Introduction

Sarin (GB; O-isopropylmethylfluorophosphonate), tabun (GA; O-ethyldimethylamidocyanophosphate), soman (GD; O-pinacolylmethylfluorophosphonate), cyclosarin (GF; O-cyclohexylmethylfluorophosphonate) and VX (O-ethyl-S-(2–diisopropylaminoethyl)-methylthiophosphonate) belong among the best known nerve agents (20).

These compounds cause longterm inactivation of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) by phosphorylating a serine residue at the active site of the enzyme (8,18).

The presently used antidotes against nerve agent intoxications, such as pralidoxime or obidoxime (functional antidotes) in combination with atropine (causal antidote), do not appear as suitable antidotes against poisonings with all kinds of nerve agents (6,7,9,19). For example, pralidoxime has no reactivation ability in treatment of cyclosarin and tabun-inhibited AChE (11,17). Obidoxime is not able to reactivate AChE inhibited by cyclosarin and its ability to re-activate tabun inhibited AChE is poor (1).

In this work, we are interested in the comparison of four new potential AChE reactivators – K005 (1,3-bis(2-hydroxyiminomethylpyridinium) propane dibromide), K033 (1,4-bis(2-hydroxyiminomethylpyridinium) butane dibromide), K027 (1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium) propane dibromide) and K048 (1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium) butane dibromide) as the potential reactivators of AChE inhibited by cyclosarin. Their reactivation potencies were studied using standard in vitro reactivation test. Rat brain homogenate was used as the source of the enzyme. Oxime K033 seems to be the most potent reactivator of cyclosarin-inhibited AChE. Its reactivation potency is significantly higher than the efficacy of all other tested AChE reactivators.

Material and methods

All new oximes were synthesized previously at our department (10,11,13). Cyclosarin [GF; O-cyclohexylmethyl-fluorophosphonate] of 95% purity was obtained from the Military Technical Institute (Zemianské Kostolany, Slovak Republic). All other chemicals of a reagent grade were obtained from commercial sources.
Standard in vitro method was used for the evaluation of reactivation potencies of these new drugs (14,15). General conditions were taken as follows. Rat brain homogenate diluted in distilled water (10%, w/v) was used as a source of AChE. Measurement was taken at 25 °C, pH 8, and concentration of the AChE reactivators in the scale from 10⁻⁸ to 10⁻² M. The activity of AChE was determined by pH static titration of acetic acid released from acetylcholine iodide using the autotitrator (Copenhagen, Danmark).

**Results**

**Affinity to the acetylcholinesterase**

Values of the constant $K_{DIS}$ characterizing the affinity of newly synthesized oximes to the intact AChE are shown in Table 1. As lower its value is as higher the affinity of tested oximes towards the AChE is. As indicated in the Table 1, oxime K005 has the lowest value of the constant $K_{DIS}$. Due to this fact, its affinity towards the enzyme is the highest among all the tested oximes. Value of the affinity of the oxime K033 is comparable with that of oxime K005.

**Tab. 1:** Affinity of the tested oximes towards acetylcholinesterase.

| Oxime | $K_{DIS}$ [µM] |
|-------|---------------|
| K005  | 53            |
| K033  | 65            |
| K027  | 5888          |
| K048  | 228           |

**Reactivation of the acetylcholinesterase**

Kinetics parameters (dissociation constant: $K_R$ and rate constants: $k_R$ and $k_r$) shown in Table 2 characterize the ability of the new oximes to reactivate cyclosarin-inhibited AChE in vitro. We were not able to measure rate constants of the compounds K027 and K048, due to the very low ability of these oximes reactivate cyclosarin-inhibited AChE.

The values of the constant $K_R$ characterizing the affinity of oximes to cyclosarin-inhibited AChE indicate that the affinity of the compound K005 to the enzyme-inhibitor complex is the highest in the comparison to all new oximes tested.

**Tab. 2:** Kinetic constants of the reactivation potency of tested oximes.

| Oxime | $K_R$ [µM] | $k_R$ [min⁻¹] | $k_r$ [M⁻¹.min⁻¹] |
|-------|------------|---------------|---------------------|
| K005  | 5          | 0.010         | 2196               |
| K033  | 20         | 0.095         | 4872               |
| K027  | -          | -             | -                  |
| K048  | -          | -             | -                  |

(-) = not measurable values

The values of the constant $k_r$ express the breakdown of the intermediate complex. The highest value of this constant was obtained for oxime K033. The values of this constant decrease as follows: K033 > K005 > K027 = K048.

Oxime K033 has the highest bimolecular constant of reactivation ($k_r$) representing overall reactivation ability.

The potency of tested oximes to reactivate cyclosarin-inhibited AChE is demonstrated in Figure 2.

In the case of oxime K033, 46% reactivation of cyclosarin-inhibited AChE was obtained at the concentration 10⁻⁴ M. Maximal reactivation potency for the oxime K005 (9 %) was obtained at the same concentration of the reactivators. Oxime K048 reached the maximum reactivation potency 11 % at the concentration 10⁻² M. Unfortunately, this concentration is not suitable for human use. Oxime K027 was not able to reactivate AChE inhibited by cyclosarin. In the case of human relevant concentration [10⁻⁵], the percentage of reactivation of cyclosarin-inhibited AChE does not reach 30% regardless of the oxime used.

According to our evaluation of all kinetic constants and concentration-reactivation relationship, we can confirm that the best reactivator from the new oximes seems to be oxime K033.

**Fig. 2:** Concentration versus reactivity relationship of the tested oximes.

**Discussion**

Thanks to the fact, that currently used AChE reactivators do not reactivate AChE inhibited by all kinds of nerve agents, searching for new AChE reactivator, which could reactivate AChE inhibited by more nerve agents, is needed. In our work, we have presented reactivation potency of new bisquaternary pyridinium AChE reactivators – K005, K033, K027 and K048 in cyclosarin intoxications. Although, this work presents just in vitro experiments our results are important because of the close relationship between in vitro and in vivo results (4,5).
It is very difficult to find an ideal broad-spectrum reactivator of nerve agents-inhibited AChE. It should have the value of $K_R$ from 10 to 100 times lower than $K_m$ of the native substrate ($K_m$ for AChE + acetylcholine iodide is 200 $\mu$M) and the value of $K_{DIS}$ from 10 to 100 times higher than $K_m$ (3.4). Thus, the affinity of the AChE reactivator to the inhibited enzyme should be higher than the affinity to the reactivated, respectively, intact enzyme. Both oximes K005 and K033 fulfil this rule.

In the case of the oximes K005 and K033, the rate constant $k_R$ is not affected by pKa of these oximes because of their similar values of this constant (K005 – 7.8; K033 – 7.7). Their differences (rate constant $k_R$) are caused just thanks to the length of the connecting chain between both pyridinium rings.

Our results presented in this work confirm the fact that the reactivation of inhibited AChE depends not only on the inhibitors but also on the chemical structure of the reacti-vators (10). As you can notice in Figure 1, chemical structures of the AChE reactivators K005 and K033 differ in the symmetry from the AChE reactivators K027 and K048. Bisquaternary symmetric pyridinium aldoximes K005 and K033 with functional aldoxime groups at both pyridinium rings are better reactivators of AChE inhibited by cyclosar-in. On the other hand, asymmetric AChE reactivators K027 and K048 are not able to reactivate AChE inhibited by this nerve agent. As published earlier, these AChE reactivators (K027 and K048) are potent reactivators of AChE inhibited by tabun and sarin (11,15).

Our data confirm earlier mentioned results presented by Dohnal et al. that higher symmetry of the AChE reactivators increases reactivation ability of the oximes (2). These results are in good agreement with work presented by Kassa and Cabal (3). They have evaluated MMC-4 (1,1-bis-(4-hydroxyiminomethylpyridinium) methane dibromide), symmetric bisquaternary pyridinium salt with aldoxime groups at the position four, as the best oxime for the reactivation of AChE inhibited by cyclosarin-inhibited AChE. K027 and K048 are not able to reactivate AChE inhibited by this nerve agent. As published earlier, these AChE reactivators (K027 and K048) are potent reactivators of AChE inhibited by tabun and sarin (11,15).

In conclusion, we have tested in vitro four new AChE reactivators (K005, K033, K027 and K048). Their reactivation potency was studied using cyclosarin-inhibited AChE. K033 seems to be promising reactivator of cyclosarin-inhibited AChE. Its reactivation potency is significantly higher than the efficacy of all other tested AChE reactivators. Thanks to the high reactivation potency of this AChE-reactivator, oxime K033 will be soon evaluated in vivo using standard reactivation test (19).

Acknowledgement

The authors are grateful to Mrs. I. Ježková and Mrs. M. Hrabinová for their skilful technical assistance. This work was supported by the grants of Ministry of Defense No. OBVLAJEP20032.

References

1. Cabal J, Bajgar J. Tabun - Reappearance 50 years later. Chem Listy 1999;93: 27-9. (in Czech)
2. Dohnal V, Kuca K, Havel J. Cabal J. Prediction of novel structures of efficient re-activators of cyclosarin-inhibited acetylcholinesterase using method of artificial neural networks. LBILICE 2004 - Progresses in organic, bioorganic and phar-maceutic chemistry, Nymburk 28-30, 11. 2003. Poster presentation.
3. Kassa J, Cabal J. A comparison of the efficiency of acetylcholinesterase reactiva-tors against cyclohexyl methylphosphonofluoridate (GF agent) by in vitro and in vivo methods. Pharmacol Toxicol 1999;84:41-5.
4. Kassa J, Cabal J. A comparison of the efficiency of a new asymmetric bispyridinium oxime by currently available oximes and H oximes against soman by in vitro and in vivo methods. Hum Exp Toxicol 1999;18:560-5.
5. Kassa J, Cabal J. A comparison of the efficiency of a new asymmetric bispyridinium oxime Bl-6 with presently used oximes and H oximes against sarin by in vitro and in vivo methods. Hum Exp Toxicol 1999;18:560-5.
6. Kassa J, Krejčová G. Neuroprotective Effects of Currently Used Antidotes in Tabun-Poisoned Rats. Pharmacology and Toxicology 2003;92:258-64.
7. Kassa J, Krejčová G, Samsalý J. A comparison of the neuroprotective efficiency of pharmacological pretreatment and antidotal treatment in soman-poisoned rats. Acta Med (Hradec Králové) 2003;46(3):101-7.
8. Kassa J, Krozová Ž, Ševelová L, Sheshko V, Kasalová I, Neubauerová V. Low-le- vel sarin-induced alteration of immune system reaction in inbred BALB/c mice. Toxicology 2003;187:195-203.
9. Krejčová G, Kassa J. Neuroprotective efficacy of pharmacological pretreatment and antidotal treatment in tabun-poisoned rats. Toxicology 2003;185:128-39.
10. Kuča K, Biełavský J, Cabal J, Biełavavá M. Synthesis of a potentisot of a potent ef-ficient acetylcholinesterase - 1-(4-hydroxyiminomethylpyridinium)-3-(carbamoylpyridinium)-propene dibromide. Tetrahedron Lett 2003;44:3123-5.
11. Kuča K, Biełavský J, Cabal J, Kassa J. Synthesis of a new reactivator of tabun-in- hibited acetylcholinesterase. Biorg Med Chem Lett 2003;13:3545-7.
12. Kuča K, Cabal J, Kassa J. A comparison of the efficacy of a bispyridinium oxime - 1,4-bis-(2-hydroxyiminomethylpyridinium) butane dibromide and currently used oximes to reactivate sarin-inhibited acetylcholinesterase by in vitro methods. Pharmacazie 2004 In press.
13. Kuča K, Cabal J, Patocka J. Kassa J. Synthesis of bisquaternary symmetric - p, p- bis(2-hydroxyiminomethylpyridinium)alkane dibromides and their reactivation of cyclosarin-inhibited acetylcholinesterase. Lett Org Chem 2004;1:84-6.
14. Kuča K, Dohnal V. 1,3-bis-(2-hydroxyiminomethylpyridinium) propane as the poten-tial reactivator of the acetylcholinesterase inhibited by nerve agents. Acta Med (Hradec Králové) 2004, In press.
15. Kuča K, Kassa J. A comparison of the ability of a new bispyridinium oxime - 1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)butane dibro-mide and currently used oximes to reactivate nerve agent-inhibited rat brain acetylcholinesterase by in vitro methods. J Enzyme Inhib Med Chem 2003;18: 529-35.
16. Kuča K, Kassa J. In Vitro reactivation of acetylcholinesterase using the oxime K027. Vet Hum Toxicol 2004;46:15-18.
17. Kuča K, Patocka J, Kassa J. Reactivation of cyclosarin-inhibited rat brain acetylcholine-sterase by pyridinium-oximes. J Enzyme Inhib Med Chem 2004;19:39-43.
18. Kuča K, Patocka J, Cabal J. Reactivation of organophosphate inhibited acetylcholinesterase activity by t-orbi(4-hydroxyiminomethylpyridinium)alkanes in vitro. J Appl Biomed 2003;1:207-11.
19. Ševelová L, Vacek J. Effect of methoxime combined with anticholinergic, anti-convulsant or anti-HCN drugs in tabun-poisoned mice. Acta Medica (Hradec Králové) 2003;46:109-12.
20. Zajtchuk R, Bellamy RF. Medical aspects of chemical and biological warfare. Office of the Surgeon General, Department of the Army, United states of America, 1997.

Submitted February 2004. Accepted April 2004.

Ing. Kamil Kuča,
Purkyně Military Medical Academy,
Department of Toxicology,
Třešťská 1575, 500 01 Hradec Králové,
Czech Republic.
e-mail: kucakam@pmfhk.cz