±1.1	10.4±2.1	9.9±0.8	2.95	-0.18
8a	9.0±0.5	8.1±0.6	10.0±0.9	7.0±0.7	3.55	-0.16
9a	10.3±0.1	10.4±0.9	12.5±0.1	10.8±1.5	3.32	-0.14
7b	14.4±0.1	14.1±0.7	16.3±2.0	15.5±0.8	2.72	-0.13
8b	20.1±0.5	19.0±2.1	22.4±0.8	19.7±1.0	3.39	-0.18
9b	13.9±0.8	13.4±0.8	16.2±0.3	13.4±0.3	3.11	-0.09
7c	11.1±0.6	11.1±0.0	14.8±0.5	11.0±0.4	2.97	-0.31
8c	7.7±0.4	9.2±0.8	10.8±0.7	8.9±1.1	3.35	-0.27
9c	9.2±0.8	10.0±0.5	11.0±0.2	9.9±0.2	3.56	-0.29