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New group of xylene linker-containing acetylcholinesterase reactivators as antidotes against the nerve agent cyclosarin

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Abstract
Nerve agents such as sarin, cyclosarin and tabun are organophosphorus substances able to inhibit the enzyme acetylcholinesterase (AChE; EC 3.1.1.7). AChE reactivators and anticholinergics are generally used as antidotes in the case of intoxication with these agents. None of the known AChE reactivators is able to reactivate AChE inhibited by all nerve agents used. In this work, reactivation potency of nine newly developed AChE reactivators with an incorporated xylene ring in their structure was measured *in vitro*. Cyclosarin was chosen as an appropriate member of the nerve agent family. Reactivation potency of the tested AChE reactivators was compared with the gold standard of AChE reactivators – pralidoxime. Two oximes (K107 and K108) surpassed the reactivation potency of pralidoxime. Moreover, from the obtained results it could be deduced that AChE reactivators with a functional oxime group in position-2 are the most potent AChE reactivators in the case of cyclosarin intoxications.

Keywords: *Nerve agent, cyclosarin, reactivation, acetylcholinesterase, oxime, inhibition*

Introduction

Organophosphorus compounds are needed in industry (softening agents, lubricants), veterinary and human medicine (drugs), agriculture (pesticides) etc. A special aspect of their utilization is military use as nerve agents [1]. History of nerve agents began before World War II in Germany. The first synthesized nerve agent – tabun (O-ethyl-N, N-dimethyl phosphoramidocyanidate) was synthesized in 1936 (Germany) and later, many other nerve agents such as sarin, cyclosarin, soman and VX were developed, stored and prepared for potential military use [2]. Fortunately, these substances were not used until recently in world conflicts. They were misused by Sadam Husain in Iraq in the Kurdish village of Birjinni (1988) and by the Japanese Aum Shinrikyo sect in Matsumoto (1994) and Tokyo (1995) [3,4].

Their toxic effect is based on the irreversible inhibition of an enzyme acetylcholinesterase (AChE; EC 3.1.1.7) by covalent binding of the nerve agent in its active site. Due to this inhibition, neuromediator acetylcholine accumulates at the synaptic clefts and overstimulates nerve receptors. Afterwards, cholinergic crisis occurs and in severe intoxications the affected organism could die due to respiratory failure [5].

Owing to the high toxicity of nerve agents, rapid use of antidotal treatment is necessary. Antidotal mean consists of anticholinergics (atropine mainly) to counteract muscarinic signs and AChE reactivators to liberate the free enzyme, which is again able to fullfill its physiological role in the organism [6].

Pralidoxime, obidoxime and HI-6 are currently the most used reactivators in the case of nerve agent intoxications. However, none from these is able to reactivate AChE inhibited regardless of nerve agent

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used [7]. For this reason, new AChE reactivators are still being developed [8–10].

In 2005, we prepared a new class of AChE reactivators with a xylene moiety introduced into the AChE reactivator structure. Due to the presence of the xylene linker, lipophilicity of this reactivator increased and, moreover, the rigidity in the reactivator structure increased, too. These substances were tested firstly as potential reactivators of AChE inhibited by the pesticide chlorpyrifos. The results showed that several of them were promising AChE reactivators [11].

In this work, we are interested in the *in vitro* evaluation of their potency in the case of reactivation of AChE inhibition using a real nerve agent. For this purpose, we have chosen cyclosarin as an appropriate member of the nerve agent family [12]. We have compared the potency of tested substances with pralidoxime, which is currently considered as gold standard for AChE reactivators [13].

Material and methods

Chemicals

All compounds were prepared as described earlier by Musilek et al [11]. Their structures are listed in Table I. Pralidoxime (2-hydroxyiminomethyl-1-methylpyridinium iodide) was used for comparison as the currently used AChE reactivator. Purities of the tested AChE reactivators were confirmed using TLC (DC-Alufolien Cellulose F; Merck, Germany; mobile phase BuOH-CH_3COOH-H_2O 5: 1: 2; detection by solution of Dragendorff reagent) and by NMR (Varian Gemini 300; ^1H 300 MHz, ^{13}C 75 MHz, Palo Alto CA, USA) [11,14]. The nerve agent cyclosarin (GF; O-cyclohexylmethylflourophosphate) was obtained from the Military Facility Brno (97% purity). All other chemicals used were of reagent grade (Sigma-Aldrich, Czech Republic).

Enzyme

Rat brain cholinesterase was chosen as an appropriate source of cholinesterases. Its preparation was as follows. Lightly ether-narcotized animals were killed by bleeding from a carotid artery and then the brains were removed, washed with saline and homogenized using an Ultra-Turrax homogenizer in distilled water to make a 10% homogenate (w/v). The animals used in this study were handled under the supervision of the Ethics Committee of the Medical Faculty of Charles University and the Faculty of Military Medical Academy in Hradec Kralove, Czech Republic.

In vitro *measurement*

Reactivation efficacy of the newly developed oximes was tested *in vitro* on the model of cholinesterases inhibited by cyclosarin using the standard reactivation test with electrometric instrumentation [15]. The rat brain homogenate (0.5 ml) was mixed with 0.5 ml of 0.01 μM cyclosarin solution in dry isopropanol and then incubated for 30 min at room temperature to reach 95% inhibition. Then the cyclosarin-inhibited AChE was incubated for 10 min with a solution of reactivator (1 ml) of given concentration (10^{-3} M or 10^{-5} M). Afterwards, 2.5 ml of 3M NaCl was added and the mixture diluted with distilled water to a volume of 23 ml. Then, 2 ml of 0.02 M acetylcholine bromide was added and enzyme activity was measured titrimetrically at pH 8.0 and 25 °C on an Autotitrator RTS 822 (Radiometer, Denmark).

The activities of intact (a_o), cyclosarin-inhibited (a_i) and reactivated AChE (a_r) were calculated from the slopes of the initial part of the titration curves. Each value represents the arithmetic mean from two independent measurements.

The percentage of reactivation (%) was calculated from measured data according to the formula:

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

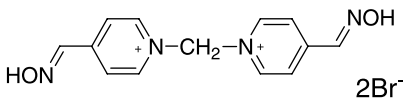
Results

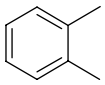
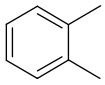
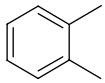
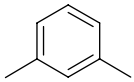
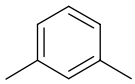
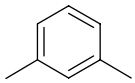
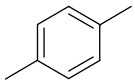
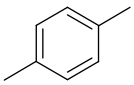
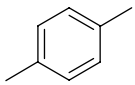
All results and the new AChE reactivators structures are summarized in Table I. Visualization of the obtained data is shown in Figure 1. As can be clearly seen, pralidoxime is not effective in the case of cyclosarin-inhibited AChE reactivation for both reactivator concentrations 10^{-5} M and 10^{-3} M. On the contrary, there are several new AChE reactivators with promising efficacy to reactivate cyclosarin-inhibited AChE; especially reactivators K107 and K108 which reached a very good reactivation potency (over 15% at concentration 10^{-5} M), which is believed to save life in the case of severe intoxications[1,16]. Other reactivators tested were able to reactivate AChE at the human safe concentration of 10^{-5} M just up to 10% (K112, K110, K113, and K114) or were ineffective (K106, K109 and K111) like pralidoxime.

Discussion

Development of new AChE reactivators is still a very important aim, due to the fact that there is no single reactivator able to reactivate all nerve agents regardless of their structure, and many laboratories throughout the world are interested in this area of research[8–10,17–20]. Pralidoxime, an AChE reactivator currently established in the USA, is generaly designated as the gold standard AChE reactivator [13], however, its potency to reactivate almost all nerve agent intoxications is poor [21–23].

Table I. Potency of newly developed acetylcholinesterase reactivators to reactivate cyclosarin-inhibited rat brain cholinesterases *in vitro*.



Name	R	Oxime position	Reactivation [%] (10^{-5} M)	Reactivation [%] (10^{-3} M)
Pralidoxime	–	2	0	0
K106		2,2	0	0
K109		3,3	3	9
K112		4,4	8	0
K107		2,2	29	0
K110		3,3	8	4
K113		4,4	7	0
K108		2,2	21	0
K111		3,3	2	0
K114		4,4	9	0

Source of the enzyme – rat brain homogenate; time of inhibition – 30 min; time of reactivation – 10 min, reactivation measured at two different reactivator concentrations – 0.00001 M and 0.001 M; pH 7.6; 25 °C

Our results confirm that pralidoxime is ineffective in the case of cyclosarin intoxications. For these purposes, new AChE reactivators are currently being synthesized [8–10].

All new AChE reactivators presented in this article were synthesized in 2005. Firstly, we have tested their in vitro potency to reactivate AChE inhibited by the pesticide chlorpyrifos and almost all these AChE reactivators (except K109, K110 and K111) had promising reactivation potency [11]. On the contrary, our new findings for the real nerve agent cyclosarin inhibition show that only two AChE reactivators (K107 and K108) seem to be promising. Moreover, their promising reactivation potency was reached at a concentration of 10^{-5} M, which is believed to be attainable in human [1,16]. Higher reactivation

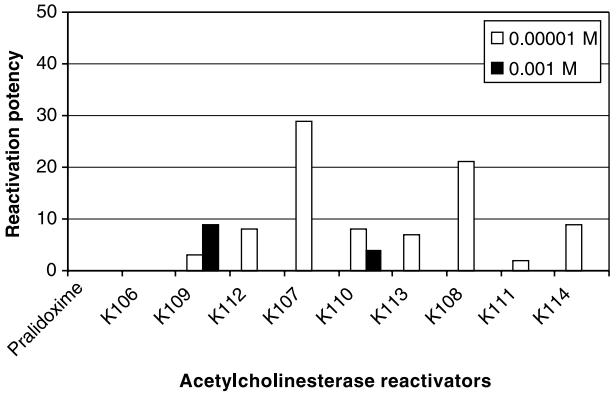


Figure 1. Efficacy of tested oximes in reactivation of cyclosarin-inhibited AChE.

potency at a lower concentration of AChE reactivators (10^{-5} M) is caused, as was described earlier in this journal, by inhibition of reactivated AChE by the reactivator itself [11].

Generally, the presence of the xylene moiety in the reactivator's structure increases the lipophilicity in comparison with currently used AChE reactivators. Possibly due to this fact, these reactivators could theoretically penetrate the human blood brain barrier (BBB) in higher amount than pralidoxime, which goes through the BBB to the extent of 10% [24].

From the structural point of view, the only promising reactivators (K107 and K108) have a functional oxime group in position-2. This result is in very good agreement with the general rule that cyclosarin-inhibited AChE is preferably reactivated by reactivators with an oxime group in position-2 of the pyridinium ring [7,25].

The third compound (K106), having an oxime group in position-2 of the pyridinium ring, was not potent due to its steric properties; the ortho-xylene linking chain probably does not enable attack on the phosphorus atom of the cyclosarin connected to the enzyme.

In conclusion, synthesis of new AChE reactivators and subsequent evaluation of their in vitro potency to reactivate nerve agent-inhibited AChE are generally first steps in the development of new AChE reactivators [26]. The obtained results shows that two AChE reactivators (K107 and K108) seem to be promising AChE reactivators which should be tested in detail using other aspects of our development process such as in vivo and neurotoxicity studies [27–29].

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References

[1] Bajgar J. Organophosphates/nerve agent poisoning: Mechanism of action, diagnosis, prophylaxis, and treatment. Adv Clin Chem 2004;38:151–216.
[2] Robinson PJ. The problem of chemical and biological warfare agents. CB weapons today. Vol 2. SIPRI: Almqvist and Wiksell; 1973.
[3] Black RM, Clarke RJ, Read RW, Reid MT. Application of gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry to the analysis of chemical warfare samples, found to contain residues of the nerve agent sarin, sulphur mustard and their degradation products. J Chromatogr A 1994;662:301–321.
[4] Bolz F, Dudonis KJ, Schulz DP. The counterterrorism Handbook – Tactics, Procedures, and Techniques. CRC Press; 2002.
[5] Marrs TC. Organophosphate poisoning. Pharmacol Ther 1993;58:51–66.
[6] Taylor P. The Pharmacological Basis of Therapeutics. McGraw Hill; 1996.
[7] Kuca K, Jun D, Musilek K. Structural requirements of acetylcholinesterase reactivators. Mini Rev Med Chem 2006;6:269–277.
[8] Kim TH, Kuca K, Jun D, Jung YS. Design and synthesis of new bis-pyridinium oximes as cyclosarin-inhibited acetylcholinesterase reactivators. Bioorg Med Chem Lett 2005;15:2914–2917.
[9] Yang GY, Yoon JH, Seong CM, Park NS, Jung YS. Synthesis of bis-pyridinium oxime antidotes using bis(methylsulfonoxymethyl) ether for organophosphate nerve agents. Bull Korean Chem Soc 2003;24:1368–1370.
[10] Picha J, Kuca K, Kivala M, Kohout M, Cabal J, Liska F. New group of monoquaternary reactivators of the acetylcholinesterase inhibited by nerve agents. J Enz Inhib Med Chem 2005;20:233–237.
[11] Musilek K, Kuca K, Jun D, Dohnal V, Dolezal M. Synthesis of the novel series of bispyridinium compounds bearing xylene linker and evaluation of their reactivation activity against chlorpyrifos-inhibited acetylcholinesterase. J Enz Inhib Med Chem 2005;20:409–415.
[12] Krejcova G, Kuca K, Sevelova L. Cyclosarin - current position of an organophosphate nerve agent. Def Sci J 2005;55:105–115.
[13] Petroianu GA, Hasan MY, Nurulain SM, Arafat K, Shafiullah M, Naseer O. Protective agents in acute high-dose organophosphate exposure: Comparison of ranitidine with pralidoxime in rats. J Appl Toxicol 2005;25:68–73.
[14] Palecek J, Cirkva V, Relich S, Slavetinska L, Kuca K, Jun D. Assignment of organophosphorus-inhibited acetylcholinesterase reactivators structures on the basis of nuclear magnetic resonance spectra. Zprav Voj Farm 2005;15:14–25 (In Czech).
[15] Kuca K, Cabal J. Evaluation of newly synthesized reactivators of the brain cholinesterase inhibited by sarin-nerve agent. Toxicol Mech Meth 2005;15:247–252.
[16] Kuca K, Kassa J. A comparison of the ability of a new byispyridinium oxime – 1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)butane dibromide and currently used oximes to reactivate nerve agent-inhibited rat brain acetylcholinesterase by in vitro methods. J Enz Inhib Med Chem 2003;18:529–535.
[17] Petroianu GA, Arafat K, Kuca K, Kassa J. Five oximes (K-27, K-33, K-48, BI-6 and methoxime) in comparison with

pralidoxime: in vitro reactivation of red blood cell acetylcholinesterase inhibitied by paraoxon. J Appl Toxicol 2006;26:64–71.

[18] Calic M, Vrdoljak AL, Radic B, Jelic D, Jun D, Kuca K, Kovarik Z. In vitro and in vivo evaluation of pyridinium oximes: Mode of interaction with acetylcholinesterase, effect on tabun- and soman-poisoned mice and their cytotoxicity. Toxicology 2006; 219:85–96.

[19] Worek F, Reiter G, Eyer P, Szinicz L. Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. Arch Toxicol 2002;76:523–529.

[20] Luo C, Leader H, Radic Z, Maxwell DM, Taylor P, Doctor BP, Saxena A. Two possible orientations of the HI-6 molecule in the reactivation of organophosphate-inhibited acetylcholinesterase. Biochem Pharmacol 2003;66:387–392.

[21] Worek F, Widmann R, Knopff O, Szinicz L. Reactivating potency of obidoxime, pralidoxime, HI 6 and HLo 7 in human erythrocyte acetylcholinesterase inhibited by highly toxic organophosphorus compounds. Arch Toxicol 1998;72:237–243.

[22] Clement JG, Erhardt N. In vitro oxime-induced reactivation of various molecular forms of soman-inhibited acetylcholinesterase in striated muscle from rat, monkey and human. Arch Toxicol 1994;68:648–655.

[23] Cabal J, Kuca K, Kassa J. Specification of the structure of oximes able to reactivate tabun inhibited acetylcholinesterase. Bas Clin Pharmacol Toxicol 2004;95:81–86.

[24] 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.

[25] Kuca K, Patocka J. Reactivation of cyclosarin-inhibited rat brain acetylcholinesterase by pyridinium-oximes. J Enz Inhib Med Chem 2004;19:39–43.

[26] Kuca K, Cabal J, Jun D, Kassa J, Bartosova L, Kunesova G, Dohnal V. Development of new acetylcholinesterase reactivators – antidotes used for treatment of nerve agent poisonings. Biomed Papers 2005;149:429–431.

[27] Bartosova L, Kuca K, Jun D, Kunesova G. Bispyridinium oximes as antidotal treatment of cyclosarin poisoning – *in vitro* and *in vivo* testing. Inter J Toxicol 2005;24:399–402.

[28] Sevelova L, Kuca K, Krejcova G. Antidotal treatment of GF-agent intoxication in mice with new bispyridinium oximes. Toxicology 2005;207:1–6.

[29] Krejcova-Kunesova G, Bartosova L, Kuca K. Signs of cyclosarin-induced neurotoxicity and its pharmacological treatment with quaternary pyridinium-oximes reactivators. Toxicology 2005;216:32–40.