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ORIGINAL ARTICLE

Synthesis of the three monopyridinium oximes and evaluation of their potency to reactivate acetylcholinesterase inhibited by nerve agents

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Summary
Three potential reactivators of nerve agents-inhibited acetylcholinesterase: 2-[(hydroxyimino)phenylmethyl]-1-methylpyridinium iodide **3a**, 2-[(hydroxyimino)pyridin-2-ylmethyl]-1-methylpyridinium iodide **3b** and 2-[(1-hydroxyimino) ethyl]-1-methylpyridinium iodide **3c** were synthesized. Their reactivation potency was examined using a standard in vitro reactivation test. A rat brain homogenate was used as the source of acetylcholinesterase. Their reactivation potency was compared with a currently used acetylcholinesterase reactivator – 2-PAM (pralidoxime) **4**. All tested reactivators were less effective acetylcholinesterase reactivators compared to 2-PAM. In this study, we also tested the reactivation potency of the oxime 2-PAM against inhibition of acetylcholinesterase by sarin, cyclosarin, VX and tabun. Satisfactory results are shown only for the reactivation sarin- and VX-inhibited acetylcholinesterase.

Keywords: VX – reactivation – acetylcholinesterase – oximes – sarin – tabun – cyclosarin

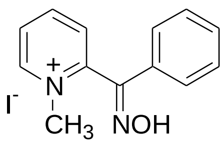
INTRODUCTION

Organophosphate (OP) compounds have largely been used as pesticides in many parts of the world and were also applied as chemical warfare agents (Worek et al. 1999, Thiermann et al. 1999). In the past, they were also misused in a terroristic attack in Tokyo city (Maekawa 1995). Therefore, the threat of intoxication with OPs is relatively high. The toxic effect of these substances is based on phosphonylation or phosphorylation of the hydroxy group in serine at the so-called esteratic site of the active center of the enzyme. Acetylcholinesterase (AChE) plays an important physiological role in the cholinergic nervous system and, therefore, its inhibition is a life-endangering factor (Marrs 1993, Taylor 1996).

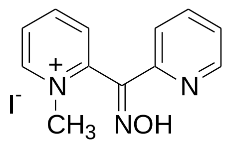
Oximes such as 2-PAM, obidoxime and HI-6 are active in the prevention and treatment of nerve agent poisonings (Bajgar 1994, Kassa 2002). Although the oxime HI-6 is currently regarded to be the most promising reactivator of inhibited AChE (Kassa and Bajgar 1995, Worek et al. 1998, Kuča and Cabal 2002), many laboratories throughout the world have decided to synthesize new reactivators of inhibited AChE (Patočka et al. 1970 , Bielavský et al. 1997, Kuča et al. 2003a, Kuča et al. 2003b).

The reactivation potency of the currently used reactivators depends on many factors. The number of pyridinium rings, the length and shape of the connecting chain between pyridinium rings, the number and position of the oxime groups at the pyridinium rings are among the main factors (Petrova et al. 2001).

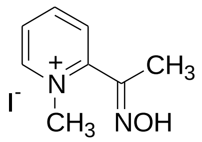
In our work, we decided to synthesize three monoquaternary ketoximes –2-[(hydroxyimino)phenylmethyl]-1-methylpyridinium iodide **3a**, 2-[(hydroxyimino)pyridin-2-ylmethyl]-1-methylpyridinium iodide **3b** and 2-[(1-hydroxyimino) ethyl]-1-methylpyridinium iodide **3c**.



3a



3b



3c

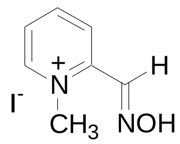


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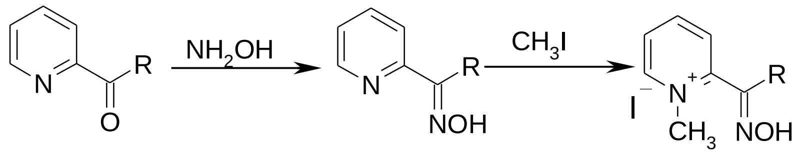
Fig. 1. **Structures of the tested oximes**

These compounds differ from currently used reactivators of AChE in the type of oxime group. The aldoxime group is replaced by ketoxime group. Phenyl, pyridin-2-yl or methyl group are used as the side chain in the ketoxime. Thanks to the presence of these moieties, we expected either higher or lower reactivation potency of the tested oximes to reactivate AChE inhibited by nerve agents. Pralidoxime **4** (2-PAM; 1-methyl-2-hydroxyiminomethylpyridinium chloride) was chosen as the reactivator for comparison of their reactivation potency.



5a,b,c,d *2a,b,c,d* *3a,b,c,4*

In formulae	**2a, 3a,5a**	R = phenyl
	2b,3b, 5b	R = pyridin-2-yl
	2c, 3c, 5c	R = CH_3
	2d, 5d, **4**	R = H

Fig. 2. **Synthetic part**

MATERIAL AND METHODS

Synthetic part

Methyl(pyridine-2-yl)ketone **5a**, phenyl(pyridine-2-yl)ketone **5b** , di(pyridine-2-yl)ketone **5c**, pyridine-2-carboxaldehyde **5d** and methyl iodide are products of Aldrich.

Ketoximes **2a-c** and aldoxime **4** were prepared from corresponding ketones **5a-c** and aldehyde **5d** using the procedure described by Hampl (Hampl et al. 1995). Quaternary pyridinium ketoximes **3a-c** and aldoxime **4** were prepared by heating **2a-d** with methyl iodide (5 molar excess) in methanol (Scheme 1). Evaporation of the solvent under reduced pressure afforded crude products which were purified by crystallization from ethanol-ether. Melting points of compounds **3a**, **3b**, **3c**, **4** correspond with those reported in the literature (Ginsburg and Wilson 1957, Daroszewski et al. 1986, Hampl et al. 1995).

Biochemical part

The reactivation efficacy of the tested oximes has been assayed in vitro on a model of AChE inhibited by VX, sarin, cyclosarin and tabun using a standard reactivation test (Kassa and Cabal 1999a, Kassa and Cabal 1999b). As a source of AChE, a homogenate of rat brains (rats of Wistar strain) and of individuals weighing 200-240 g without sex preference was used. The animals were killed in ether narcosis by cutting the carotids, the brains were removed, rinsed in physiological saline and homogenized in an Ultra-Turrax (Germany) homogenizer in distilled water to make a 10% homogenate. Activity of the inhibited enzyme: the AChE homogenate (0.5 ml) was mixed with 20 μl of 10^{-5} M solution of nerve agent (in dry isopropylalcohol) and incubated at 25 °C for 30 min. Then, 2.5 ml of 3M NaCl was added and filled with distilled water to a final volume of 23 ml. Then, 2 ml of 0.02 M acetylcholine bromide was added and the enzyme activity was assayed titrimetrically at pH 8.0 and 25 °C using an Autotitrator RTS 822 (Radiometer, Copenhagen).

The activity of non-inhibited enzyme was measured in the same way (without the nerve agent).

Reactivation of the enzyme inhibited by nerve agents was performed immediately after the inhibition (as above). A solution of the reactivator of given concentration (concentration range from 10^{-6} to 10^{-1}M; 1.0 ml) was added to the enzyme and, immediately afterwards, the activity of the reactivated enzyme was determined using the same method as described in the previous experiments.

Table 1. Kinetic constants of the reactivation of VX-inhibited AChE

Oxime	K_R [μM]	k_R [min^{-1}]	k_r [min-1M^{-1}]
4	127	0.047	370
3a	120	0.012	103
3b	1060	0.006	6
3c	940	0.010	11

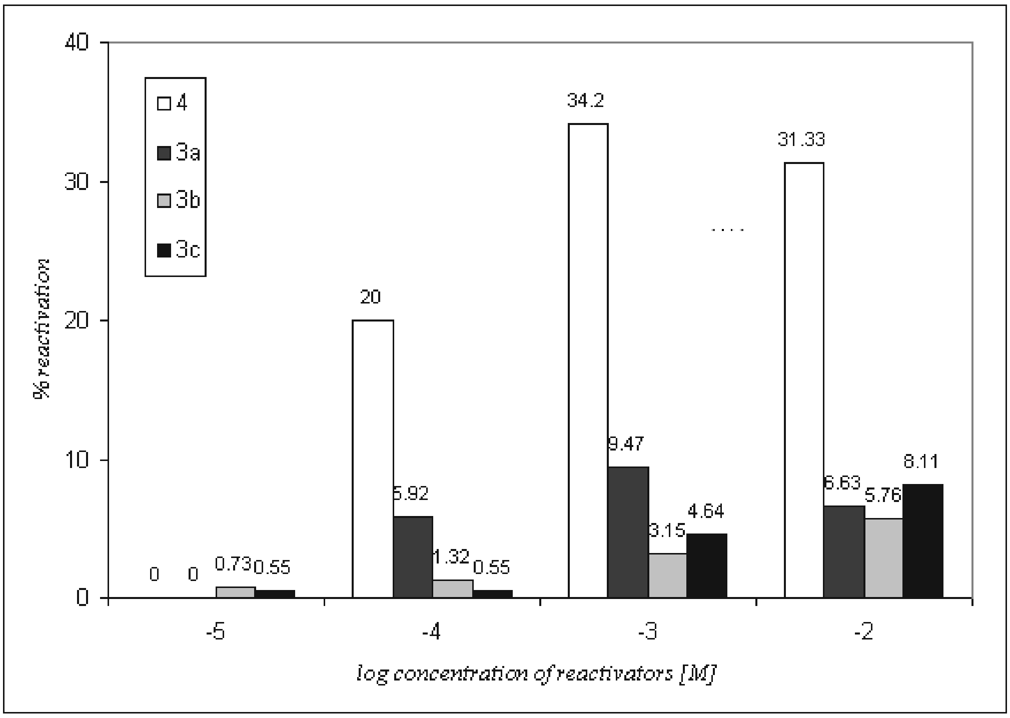


Fig. 3. **Concentration-reactivation relationship of oximes to VX-inhibited AChE**

RESULTS AND DISCUSSION

The reactivation potency of the tested oximes is characterized by several constants. Dissociation constant K_R describes a creation of the reactivator-inhibited enzyme complex. Compounds with lower values of this constant have higher affinity to the inhibited enzyme.

Rate constants k_R and k_r describe a velocity of the reactivation of inhibited AChE. The first order rate constant k_R characterizes the creation of the inhhibited enzyme-reactivator complex. Second order rate constant k_r characterizes the velocity of the overall reaction and it is derived from the equation (Patočka et al. 1970):

$$k_r = k_R / K_R.$$

Newly synthesized oximes were able to reactivate VX-inhibited AChE only. Kinetic constants of the reactivation of VX-inhibited AChE are shown in the Table 1.

The oxime **3a** has a dissociation constant K_R comparable with the oxime **4** which was used as compound for comparison of reactivation ability. The other two oximes have ten times lower affinity to the inhibited AChE.

The first order constant k_R is the highest for the oxime **4**. Oximes **3a** and **3c** have values of this constant four times lower and oxime **3b** about eight times lower. The value of the second order rate constant k_r which describes the velocity of the overall reaction is also the highest for oxime **4**. The rate constants for the other oximes decrease in the slope **3a** > **3b** > **3c**.

The ability of studied oximes to reactivate VX-inhibited AChE is shown in Figure 2.

All tested reactivators were able to reactivate VX-inhibited AChE. The best reactivator of VX-inhibited AChE was again 2-PAM. 10^{-3} M concentration of the 2-PAM is necessary to reach 34% reactivation of VX-inhibited AChE. Other tested oximes were not able reach more than 10% reactivation potency in the concentration range from 10^{-5} to 10^{-2} M.

We have also tested the reactivation potency of the newly synthesized oximes against sarin-, cyclosarin- and tabun-inhibited AChE. None of the tested reactivators (except the oxime 2-PAM, **4**) was able to satisfactorily reactivate inhibited AChE. Their reactivation constants could not be measured. On the other hand, the reactivation constants for oxime 2-PAM (**4**) were calculated and are written in the Table 2. The reactivation constants for all used nerve agents are compared in this table.

Table 2. **Kinetic constants for 2-PAM**

Nerve agents	K_R [μM]	k_R[min^{-1}]	k_r[min-1M^{-1}]
Sarin	354	0.140	403
Cyclosarin	12000	0.04	3.33
VX	127	0.047	370
Tabun	575	0.006	10

The affinity of the oxime 2-PAM (**4**) to the inhibited AChE is the highest for AChE inhibited by VX and sarin. The described results were expected because of the same electron effect of the phosphorylated enzyme, which differs only in one methyl group [EtO- by VX, iPrO- by sarin] (Cabal 1992). On the other hand, the lowest affinity to the cyclosarin-inhibited AChE was demonstrated, because of the presence of a sterically large cyclohexyl group.

The first order rate constant is the highest in the case of the reactivation of sarin-inhibited AChE. The lowest value of this constant (23 times lower in comparison with sarin-inhibited AChE) is calculated for tabun-inhibited AChE. The differences of the values of the first order rate constant (k_R) depend on the type of difficulties with nucleophilic attack (Wilson and Sondheimer 1957). The low values of k_R for tabun-inhibited AChE, demonstrated in this paper, were expected (Cabal and Bajgar 1999).

The second order rate constants k_r for the overall reaction favour 2-PAM (**4**) for reactivation of sarin and VX-inhibited AChE. This fact confirms the above mentioned rule, which describes the same electron effect of the phosphorylated enzyme (Cabal 1992).

The concentration-reactivation relationship of 2-PAM (**4**) to sarin-, cyclosarin-, VX- and tabun-inhibited AChE is showed in Figure 3.

The oxime 2-PAM (**4**) is able to reactivate AChE inhibited by all tested nerve agents except tabun. This efficacious reactivation ability was demonstrated at the concentration between 10^{-3} and 10^{-2} M. Nevertheless, this is not a concentration acceptable for human use. So that, 2-PAM (**4**) can be only used for reactivation of AChE inhibited by sarin and VX.

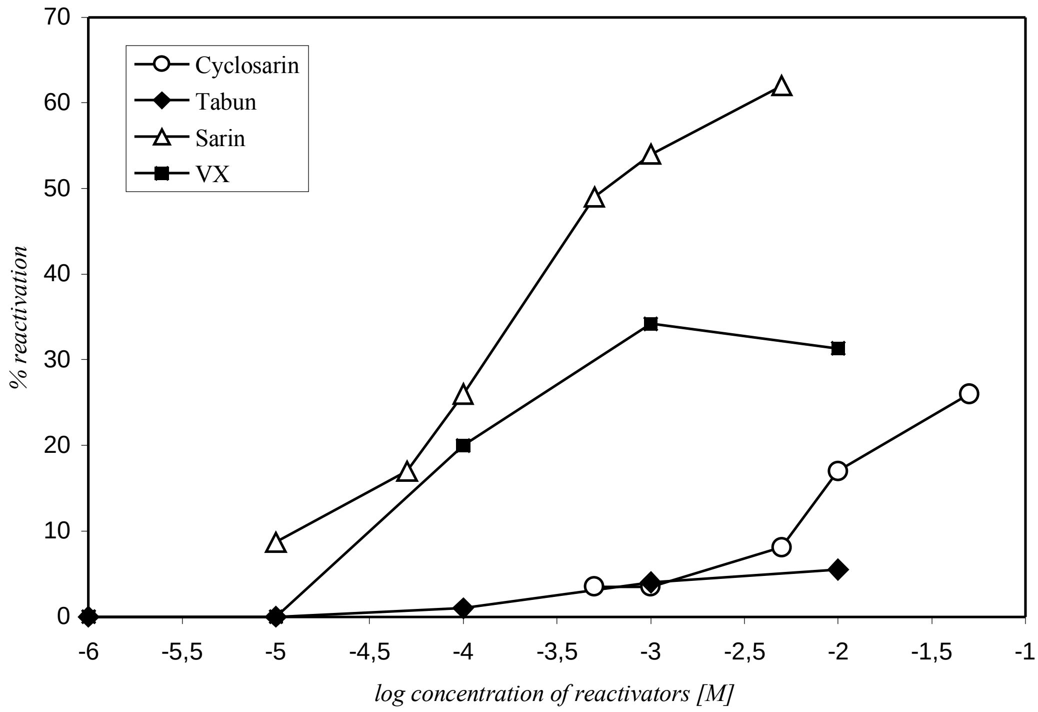


Fig. 4. **Concentration-reactivation relationship of oxime 2-PAM to OPs-inhibited AChE**

In conclusion, we have synthesized three potential reactivators of nerve agents-inhibited AChE. Their reactivation ability was compared to 2-PAM (**4**) using the standard reactivation method. All three tested reactivators were able to reactivate VX-inhibited AChE only. None of the new AChE reactivators was able to reactivate nerve agents inhibited AChE better than 2-PAM. The reason for this too low reactivation potency of the new synthesized AChE reactivators is probably the presence of the ketoxime group instead of the aldoxime group. The aldoxime group is currently the most prefered functional group of the AChE reactivators (Kassa 2002). The reactivation potency of the newly synthesized oximes is increased due to the sterical demand of the second part of the ketoxime group (in our case methyl, phenyl, pyridinyl). On the other hand, currently used reactivators (2-PAM, obidoxime or HI-6) have the aldoxime group as the functional group. In the case of the aldoxime group, there is only hydrogen in place of methyl, phenyl and pyridinyl. Its sterical demand factor is almost negligible in comparison with methyl, phenyl and pyridinyl.

We also compared the reactivation potency of the oxime 2-PAM (**4**) against sarin-, cyclosarin-, VX- and tabun-inhibited AChE. 2-PAM (**4**) was able to satisfactorily reactivate sarin- and VX-inhibited AChE. Its very low reactivation ability in the tabun and cyclosarin intoxications is well known (Kuča and Cabal 2002, Kuča et al. 2003b).

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