Oxime Reactivators for Paraoxon-inhibited AChE

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In vitro Screening of Oxime Reactivators on the Model of Paraoxon-inhibited Acetylcholinesterase-SAR Study

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Acetylcholinesterase reactivators are crucial antidotes for the treatment of organophosphate intoxication. Standard *in vitro* test was chosen using a rat brain homogenate as the source of AChE. Screening of reactivation potency was performed with two concentration of reactivator (1000 μM and 10 μM). Results were compared to established reactivators pralidoxime, methoxime, HI-6, trimedoxime and obidoxime. More than 30 novel reactivators performed equal or better reactivation ability of POX-inhibited AChE compared to currently used reactivators. The structure-activity relationship for reactivators of paraoxon-inhibited AChE was developed.

Key Words: Acetylcholinesterase, Pesticide, Reactivator, Structural requirements

Introduction

Acetylcholinesterase (AChE 3.1.1.7) belongs to the family of serine hydrolases. It plays a crucial role in the organism.[1] AChE terminates a nervous transmission *via* degrading a neurotransmitter acetylcholine within the synaptic cleft.[2] Cavity of the enzyme is located in 20 Å (20 nm) deep gorge with active (A) site and peripheral (P) site.[3,4] A-site contains amino-acids residues of catalytic triad (S203, E334 and H447).[5,6] P-site plays important role in allosteric modulation of the enzyme activity, it contains residues of Tyr72, Tyr124, Trp286, Tyr341 and Asp74.[5] Organophosphorus compounds (OPCs) are able to inhibit AChE irreversibly.[7] Many organophosphorus AChE inhibitors were synthesized and widely used as pesticides (e.g. parathion, paraoxon, chlorpyrifos, and diazinon)[8,9] or industrial compounds (e.g. plasticizers and flame retardants). Some of them were developed for

military purposes and they are known as nerve agents[10] (e.g. sarin, soman, tabun, and VX). Some OPCs are shown in Figure 1. OPCs irreversibly inhibit AChE that cannot fulfil its physiological role. Intoxication is manifested by bronchial hypersecretion, salivation, muscle spasms and fasciculation leading to paralysis, depression of breathing centre followed by death.[11,12]

Moreover, inhibited enzyme undergoes a process called "aging".[13] During this process, OPC-AChE complex is dealkylated. None of currently known reactivators is able to restore the AChE function after aging process.[14]

Standard treatment of OPC intoxication consists of AChE reactivators, anticholinergic drug (e.g. atropine) and anticonvulsive (e.g. diazepam).[15] AChE reactivators are able to counteract consequences of OPC intoxication and they are an essential part of the standard treatment.[16] Reactivators are monoquaternary or bisquaternary pyridinium salts, which bear an oxime (hydroxy-
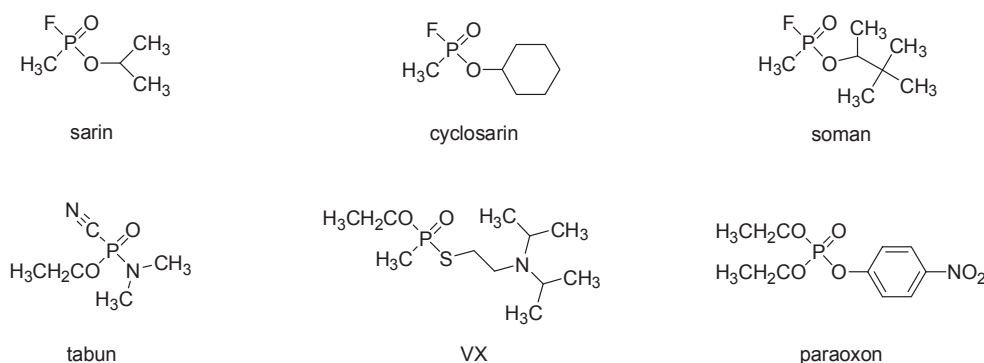

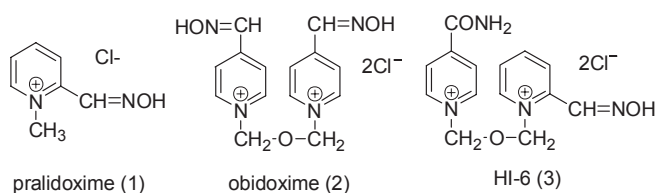**Figure 1.** Organophosphorus inhibitors of AChE.

Figure 2. Commercially available oxime reactivators.

iminomethyl) group in the molecule.[17,18] Dissociated oximate anion is able to attack to the covalent bond in the AChE-OPC complex and restores the enzyme function.[19] The pralidoxime (**1**, 2-hydroxyiminomethyl-1-methylpyridinium chloride), HI-6 (**2**, 1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride) obidoxime (**3**, 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride) are commercially available oxime reactivators[20] (Figure 2). Atropine is used for binding to muscarinic acetylcholine receptors (AChR) with consequent neuronal transduction, therefore prevents from hyperstimulation with ACh and subsequent cholinergic crisis.[21] Diazepam is used for anticonvulsive treatment.[10]

OP pesticides are easily available and used globally. Moreover, at least 200 000 people annually die of OP pesticide self-poisoning.[8,22] Since there is a wide variety of OPCs, none of currently used compounds is able to fairly reactivate AChE inhibited by various pesticides.[23] Additionally, commercial AChE re-activators were originally designed against nerve agents. Hence, new reactivators of OP pesticides are currently requested.[24]

In this study, paraoxon was chosen as model OPC for our *in vitro* screening. Though paraoxon is prohibited OP compound for several decades for its increased toxicity in human, its thio-analogue parathion may still be in use. Parathion is easily bio-activated *via* CYP-450 to paraoxon.[25]

Results and Discussion

The previously known compounds (**1-7**) and over 50 new re-activators (**8-63**) were assayed for their reactivation ability using rat brain homogenate inhibited by paraoxon. The reactivation results are listed in Table 1-9.

A reactivation *in vitro* should exceed 10% to suggest a promising compound warranting further testing.[17,26] Apparently, not all of the tested compounds were able to fulfil this requirement. Moreover, maximal attainable plasma concentration of reactivator is 100 μM.[27] Consequently, reactivation ability at 10 μM is more relevant concerning further testing and plausible use. Reactivation potency of oximes is better for POX-inhibited AChE comparing to nerve agents, because they undergo slower or no "aging" process.[28]

The best reactivation results among standard compounds were obtained for compounds **2**, **4**, **6** and **7** with concentration 1000 μM and **2** and **5** (obidoxime and trimedoxime) with con-

Table 1. Reactivation potency of commercial and standard reactivators

Reactivator	R_1	R_2	A	X	% reactivation ± SD	
					1000 μM	10 μM
1 (Pralidoxime)	2-CH=NOH	-	CH$_3$	I	42 ± 1	0
2 (Obidoxime)	4-CH=NOH	4-CH=NOH	CH$_2$OCH$_2$	2Cl	76 ± 2	37 ± 2
3 (HI-6)	2-CH=NOH	4-CONH$_2$	CH$_2$OCH$_2$	2Cl	35 ± 2	0
4	4-CH=NOH	4-CH=NOH	CH$_2$	2Br	71 ± 3	0
5	4-CH=NOH	4-CH=NOH	(CH$_2$)$_3$	2Br	46 ± 1	50 ± 4
6	4-CH=NOH	4-CONH$_2$	(CH$_2$)$_3$	2Br	59 ± 4	21 ± 1
7	4-CH=NOH	4-CONH$_2$	(CH$_2$)$_4$	2Br	57 ± 4	5 ± 2

Table 2. Reactivation potency of bisoxime xylene linked reactivators

Reactivator	Oxime position	Linker (A)	% reactivation ± SD	
			1000 μM	10 μM
8	2,3'	*o*-phenylene	0	0
9	2,4'	*o*-phenylene	0	0
10	3,4'	*o*-phenylene	0	0
11	2,3'	*m*-phenylene	0	49 ± 4
12	2,4'	*m*-phenylene	0	53 ± 3
13	3,4'	*m*-phenylene	0	0
14	2,3'	*p*-phenylene	0	46 ± 1
15	2,4'	*p*-phenylene	0	53 ± 6
16	3,4'	*p*-phenylene	0	0

Table 3. Reactivation potency of bisoxime xylene linked reactivators
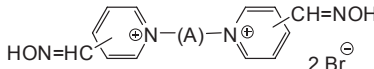
Reactivator	HON=HC⊕N—(A)—N⊕ CH=NOH 2 Br⊖		% reactivation ± SD	
	Oxime position	A	1000 μM	10 μM
17	2,2'	(CH$_2$)$_3$	16 ± 2	0
18	3,3'	(CH$_2$)$_3$	9 ± 0	0
19	2,3'	(CH$_2$)$_3$	33 ± 1	12 ± 0
20	2,4'	(CH$_2$)$_3$	25 ± 0	38 ± 0
21	3,4'	(CH$_2$)$_3$	41 ± 1	43 ± 2

Table 4. Reactivation potency of monooxime-monocyano propane linked reactivators
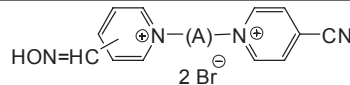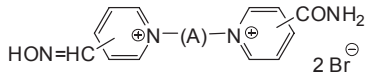
Reactivator	HON=HC⊕N—(A)—N⊕ CN 2 Br⊖		% reactivation ± SD	
	Substituent position	A	1000 μM	10 μM
22	3,4'	(CH$_2$)$_3$	4 ± 0	25 ± 0
23	2,4'	(CH$_2$)$_3$	0	0
24	4,4'	(CH$_2$)$_3$	32 ± 3	3 ± 1

Table 5. Reactivation potency of monooxime-monocarbamoyl (*E*) but-2-en linked reactivators

Reactivator	HON=HC⊕N—(A)—N⊕ CONH$_2$ 2 Br⊖		% reactivation ± SD	
	Substituent position	A	1000 μM	10 μM
25	2,4'	(*E*) CH$_2$CH=CHCH$_2$	44 ± 1	39 ± 3
26	3,4'	(*E*) CH$_2$CH=CHCH$_2$	16 ± 0	0
27	4,4'	(*E*) CH$_2$CH=CHCH$_2$	64 ± 3	23 ± 1
28	2,3'	(*E*) CH$_2$CH=CHCH$_2$	18 ± 1	33 ± 4
29	3,3'	(*E*) CH$_2$CH=CHCH$_2$	0	25 ± 0
30	4,3'	(*E*) CH$_2$CH=CHCH$_2$	54 ± 0	49 ± 0

centration 10 μM. Other commercial reactivators (**1**, **3** and **4**) were not convenient for reactivation of POX-inhibited AChE *in vivo*.

Some newly tested reactivators showed promising results, mainly for concentration 10 μM. Promising compounds, which showed better results compared to the group of standards, were mainly the reactivators with xylene and but-2-ene linkage (**11**, **12**, **14**, **15**, **25**, **27**, **33**, **44**, **56**, **62**). Hence, some structural features were found more beneficial for reactivation of POX-inhibited AChE. In addition, interesting phenomenon was observed, where some compounds showed higher reactivation ability for lower concentration of the reactivator (e.g. **11-12**, **14-15**, **20-22**, **28-29**, **32-36**, **58**, **61-63**). This was probably caused by coincident reactivation and inhibition of the enzyme by reactivator itself as was described earlier.[24]

Structural requirements for oxime reactivators were previously described.[19,29,30] Some authors used molecular modelling to predict the most effective structure of oxime reactivators, too.[4,13] The main structural features that influence the reactivation abili-

ty are the oxime functional group (its position and amount), number of quaternary nitrogen, varying functional group on non-oxime heteroaromatic ring and the connecting linker (its length and structure) for bisquarternary reactivators.[24,31]

Oxime functional group is essential for reactivation process. For POX-inhibited AChE, at least one oxime group in position four (**30**) seemed to be beneficial, when compared to related compounds with the oxime in position two (**17**, **28**) or three (**18**, **29**), especially for 10 μM. Compounds with oxime group in position 3 were much less effective (**26** compared to **25** and **27**) that was formerly explained by increased pK_a of 3-positioned oxime.[17] Furthermore, the reactivation results showed that one oxime group was essential and introduction of second oxime didn't lead to significant increase of reactivation ability (**25-30** compared to **56-58**).

Differently, the non-oxime functional group may extend the hydrophilic/hydrophobic interactions within the enzyme active sites.[32] From this point of view, the best results were obtained for the isoquinolinium-monooxime reactivator (**62**; Table 9). In this

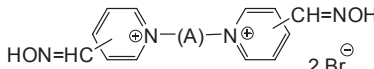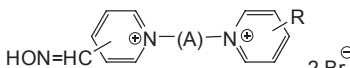**Table 6.** Reactivation potency of bisoxime (Z) but-2-ene linked reactivators

Reactivator	HON=HC⊕N—(A)—N⊕CH=NOH 2 Br⊖		% reactivation ± SD	
	Oxime position	A	1000 μM	10 μM
31	2,2'	(Z) $CH_2CH=CHCH_2$	26 ± 1	13 ± 0
32	3,3'	(Z) $CH_2CH=CHCH_2$	5 ± 1	8 ± 1
33	4,4'	(Z) $CH_2CH=CHCH_2$	40 ± 6	46 ± 1
34	2,3'	(Z) $CH_2CH=CHCH_2$	0	14 ± 0
35	2,4'	(Z) $CH_2CH=CHCH_2$	19 ± 2	29 ± 1
36	3,4'	(Z) $CH_2CH=CHCH_2$	3 ± 0	18 ± 1

Table 7. Reactivation potency of monoxime (*E*) but-2-ene linked reactivators

Reactivator	HON=HC⊕N—(A)—N⊕R 2 Br⊖			% reactivation ± SD	
	Oxime position	R	A	1000 μM	10 μM
37	4	4-COOH	(*E*) $CH_2CH=CHCH_2$	63 ± 1	15 ± 5
38	4	4-COOCH₃	(*E*) $CH_2CH=CHCH_2$	68 ± 2	36 ± 2
39	4	4-COOEt	(*E*) $CH_2CH=CHCH_2$	68 ± 2	30 ± 2
40	4	4-fenyl	(*E*) $CH_2CH=CHCH_2$	13 ± 0	22 ± 0
41	4	4-benzyl	(*E*) $CH_2CH=CHCH_2$	0	14 ± 1
42	4	-	(*E*) $CH_2CH=CHCH_2$	57 ± 0	22 ± 2
43	4	4-CN	(*E*) $CH_2CH=CHCH_2$	28 ± 2	20 ± 0
44	4	4-CH₂OH	(*E*) $CH_2CH=CHCH_2$	50 ± 0	42 ± 0
45	4	4-C₂H₄SO₃H	(*E*) $CH_2CH=CHCH_2$	67 ± 2	6 ± 4
46	4	4-SCH₂COOH	(*E*) $CH_2CH=CHCH_2$	13 ± 1	6 ± 0
47	4	4-CH₃	(*E*) $CH_2CH=CHCH_2$	61 ± 1	26 ± 2
48	4	4-*t*-butyl	(*E*) $CH_2CH=CHCH_2$	52 ± 1	14 ± 1
49	4	4-N(CH₃)₂	(*E*) $CH_2CH=CHCH_2$	0	8 ± 1
50	4	4-COOH	(*E*) $CH_2CH=CHCH_2$	63 ± 1	15 ± 5
51	4	4-(NH₂)NOH	(*E*) $CH_2CH=CHCH_2$	52 ± 2	32 ± 1

case, the weak interactions (e.g. cation-π or π-π) and spatial orientation of the molecule **62** are probably essential for good reactivation ability, if compared to very similar quinolinium compound **61**. On the other hand, it is not clear, if hydrophilic or hydrophobic moiety had increased influence on reactivation of POX-AChE complex. Among reactivators with lipophilic moieties, the methyl and phenyl derivatives (**40** and **47**) exceeded the properties of *t*-butyl and benzyl (**41** and **48**) reactivators at 10 μM. In the group of hydrophilic substituted reactivators, the best results were obtained for structures with methylcarbonyl, ethylcarboxyl and hydroxymethyl functional groups (**38**, **39** and **44**).

Additionally, compounds with two quaternary pyridinium rings had better reactivation ability compared to monoquaternary reactivators (**1**, **53-54** compared to **5**, **12**, **15**, **30**, **44**, **56**, **62**).[5,33] This fact arises from cation-π interactions in the enzyme active sites. Besides this, monoquaternary reactivators significantly improved penetration through BBB compared to bisquaternary compounds, where small and only mono-charged molecules are more valuable.[34,35]

Regarding the connecting linker, an optimal length of the aliphatic linker for POX-inhibited AChE ranges from 3 to 4 methylene units (**5**, **6**, **20-21**) as was formerly reported.[19] This structural requirement may be described by spatial distance of two pyridinium moieties that are responsible for crucial cation-π interactions within the AChE active sites and thus higher affinity towards AChE. Among the reactivators with propane linker, the best results were obtained with standard reactivator **5** and **6** and with new reactivators **19-21** for concentration 1000 μM. Same reactivators performed best results for concentration 10 μM with exception of compound **19**. Reactivators with (*E*)-but-2-ene linker showed promising reactivation potency mostly for oximes **25**, **30**, **38**, **44**, **56** and **62** for both concentrations. In the group of (*Z*)-but-2-ene linked reactivators, the best results were obtained for oximes **31** and **33** for concentration 1000 μM and **33** and **35** for 10 μM concentration. (*E*)-But-2-ene linked compounds resulted as more valuable when compared to (*Z*)-but-2-ene linked reactivators (**56** and **58** to **33-34**).[31,36] The reactivation ability of the xylene linked compounds was influenced by higher linker rigidity, where xylene linker is spatially plain structure

Table 8. Reactivation potency of monoquaternary and bisquaternary oxime reactivators
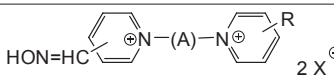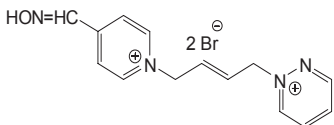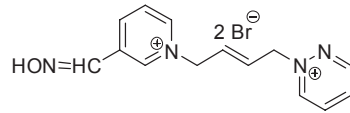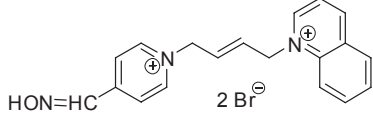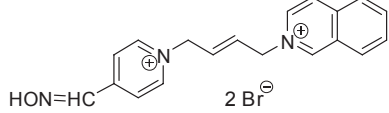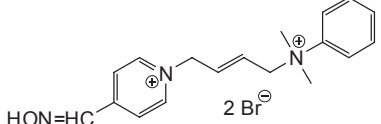
| Reactivator | HON=HC⊕N—(A)—N⊕ R, 2 X⊖ | | | | % reactivation ± SD | |
	Oxime position	R	A	X	1000 μM	10 μM
52	4-CH=NOH	4-(NH₂)NOH	(*E*) CH₂CH=CHCH₂		52 ± 2	32 ± 1
53	3-CH=NOH	-	-CH₃	I⁻	12 ± 3	11 ± 0
54	4-CH=NOH	-	-CH₃	I⁻	40 ± 0	0
55	2-CH=NOH	2-CH=NOH	(CH₂)₄	2Br⁻	32 ± 3	33 ± 2
56	4-CH=NOH	4-CH=NOH	(*E*) CH₂CH=CHCH₂	2Br⁻	60 ± 1	46 ± 2
57	2-CH=NOH	2-CH=NOH	(*E*) CH₂CH=CHCH₂	2Br⁻	0	8 ± 2
58	2-CH=NOH	3-CH=NOH	(*E*) CH₂CH=CHCH₂	2Br⁻	28 ± 2	34 ± 2

Table 9. Reactivation potency of monooxime reactivators – different heteroaromatic structures

| Reactivator | Structure | % reactivation ± SD | |
		1000 μM	10 μM
59		35 ± 6	23 ± 0
60		34 ± 0	0
61		0	15 ± 1
62		43 ± 0	48 ± 4
63		14 ± 1	30 ± 1

with limited possibilities of free rotation in comparison to aliphatic linkers (e.g. propane). Thus, the only bonds accessible for free rotation in xylene compounds are methylene junctions. These cause the non-coplanar formation of each pyridinium ring *versus* the xylene ring. Subsequently, the spatial orientation of xylene molecule is shifted, when compared to compound with aliphatic linker. In addition, better results (namely at 10 μM) for xylene reactivators were obtained for compounds with spatially more opened molecules with *m*- or *p*-xylene linker (**11**, **12**, **14** and **15**).[37] The explanation of these results consist in entering the narrow AChE active gorge (about 5 Å), where *o*-xylene linked molecules are too bulky for going inside and consequently for successful reactivation. Generally, reactivators with double bond or xylene presented better reactivation ability compared to compounds with aliphatic linkers (**7** compared to **27**, **7** compared to **12** and **15**). These differences were plausibly caused by the presence of the π-electrons in the connection chain and subsequent interactions with aromatic residues (His, Phe, Trp, Tyr) from AChE active sites.[38]

Conclusions

56 new potential AChE reactivators were tested *in vitro* and compared to the commercial compounds on the model of paraoxon-inhibited rat brain AChE. Structure-activity relationship was studied within this series compounds. More than 30 tested

reactivators showed promising results at 10 μM that is attainable concentration after *in vivo* administration. The best reactivation results were obtained for bisquarternary molecules with at least one oxime group in position 4 and with connecting linker ranging from 3 to 4 C-C bonds.

Material and Methods

All chemicals were purchased from Fluka or Sigma-Aldrich and used without further purification. Commercial and novel reactivators were synthesized in the Department of Toxicology (Faculty of Military Health Sciences, University of Defence). Their purity was verified by NMR and HPLC-MS methods. 10% rat brain homogenate was used as a source of AChE.

In vitro testing of reactivators was formerly described in detail.[17] This method was chosen instead of Ellman´s method to prevent oximolysis at higher oxime concentrations. Briefly, the 10% rat brain homogenate in distilled water was used as a source of AChE. The brain homogenate (0.5 mL) was mixed with of 1 mM isopropanol solution paraoxon (20 μL; *O,O*-diethyl-*O*-(4-nitrophenyl)phosphate, analytical standard 99.2% from Sigma-Aldrich) and distilled water (0.5 mL). The mixture was incubated at 25 $^{\circ}$C for 30 min and 95% inhibition of AChE was achieved. The mixture was filled in assay vessel to the volume 23 mL with distilled water and sodium chloride (3 M, 2.5 mL) was added. Finally, acetylcholine iodide (0.02 M, 2 mL; substrate for enzymatic reaction) was added. The enzyme activity (analyzed by potentiometric titration of decomposed acetylcholine iodide) was measured at pH 7.6 and 25 $^{\circ}$C using an autotitrator RTS 822 (Radiometer, Denmark).

The same procedure was repeated with inhibited homogenate (0.8 mL) further subjected to 10 min incubation with an aqueous solution of reactivator (0.2 ml of 1 mM or 10 μM), which replaced 0.2 mL of water. All measurements were done in triplicate. Activities of intact AChE (a_0), inhibited AChE (a_i) and reactivated AChE (a_r) were deduced from the consumption of NaOH solution (0.01 M) in time. The percentage of reactivation (%) was calculated from the measured data according to the formula:

$$ x = \left(1 - \frac{a_0 - a_r}{a_0 - a_i}\right) \cdot 100 \left[\%\right] $$

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