In vitro Evaluation of New Acetylcholinesterase Reactivators as Casual Antidotes against Tabun and Cyclosarin

Kamil Kuca,[*] Daniel Jun, Tae-Hyuk Kim,[†] Jiri Cabal, and Young-Sik Jung[†,*]

Department of Toxicology, Faculty of Military Health Sciences, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
[*]E-mail: kucakam@pmfhk.cz
[†]Medicinal Science Division, Korea Research Institute of Chemical Technology, P.O. Box 107, Yusong, Daejeon 305-606, Korea
[*]E-mail: ysjung@krict.re.kr
Received November 1, 2005

Nerve agents (sarin, tabun, soman and VX) are class of military important substances able to cause many severe intoxications during few minutes. Currently, the threat of misuse of these agents is daily discussed. Unfortunately, there is no single antidote able to treat intoxication caused by all of these agents. Owing to this fact, new generation of antidotes, especially acetylcholinesterase (AChE; EC 3.1.1.7) reactivators, is still developed. In this study, we have tested four newly developed AChE reactivators: 1-(4-hydroxyiminomethylpyridinium)-5-(4-carbamoylpyridinium)-3-oxa-pentane dibromide (**1**), 1-(3-hydroxyiminomethylpyridinium)-5-(4-carbamoylpyridinium)-3-oxa-pentane dibromide (**2**), 1,5-bis(2-hydroxyiminomethylpyridinium)-3-oxa-pentane dichloride (**3**) and 1,5-bis(4-hydroxyiminomethylpyridinium)-3-oxa-pentane dibromide (**4**) for their potency to reactivate *in vitro* tabun and cyclosarin-inhibited AChE. Their reactivation efficacy was compared with currently the most promising oxime HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride). According to obtained results, two AChE reactivators **1** and **4** were able to reactivate tabun-inhibited AChE. On the contrary, there was no better AChE reactivator than HI-6 able to reactivate cyclosarin-inhibited AChE.

Key Words : Acetylcholinesterase, Reactivators, Antidote, Organophosphorus intoxication

Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a crucial enzyme in the human body. AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine - it terminates impulse transmission at cholinergic synapses. Nerve agents and pesticides are organophosphorus compounds with moderate (pesticides) and high (nerve agents) toxicity.[1] Their acute toxicity is based on the irreversible inhibition of the enzyme AChE and subsequent accumulation of the neuromediator acetylcholine at peripheral and central cholinergic sites.[2] The inhibitory effect of organophosphorus compounds is based on phosphorylation or phosphonylation of serine hydroxy group at the active site of the enzyme.[3]

Protection against organophosphorus intoxication consists of prophylaxis[4] and decontamination.[5] After the intoxication, there is need of immediate and appropriate treatment. For this purpose, antidotal mean consisting of anticholinergics (atropine mainly) and AChE reactivators (called oximes) is used. Pralidoxime (2-PAM, 1-methyl-2-hydroxyiminomethylpyridinium chloride), obidoxime (Toxogonine®; 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride) and HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride) are worldwide currently the most used AChE reactivators.[6] Unfortunately, none of these oximes is able to reactivate AChE inhibited by all nerve agents used. For this reason, structures of new AChE reactivators are predicted and then synthesized using standard or new synthetic approaches.[7] In this study, we were interested in evaluation of the reactivation potency of four newly synthesized AChE reactivators 1-(4-hydroxyiminomethylpyridinium)-5-(4-carbamoylpyridinium)-3-oxa-pentane dibromide (1), 1-(3-hydroxyiminomethylpyridinium)-5-(4-carbamoylpyridinium)-3-oxa-pentane dibromide (2), 1,5-bis(2-hydroxyiminomethylpyridinium)-3-oxa-pentane dichloride (3) and 1,5-bis(4-hydroxyiminomethylpyridinium)-3-oxa-pentane dibromide (4), which were synthesized earlier.[7d] Newly developed substances differ from the currently used oximes in the length of connecting chain between two pyridinium rings. We were interested in the difference of the reactivation potency of these reactivators (five atoms membered connecting chain) versus currently used oxime HI-6 (three atoms membered connection chain) because the length of the connecting chain is very important factor influencing the reactivation process and their specifity.[7b,8] Our preliminary screening test showed that all these above mentioned oximes are able to reactivate cyclosarin-inhibited AChE, however not enough in comparison with currently used reactivator HI-6.[7d]

In order to obtain some new information about the reactivation potency of our new AChE reactivators, in this work, we have tested their abilities to reactivate rat-brain AChE inhibited by tabun (GA) and cyclosarin (GF). Their reactivation potencies were compared with commonly used AChE reactivator - HI-6. Afterwards, we have discussed the relationship between the structure of AChE reactivators and their biological affinity.

Material and Methods

Chemicals. All tested compounds (**1**, **2**, **3**, **4**) were prepared earlier.[7d] HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride) purchased by Merck (Germany) was used for comparison as the currently used AChE reactivator. Purities of the tested AChE reactivators were estimated using ^{1}H-NMR spectra. Nerve agents (cyclosarin and tabun) were obtained from the Military Facility Brno (94% and 96% purity). All other chemicals used were of reagent grade (Sigma-Aldrich, Czech Republic).

Enzyme. Rat brain AChE was chosen as the appropriate source of the enzyme. Its preparation was as follows. Lightly ether-narcotized animals were killed by bleeding from a carotid artery and the brains were removed, washed with saline and homogenized in an Ultra-Turrax homogenizer in distilled water to make a 10% homogenate.

***In vitro* measurement.** Reactivation efficacy of the oximes was tested *in vitro* on the model of AChE inhibited by cyclosarin or tabun using standard reactivation test with electrometric instrumentation.[8] The AChE homogenate (0.5 mL) was mixed with 0.5 mL solution of cyclosarin or tabun (to obtain 95% inhibition) in dry isopropanol and incubated for 30 min (25 °C). Then 2.5 mL of 3 M NaCl was added and supplied by distilled water to a volume of 23 mL. After that, 2 mL of 0.02 M acetylcholine bromide was added and enzyme activity was assayed titrimetrically at pH 8.0 and 25 °C on the Autotitrator RTS 822 (Radiometer, Denmark).

The activities of intact (a_o) and GA or GF-inhibited (a_i) AChE were determined. When cyclosarin- or tabun-inhibited AChE was incubated 10 min with solution of reactivator, the activity of reactivated AChE (a_r) was obtained. The activity values a_0, a_i and a_r were calculated from the slopes of the initial part of titration curves. Each value represents arithmetic mean from two independent measurements.

Results

The kinetics of the reactivation process follows second-order kinetics and can be described schematically by the followed equation:

$$EI + R \underset{}{\overset{K_R}{\rightleftharpoons}} EIR \overset{k_R}{\rightleftharpoons} E + P$$

where EI is the phospohrylated enzyme, R the reactivator, EIR is the intermediary complex, P is the reaction product, and E is the enzyme. K_R is the dissociation constant of complex EIR and k_R is the decomposition rate of this complex. All results obtained are summarized in Table 1 and in Figures 2-3.

Tabun. Oxime HI-6 has the highest affinity (characterized by the dissociation constant K_R) towards the tabun-inhibited AChE (Table 1). Oximes **3** and **4** have almost similar affinities, and **1** has the lowest affinity towards the tabun-inhibited AChE. Unfortunately, we were not able to calculate this constant for **2** because of no ability of this oxime to reactivate tabun-inhibited AChE. Values of the first order rate constants characterizing splitting of the bond between inhibitor and enzyme (k_R) favour oximes **1** and **4**

Table 1. Parameters characterizing the reactivation process

AChE reactivator	Nerve agent	$K_R \pm$ s.d. [μM]	$k_R \pm$ s.d. [min^{-1}]	k_r [min^{-1} M^{-1}]
HI-6	Tabun	27 ± 5	0.020 ± 0.001	740
1	Tabun	209 ± 20	0.032 ± 0.001	153
2	Tabun	–*	–*	–*
3	Tabun	47 ± 7	0.017 ± 0.001	362
4	Tabun	41 ± 4	0.037 ± 0.001	787
HI-6	Cyclosarin	45 ± 6	0.141 ± 0.010	3133
1	Cyclosarin	–*	–*	–*
2	Cyclosarin	2692 ± 34	0.013 ± 0.000	5
3	Cyclosarin	66 ± 2	0.023 ± 0.000	348
4	Cyclosarin	–*	–*	–*

K_R, dissociation constant of inhibited enzyme-reactivator complex; k_R, the first order rate constant of reactivation; k_r, the second order rate constant of reactivation. *we were not able to calculate the appropriate constant due to the very low potency of the reactivator to reactivate nerve agent-inhibited AChE

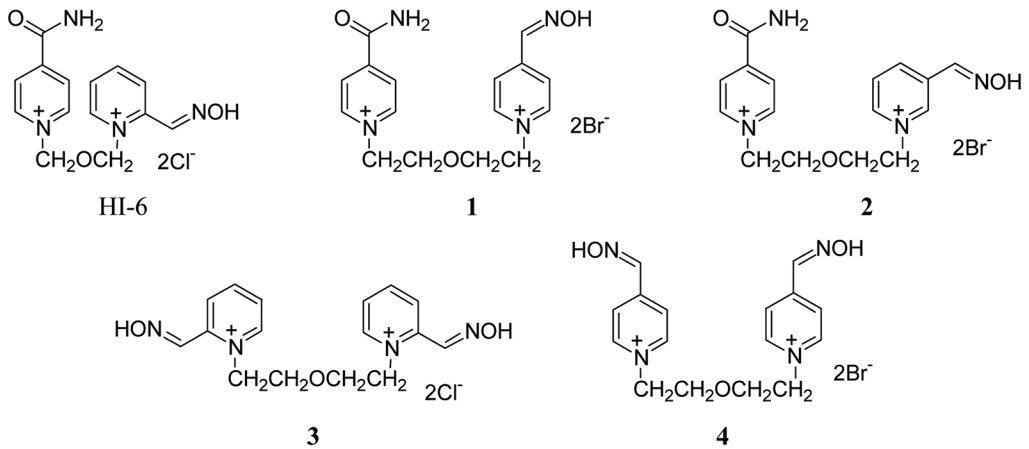

Figure 1. Structures of tested AChE reactivators.

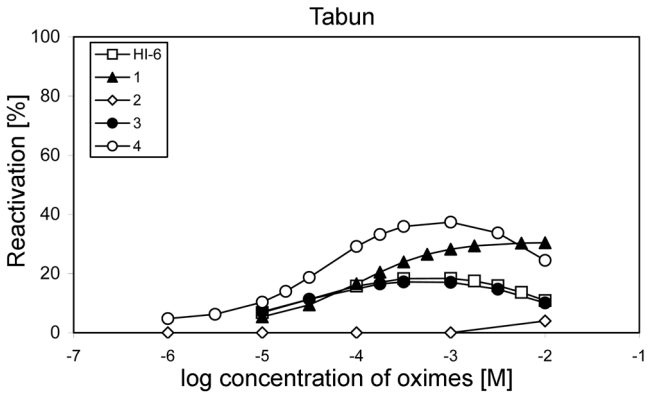

Figure 2. Concentration-reactivation relationship of oximes to tabun-inhibited AChE.

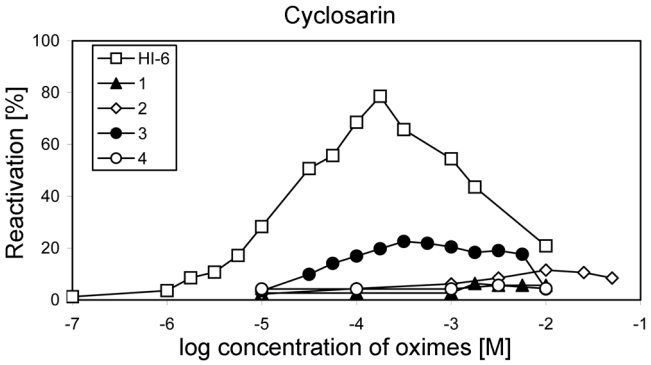

Figure 3. Concentration-reactivation relationship of oximes to cyclosarin-inhibited AChE.

prior other oximes tested. However, constants k_r (the second order rate constant, characterizing the whole reactivation process) show that the most potent AChE reactivator tested in this work is **4**. This result is also confirmed in Figure 2. As it can be seen in this figure, **4** seems to be the most potent tabun-inhibited AChE reactivator with the maximum reactivation potency at the concentration 10^{-3} M. This reactivator is able to reactivate tabun-inhibited AChE also at low concentrations 10^{-4} M (28%) and 10^{-5} M (13%), which favours this oxime for further investigation. Also **1** seems to be potent reactivator of tabun-inhibited AChE. However, its highest reactivation potency was achieved at the concentration 10^{-2} M, which is not physiologically attainable.

Cyclosarin. The highest affinity towards cyclosarin-inhibited AChE was measured for HI-6 followed by **3**. Oxime **2** had the highest value of the dissociation constant K_R and due to this fact the lowest affinity towards cyclosarin-inhibited AChE. Unfortunately, we were not able to calculate kinetic parameters for oximes **1** and **4** due to their poor ability to reactivate cyclosarin-inhibited AChE. Also constant k_R favours oxime HI-6 prior all other oximes tested. The second order rate constant k_r also confirms the highest ability of oxime HI-6 to reactivate cyclosarin-inhibited AChE.

In Figure 3, there is shown the dependence of the reactivation potency of tested oximes on their concentration. HI-6 is superior from all tested oximes. Its potency to reactivate cyclosarin-inhibited AChE is over 80% at concentration 10^{-4} M. Just **3** is able to reactivate cyclosarin-inhibited AChE over 20%. None from the other oximes surpassed 10% level of reactivation.

Discussion

Current development of prophylaxis and treatment of nerve agent intoxications is focused on cholinesterases used as scavengers.[3,4,9] Unfortunately, this approach is able to protect against just few LD_{50} of nerve agent.[9d] If the amount of nerve agent exceeds the tolerable dose, the need of AChE reactivators as the casual antidotes is still required. That is the reason that the development of new more potent AChE reactivators continues.[7,10]

In this work, we have compared in vitro potency of four new AChE reactivators (**1**, **2**, **3**, **4**) to reactivate tabun and cyclosarin-inhibited AChE. Currently the most promising oxime HI-6 was taken as appropriate AChE reactivator for comparison.

None from the oximes tested was able to reactivate both nerve agent used. Our results confirm the general rule that there is no single AChE reactivator able to sufficiently reactivate AChE inhibited by all possible nerve agents.[3b,6] Even oxime HI-6 - so potent AChE reactivator in the case of sarin, cyclosarin, VX and soman (if administered before aging) - could not be designated as broad spectrum AChE reactivator. Our present data are in a good agreement with our previous results.[9,10,11]

On the other hand, **4** seems to be the most potent reactivator of tabun-inhibited AChE. Its promising reactivation ability was achieved at the concentration 10^{-3} M. Moreover, its ability to reactivatre tabun-inhibited AChE was over 20% at concentration $10^{-4.5}$ M. This concentration could be physiologically attainable. Another promising oxime usable for treatment of tabun intoxications could be also oxime **1**. Its course of reactivation curve is almost similar to our former oximes K027 and K048.[8,10b] Although **1** has maximum reactivation potency at the concentrations 10^{-3} and 10^{-2} M (similar to oximes K027 and K048), it could be as potent reactivator *in vivo* as both oximes K027 and K048 are. To find out if this statement is true, *in vivo* investigation using standard reactivation tests should be done.[9,12]

In the case of cyclosarin reactivation, different oximes in comparison to tabun reactivation were active. HI-6 was superior to all newly synthesized oximes. This result is similar to those presented by Kassa and Cabal,[11c] and Kuca and Patocka.[13] Only **3** achived reactivation potency around 20% at relatively low, for human use achiveable concentrations.

It is known that reactivation potency of AChE reactivators depends on their chemical structure. There are several structural factors, which influence the reactivation potency-presence and number of quaternary pyridinium rings (needed for affinity towards AChE), length and rigidity of

connecting chain between two pyridinium rings, and presence and number of oxime groups (nucleophilic agent for splitting the bond between nerve agent and enzyme).[14] Our results confirm the general rule that cyclosarin inhibited AChE is very good reactivated with reactivators with oxime group at the position two and tabun-inhibited enzyme is better to reactivate with oxime group at the position four.[14] Because reactivators with oxime group at the position four are very good in reactivation of pesticides-inhibited AChE,[15] we could recommend oximes **1** and **4** for further experiments as antidotes against pesticide poisoning. Both of these oximes will be also tested in near future *in vivo* for their potency to reactivate tabun-inhibited AChE.

Acknowledgement. The authors wish to thank to Mrs. Martina Hrabinova for her skilful technical assistance. The experiments described here were carried out in compliance with the current law of Czech Republic. This work was supported by the grants of Ministry of Defence ONVLAJEP20031, OBVLAJEP20032, and from the Ministry of Science and Technology in Korea.

References

1. Patocka, J.; Kuca, K.; Jun, D. *Acta Medica (Hradec Kralove)* **2004**, *47*(4), 215-230.
2. Marrs, T. C. *Pharmacol. Ther.* **1993**, *58*, 51-66.
3. (a) Taylor, P. *Anticholinergic Agents* in *The Pharmacological Basis of Therapeutics*; Hardman, J. G.; Limbird, L. E., Eds.; McGraw Hill: New York, 1996; pp 161-176. (b) Bajgar, J. *Adv. Clin. Chem.* **2004**, *38*, 151-216.
4. Bajgar, J. *J. Med. Chem. Def.* **2004**, *1*, 1-16.
5. Cabal, J.; Kuca, K.; Sevelova, L.; Dohnal, V. *Acta Medica (Hradec Kralove)* **2004**, *47*(2), 115-118.
6. Kassa, J. *J. Toxicol. Clin. Toxicol.* **2002**, *40*, 803-816.
7. (a) Yang, G. Y.; Yoon, J. H.; Seong, C. M.; Park, N. S.; Jung, Y. S. *Bull. Korean Chem. Soc.* **2003**, *24*, 1368-70. (b) Pang, Y. P.; Kollmeyer, T. M.; Hong, F.; Lee, J. C.; Hammond, P. I.; Haugabouk, S. P.; Brimijoin, S. *Chem. Biol.* **2003**, *10*, 491-502. (c) Kuca, K.; Cabal, J.; Patocka, J.; Kassa, J. *Lett. Org. Chem.* **2004**, *1*, 84. (d) Kim, T. H.; Kuca, K.; Jun, D.; Jung, Y. S. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2914-2917. (e) Chennamaneni, S. R.; Vobalaboina, V.; Garlapati, A. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3076-3080. (f) Dohnal, V.; Kuca, K.; Jun, D. *J. Appl. Biomed.* **2005**, *3*, 139-145.
8. Kuca, K.; Kassa, J. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 529-535.
9. (a) Sevelova, L.; Bajgar, J.; Saxena, A.; Doctor, B. P. *Inhal. Toxicol.* **2004**, *16*, 531-536. (b) Sevelova, L.; Kuca, K.; Krejcova, G.; Vachek, J. *J. Appl. Biomed.* **2004**, *2*(3), 163-167. (c) Sevelova, L.; Kuca, K.; Krejcova, G. *Toxicology* **2005**, *207*, 1-6. (d) Broomfield, C. A.; Maxwell, D. M.; Solana, R. P.; Castro, C. A.; Finger, A. V.; Lenz, D. E. *J. Pharmacol. Exp. Ther.* **1991**, *259*, 633-638.
10. (a) Kuca, K.; Kassa, J. *Hum. Exp. Toxicol.* **2004**, *23*(4), 167-171. (b) Kuca, K.; Kassa, J. *Veterinary Human Toxicol.* **2004**, *46*, 15-18.
11. (a) Kassa, J.; Cabal, J. *Toxicology* **1999**, *132*, 111-118. (b) Kassa, J.; Cabal, J. *Hum. Exp. Toxicol.* **1999**, *18*, 560-565. (c) Kassa, J.; Cabal, J. *Pharmacol. Toxicol.* **1999**, *84*, 41.
12. Krejcova-Kunesova, G.; Bartosova, L.; Kuca, K. *Toxicology* **2005**, in press.
13. Kuca, K.; Patocka, J.; Cabal, J. *J. Appl. Biomed.* **2003**, *1*, 207-211.
14. Patocka, J.; Cabal, J.; Kuca, K.; Jun, D. *J. Appl. Biomed.* **2005**, *3*(2), 91-99.
15. Worek, F.; Kirchner, T.; Bäcker, M.; Szinicz, L. *Arch. Toxicol.* **1996**, *70*, 497-503.