Purkyně Military Medical Academy, Department of Toxicology, Hradec Králové, Czech Republic

A comparison of the efficacy of a bispyridinium oxime – 1,4-bis-(2-hydroxyiminomethylpyridinium) butane dibromide and currently used oximes to reactivate sarin, tabun or cyclosarin-inhibited acetylcholinesterase by *in vitro* methods

K. KUČA, J. CABAL, J. KASSA

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Kamil KUČA, Třebešská 1575, Purkyně Military Medical Academy, 50001 Hradec Králové, Czech Republic
kucakam@pmfhk.cz

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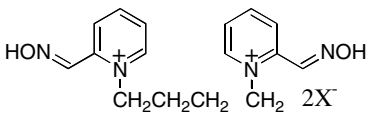
The efficacy of a bispyridinium oxime 1,4-bis(2-hydroxyiminomethylpyridinium) butane dibromide, called K033, and of currently used oximes (pralidoxime, obidoxime, oxime HI-6), to reactivate acetylcholinesterase inhibited by various nerve agents (sarin, tabun cyclosarin) was tested by *in vitro* methods. The new oxime K033 was found to be a more efficacious reactivator of sarin or cyclosarin-inhibited acetylcholinesterase than pralidoxime and obidoxime but it did not reach the efficacy of oxime HI-6 in the case of the inhibition of acetylcholinesterase by sarin or cyclosarin. On the other hand, oxime K033 was more efficacious than oxime HI-6 in reactivating tabun-inhibited acetylcholinesterase. Thus, oxime K033 seems to be a relatively efficacious broad spectrum acetylcholinesterase reactivator and, therefore, could be useful if no information about the type of nerve agent used was available.

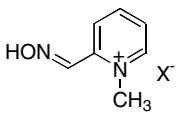
1. Introduction

The highly toxic organophosphorus compounds known as nerve agents (sarin, soman, tabun, cyclosarin or agent VX) are among the most dangerous chemical warfare agents. Their acute toxicity is based on the irreversible inhibition of the enzyme acetylcholinesterase (AChE; EC 3.1.1.7) and subsequent accumulation of the neuromediator acetylcholine at peripheral and central cholinergic sites. AChE plays a key role in the physiological function of the cholinergic nervous system and, therefore, its inhibition is a life-endangering factor. The inhibitory effect is based on phosphorylation or phosphonylation of the serine hydroxy group at the esteratic site of the active site of the enzyme (Marrs 1996).

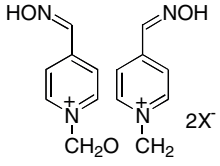
The antidotal treatment of acute poisoning with nerve agents is based on the administration of anticholinergic drugs to antagonize the overstimulation of cholinergic receptors caused by accumulated acetylcholine and acetylcholinesterase reactivators to reactivate phosphonylated or phosphorylated AChE. According to experimental data, quaternary pyridinium aldoximes have been found to be the most promising for reactivating nerve agent-inhibited AChE (Kassa 2002). Monoquaternary pralidoxime (2-PAM, 2-hydroxyiminomethyl-1-methylpyridinium chloride) or more extended bisquaternary compounds such as obidoxime (toxogonine, 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride) and H-oxime HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridi-



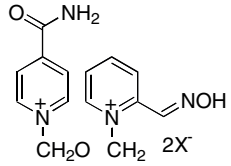
K033



2-PAM



Obidoxime



HI-6

nium)-2-oxa-propane dichloride) are particular representatives of these aldoximes (Kuča et al. 2003a).
Unfortunately, currently used oximes are not sufficiently effective against some nerve agents. They are not able to reactivate nerve agent-inhibited AChE regardless of the type of nerve agent used (Kuča et al. 2003b; Bajgar et al. 1994; Koplovitz and Stewart 1994). While pralidoxime and obidoxime have very low potency to reactivate soman and cyclosarin-inhibited AChE (Kassa and Cabal 1999a, b), the oxime HI-6 seems to be practically ineffective to reactivate tabun-inhibited AChE (Worek et al. 1998). Therefore, finding another bispyridinium oxime able to sufficiently reactivate nerve agent-inhibited AChE regardless of the type of nerve agent is still a very important task for medicine, in order to improve the efficacy of antidotal treatment of acute poisonings with nerve agents. The present study compares the reactivating efficacy of currently used oximes (pralidoxime, obidoxime, HI-6) and a new symmetric bisquaternary oxime, called K033 (1,4-bis(2-hydroxyiminomethyl-pyridinium) butane dibromide), synthesized at our Department of Toxicology (Kuča et al. 2004), against AChE inhibited by selected nerve agents (tabun, sarin, cyclosarin) by *in vitro* methods.

2. Investigations and results

The ability of the tested monopyridinium and bispyridinium oximes to reactivate AChE inhibited by chosen nerve agents *in vitro* (Kuča and Kassa 2003), characterized by percentage of reactivation and kinetic parameters, is summarized in the Table and Fig. 1–3.
As can be seen in Fig. 1–3, all the reactivation curves are bell-shaped. According to these results, we can divide the reactivation curves into two parts. In the first part, general linear dependence of the reactivation ability on the dose of the reactivator used is demonstrated. The second decreasing part of the curves is caused by the inhibiting effect of an excessive amount of an oxime.
The ability of all the oximes studied to reactivate cyclosarin-inhibited AChE *in vitro* is demonstrated by the kinetic parameters in the Table and by the concentration-reactivation relationship in Fig. 1. The values of the dissociation constant (K_{dis}) indicating the affinity of oximes towards non-inhibited AChE show that both K033 and oxime HI-6 have a higher affinity to the intact enzyme than the commonly used obidoxime and pralidoxime. The dissociation constant K_R, which characterizes the affinity of oximes to the inhibited AChE (inhibitor-enzyme complex), indicates

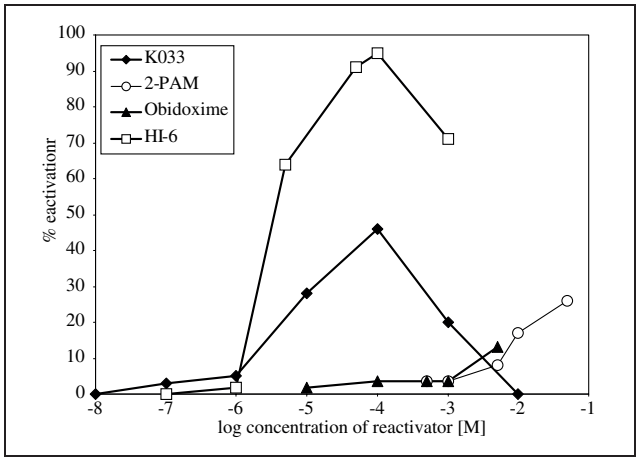


Fig. 1: Reactivation-concentration relationships of oximes to cyclosarin-inhibited AChE – semilogarithmic transformation

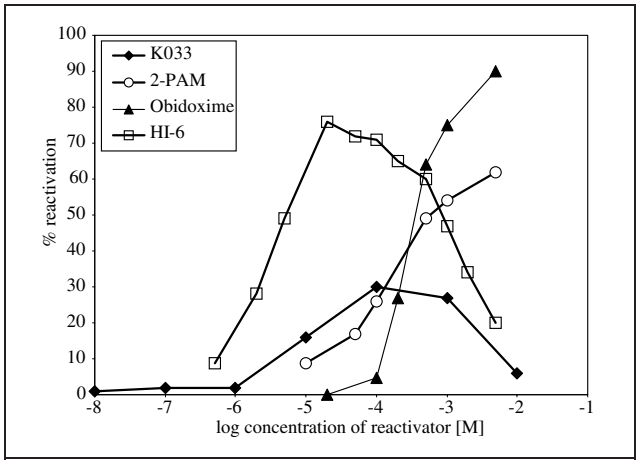


Fig. 2: Reactivation-concentration relationships of oximes to sarin-inhibited AChE – semilogarithmic transformation

that the affinity of K033 and HI-6 to the enzyme-inhibitor complex is also much higher compared to pralidoxime. The differences in the affinity of the oximes studied to intact or inhibited enzyme correspond to differences in the second-order rate constant of reactivation of cyclosarin-inhibited AChE (k_r). The kinetic parameters for obidoxime could not be measured because of its extremely low potency to reactivate cyclosarin-inhibited AChE. According to our results, shown in Fig. 1, both oxime HI-6 and K033 are sufficiently effective to reactivate cyclosarin-inhibited

Table: Kinetic parameters of the reactivation of cyclosarin-, sarin- and tabun-inhibited AChE in rat brain homogenate *in vitro*

Oxime	Nerve Agent	K_{dis} (μM)	K_R (μM)	k_R (min^{-1})	k_r ($M^{-1} \cdot min^{-1}$)
K033	GF	29 ± 3	20 ± 3	0.095 ± 0.011	4872 ± 399
2-PAM	GF	210 ± 14	12000 ± 565	0.040 ± 0.008	3 ± 1
Obidoxime	GF	280 ± 28	–	–	–
HI-6	GF	24 ± 4	12 ± 2	0.350 ± 0.042	29000 ± 589
K033	GB	29 ± 3	16 ± 3	0.042 ± 0.007	2695 ± 241
2-PAM	GB	210 ± 12	354 ± 38	0.140 ± 0.013	403 ± 23
Obidoxime	GB	280 ± 29	781 ± 57	0.380 ± 0.034	486 ± 49
HI-6	GB	24 ± 3	9 ± 2	0.210 ± 0.021	22000 ± 1100
K033	GA	29 ± 5	5 ± 1	0.012 ± 0.002	2591 ± 244
2-PAM	GA	210 ± 22	575 ± 43	0.006 ± 0.001	10 ± 3
Obidoxime	GA	280 ± 30	3 ± 1	0.020 ± 0.002	6250 ± 560
HI-6	GA	24 ± 3	6 ± 1	0.007 ± 0.002	1111 ± 133

K_{DIS}, dissociation constant of the enzyme-reactivator complex; 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; GF, cyclosarin; GB, sarin; GA, tabun

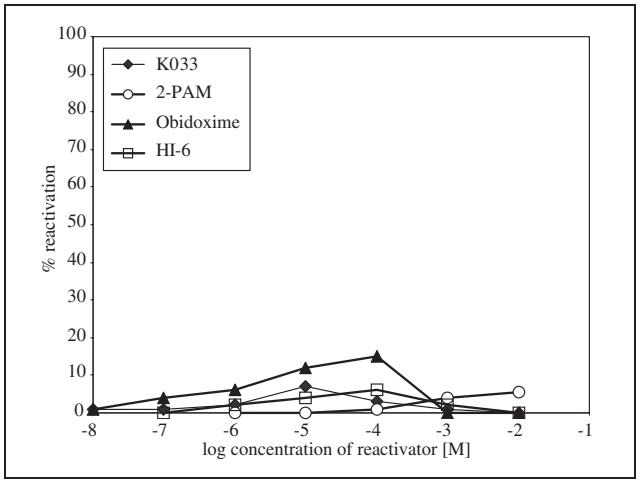


Fig. 3: Reactivation-concentration relationships of oximes to tabun-inhibited AChE – semilogarithmic transformation

AChE in concentrations from 10^{-6} to 10^{-4} M that should be safe for human use. On the other hand, both obidoxime and pralidoxime are able to reactivate cyclosarin-inhibited AChE *in vitro* only in very high concentrations (from 10^{-2} to 10^{-1} M) that would be toxic for human use.

The ability of all the oximes studied to reactivate sarin-inhibited AChE *in vitro* is demonstrated by the kinetic parameters in the Table and by the concentration-reactivation relationship expressed in Fig. 2. The differences in the potency of the tested oximes to reactivate sarin-inhibited AChE are similar to their potency to reactivate cyclosarin-inhibited AChE. The values of the dissociation constant K_R indicate that the affinity of K033 and HI-6 to the enzyme-inhibitor complex is significantly higher than that of obidoxime and pralidoxime. The differences in the affinity of the studied oximes to intact or inhibited enzyme also correspond to the differences in the second-order rate constant of reactivation of sarin-inhibited AChE (k_r). According to our results, shown in Fig. 2, both oxime HI-6 and K033 are sufficiently effective to reactivate sarin-inhibited AChE in concentrations from 10^{-6} to 10^{-4} M that should be safe for human use. On the other hand, both obidoxime and pralidoxime are able to reactivate sarin-inhibited AChE in vitro only in much higher concentrations (from 10^{-4} to 10^{-2} M).

The kinetic parameters in the Table also characterize the ability of all the oximes studied to reactivate tabun-inhibited AChE *in vitro*. The concentration-reactivation relationship is expressed in Fig. 3. While the kinetic parameters demonstrate a relatively high affinity of K033, obidoxime and the oxime HI-6 and a low affinity of pralidoxime toward tabun-inhibited AChE and a high second-order rate of reactivation of tabun-inhibited AChE by the oximes mentioned with the exception of pralidoxime, their potency to reactivate tabun-inhibited AChE is relatively low. According to the results shown in Fig. 3, obidoxime and K033 are slightly effective in reactivating tabun-inhibited AChE in concentrations from 10^{-6} to 10^{-4} M, while pralidoxime is completely ineffective and the oxime HI-6 needs to be at a higher concentration to be able to reactivate tabun-inhibited AChE.

3. Discussion

The efficacy of AChE reactivators depends on their reactivity and affinity to the nerve agent-inhibited enzyme. Their reactivity is derived from the nucleophilic activity of the oxime anion that is bound on the pyridinium ring (Shih 1993). The reactivity of all the oximes studied (k_R) is almost the same because their basic structure is very similar. They differ from each other only by the position of the oxime group on the pyridinium ring and, in the case of bispyridinium oximes, by the chemical structure of the bridge between the two pyridinium rings.

The affinity of oximes for the intact enzyme, characterized by K_{dis}, and for the nerve agent-inhibited enzyme, characterized by K_R, is derived from various physical features (steric compatibility, electrostatic effects, hydrophobic interactions) and from the shape and the size of the whole molecule as well as its functional groups (Kuča et al. 2003c; Bieger and Wassermann 1967). The affinity of reactivators for nerve agent-inhibited AChE is considered to be the most important factor for their reactivating efficacy. Our results demonstrate that the strength of reactivator binding to inhibited AChE is usually weaker than that to the native protein because of a reduction of a space in the cavity of the AChE molecule following enzyme phosphonylation or phosphorylation. Generally, nerve agents such as sarin and cyclosarin reduce the strength of binding of oximes to AChE and, thus, make it more difficult to demonstrate their nucleophilic effects. In spite of the reduction in the strength of binding to sarin- or cyclosarin-inhibited AChE, the oxime HI-6 and the bispyridinium oxime K033 seem to be very good reactivators of sarin- or cyclosarin-inhibited AChE *in vitro* because their affinity for sarin- or cyclosarin-inhibited AChE is relatively high. On the other hand, currently available oximes (pralidoxime and obidoxime) have a low affinity for sarin- or cyclosarin-inhibited AChE and, thus, their reactivating potency, determined *in vitro*, is much lower in comparison with HI-6 and K033.

The *in vitro* potency of all the oximes studied to reactivate tabun-inhibited AChE is completely different from their ability to reactivate sarin- or cyclosarin-inhibited AChE. In spite of the relatively high affinity of obidoxime, HI-6 and K033 to tabun-inhibited AChE, their potency to reactivate tabun-inhibited AChE is very low. Tabun-inhibited AChE is extraordinarily difficult to reactivate because of the existence of a lone electron pair located on an amidic group that makes the nucleophilic attack of both monopyridinium and bispyridinium oximes almost impossible (Bajgar and Cabal 1999; Eto 1976; Jokanovic et al. 1996; Koplovitz et al. 1995). The reactivating efficacy of bispyridinium oximes depends strongly on the chemical structure of the bridge connecting the both pyridinium rings, the position of the oxime groups and the chemical structure of the substituent situated on the second pyridinium ring (Worek et al. 1998). This fact can explain the relatively low efficacy of oxime HI-6, so efficacious against fluorophosphonates, against tabun, because oxime HI-6 contains an ether bridge and has a carbamide group instead of the oxime group on the second pyridinium ring. Therefore, the newly synthesized oxime K033, that differs from oxime HI-6 by the structure of the bridge connecting the two pyridinium rings (butan instead of ether) and by having oxime groups on both pyridinium rings, is a more efficacious reactivator of tabun-inhibited AChE *in vitro* than is HI-6. The potency of obidoxime to reactivate tabun-inhibited AChE is a little higher compared to the other oximes studied because of the more suitable position of the two oxime groups.

Our results confirm that is very difficult to find a broad-spectrum oxime suitable for the antidotal treatment of poisoning with all highly toxic organophosphorus agents

(Kassa 2002). The oxime HI-6 which is so promising against fluorophosphonates such as soman, sarin and cyclosarin (Kassa and Cabal 1999a, b, c) is practically ineffective to reactivate tabun-inhibited AChE (Kuča et al. 2003a; Kuča and Kassa 2003). Obidoxime, which is able to reactivate tabun-inhibited AChE to some extent, is practically ineffective at reactivating soman- or cyclosarin-inhibited AChE (Kassa and Cabal 1999a, b). Therefore, bispyridinium oxime K033, which is able to reactivate sarin- and cyclosarin-inhibited AChE more than obidoxime but less than HI-6 and is able to reactivate tabun-inhibited AChE more than HI-6 but less than obidoxime, seems to be, among the known oximes, the most promising broad-spectrum oxime for antidotal treatment of nerve agent poisoning as evaluated by *in vitro* reactivating efficacy.

4. Experimental

4.1. Animals

Male albino Wistar rats weighing 180–220 g were purchased from Biotest Konárovice (Czech Republic). They were kept in an air-conditioned room (22 ± 1 °C and $50 \pm 10\%$ relative humidity, with lights from 07.00 to 19.00) and were allowed free access to standard food and tap water ad libitum. Experiments were performed under the supervision of the Ethics Committee of the Purkyně Military Medical Academy, Czech Republic.

4.2. Enzymes and chemicals

After exsanguination of anaesthetized animals by decapitation (i.p. injection of urethane 1.5 g/kg), the rat brains were removed and used as a source of AChE after homogenization. All nerve agents (tabun – GA; sarin – GB; cyclosarin – GF) were obtained from the Military Technical Institute (Brno, Czech Republic) and were 89–95% pure. The purity was evaluated by acidimetric titration. The monopyridinium and bispyridinium oximes (pralidoxime, obidoxime, HI-6), including the new oxime K033, had been synthesized earlier at the Department of Toxicology of the Military Medical Academy (Czech Republic) (Kuča et al. 2004). Their purities were analysed using an HPLC technique. All other drugs and chemicals of analytical grade were obtained comercially and used without further purification.

Rat brain acetylcholinesterase was chosen as the source of the enzyme. Its preparation was as follows. Narcotised animals were killed by bleeding from a carotid artery and the brains were removed, washed with saline and homogenized in an Ultra-Turrax (Germany) homogenizer in distilled water to make a 10% homogenate. The resulting homogenate was divided into test-tubes and stored at −35 °C in a freezer and defrozen immediately prior the use.

4.3. In vitro experiments

The reactivation effectivity of the oximes was tested *in vitro* on the model of AChE inhibited by tabun, sarin or cyclosarin using a standard reactivation test with electrometric instrumentation (Kuča and Kassa 2003) as follows.

Activity of the intact enzyme: Rat brain homogenate (0.5 ml) was added to sodium chloride solution (3 M; 2 ml) and water (20.5 ml). Then, acetylcholine iodide solution (0.02 M; 2.0 ml) was added. The acetic acid liberated was titrated with 0.01 M sodium hydroxide on an RTS 822 titrator (Radiometer Copenhagen) in pH-stat mode (pH 8.0). The slope of the linear part of the time dependence of the sodium hydroxide used represents the activity (a_0) of the intact enzyme.

Activity of inhibited enzyme: Rat brain homogenate (0.5 ml) was treated with an isopropanol solution of nerve agent (10^{-6} M; 20 µl) for 30 min, which resulted in about 95% inhibition of the enzyme. After this, a solution of acetylcholine iodide (0.02 M; 2.0 ml) was added and the mixture was adjusted to 25.0 ml with sodium chloride solution (0.3 M). The activity of the inhibited enzyme (a_I) was immediately determined as described in the previous experiment.

Activity of reactivated enzyme: Reactivation of the inhibited enzyme was performed immediately after inhibition. A solution of the reactivator of defined concentration (concentration range from 10^{-7} to 10^{-1} M; 1.0 ml) was added to the enzyme. After 10 min of reactivation at 25 °C, acetylcholine iodide solution (0.02 M; 2.0 ml) was added. The mixture was adjusted to 25.0 ml with sodium chloride solution (0.3 M) and immediately afterwards the activity of the reactivated enzyme (a_R) was determined as described in the previous experiments.

The reactivation efficacy was calculated from the measured activities of the intact enzyme (a_0), nerve agent-inhibited enzyme (a_I), and reactivated enzyme (a_R), using the equation.

$$\% \, R = [1 - (a_0 - a_R)/(a_0 - a_I)] \cdot 100$$

4.4. Statistical evaluation

Statistical significance was determined by Student's t-test and differences were considered significant when $P < 0.05$. Statistical evaluation was determined with the relevant computer programs (Tallarida and Murray 1987).

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