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In vitro Reactivation Potency of Acetylcholinesterase Reactivators - K074 and K075 - to Reactivate Tabun-inhibited Human Brain Cholinesterases

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In this work, two oximes for the treatment of tabun-inhibited acetylcholinesterase (AChE; EC 3.1.1.7), K074 (1,4-*bis*(4-hydroxyiminomethylpyridinium) butane dibromide) and K075 ((E)-1,4-*bis*(4-hydroxyiminomethylpyridinium)but-2-en dibromide), were tested *in vitro* as reactivators of AChE. Comparison was made with currently used AChE reactivators (pralidoxime, HI-6, methoxime and obidoxime). Human brain homogenate was taken as an appropriate source of the cholinesterases. As resulted, oxime K074 appears to be the most potent reactivator of tabun-inhibited AChE, with reactivation potency comparable to that of obidoxime. A second AChE reactivator, K075, does not attain as great a reactivation potency as K074, although its maximal reactivation (17%) was achieved at relevant concentrations for humans.

Keywords: Acetylcholinesterase; Nerve agents; K074; K075; Oximes; Reactivators; Tabun

INTRODUCTION

Organophosphorus compounds are widely used in agriculture as insecticides and acaricides, in industry and technology as softening agents, and additives to lubricants. Some of them are declared as chemical warfare agents (nerve agents). Sarin, soman, tabun, cyclosarin or VX belong to the most known members of the organophosphorus nerve agent family (Bajgar, 2004). The history of these compounds began before World War II in Germany. The first known nerve agent - tabun (*O*-ethyl-*N,N*-dimethyl phosphoramidocyanidate) was synthesized in 1936 (Germany). Afterwards, many other nerve agents such as sarin, cyclosarin, soman and VX were developed, stored and prepared for potential military use. One of these nerve agents - sarin - was misused by the Japanese Aum Shinrikyo sect in Matsumoto (1994) and Tokyo (1995) (Maekawa, 1995).

The toxic effect of these compounds is based on the phosphorylation or phosphonylation of the serine hydroxyl group at the esteratic part of the active site of the enzyme acetylcholinesterase (AChE; EC 3.1.1.7). AChE plays a key role in physiological function of the cholinergic nervous system, and therefore, its inhibition is a life-endangering factor (Marrs, 1993; Bajgar, 2004).

After the irreversible inhibition of AChE by these agents, AChE is unable to fulfil its physiological role in the organism (splitting the neuromediator acetylcholine (ACh) in the synaptic cleft), and sub-

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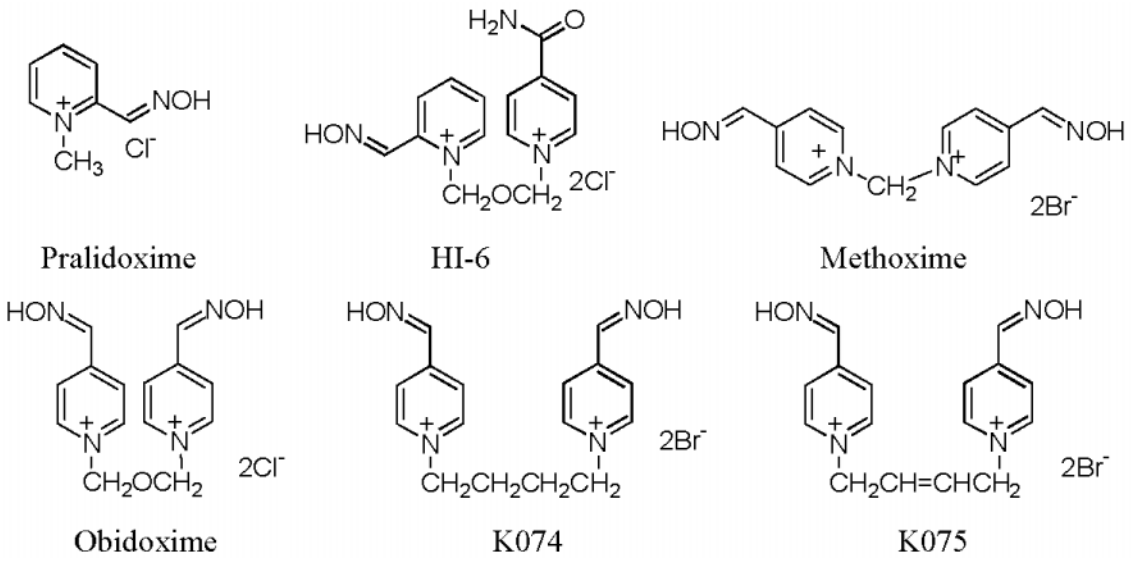

FIGURE 1 Structures of tested acetylcholinesterase reactivators.

sequently, ACh accumulates at the synaptic junction (Taylor, 1996; Kassa, 2006).

The acute toxicity of nerve agents is usually attributed to the excessive cholinergic stimulation caused by the above-mentioned accumulation of ACh and subsequent overstimulation of the cholinergic pathways followed by desensitization of the cholinergic peripheral and central receptor sites. Afterwards, cholinergic crisis occurs and in severe cases an intoxicated person can die due to respiratory failure (Marrs, 1993; Taylor, 1996; Kassa and Kunesova, 2006).

A combined regimen of therapy is now generally considered as the most effective medical approach for the treatment of nerve agent-poisonings of military personnel. In the case of intoxication, immediate therapeutic treatment with an anticholinergic drug (such as atropine sulfate) antagonizes the effects of ACh excess at peripheral muscarinic receptor sites; and administration of an AChE reactivator (termed an oxime) which renews the function of inhibited AChE (Kassa, 2002; Kuca *et al.*, 2004).

Oximes are nucleophilic substances that are able to break down the bond between enzyme and organophosphate inhibitor and liberate the enzyme, which can then fulfil its physiological role. Monoquaternary pralidoxime (2-PAM, 2-hydroxyiminomethyl-1-methylpyridinium chloride) or more extended bisquaternary compounds such as obidoxime (1,3-*bis*(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride) and H-oxime HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride) are fundamental representatives of these aldoximes (FIG. 1) (Kassa, 2002; Bartosova *et al.*, 2006).

As was mentioned above, tabun (GA; *O*-ethyl-*N,N*-dimethyl phosphoramidocyanidate) is a highly toxic organophosphorus AChE inhibitor (first synthesized before World War II), which has the potential for being used as a chemical warfare agent. This potential increased during Operation Desert Shield and Desert Storm with the possibility that this agent could be a constituent of the Iraqi chemical agent inventory (Cabal and Bajgar, 1999).

Unfortunately, currently commercially used AChE reactivators (pralidoxime, obidoxime and HI-6) are poor reactivators of AChE inhibited by this nerve agent (de Jong *et al.*, 1989; Worek *et al.*, 2004). Of these reactivators, only obidoxime could reactivate tabun-inhibited AChE. However, its potency does not surpass more than 15% (Cabal *et al.*, 2004). In 2005, we prepared two new AChE reactivators, K074 (1,4-*bis*(4-hydroxyiminomethylpyridinium)butane dibromide) and K075 ((E)-1,4-*bis*(4-hydroxyiminomethylpyridin-ium)but-2-en dibromide), derived from trimedoxime, which is currently considered as the best reactivator of tabun-inhibited AChE. These reactivators were very promising *in vitro* in comparison with all currently available oximes including trimedoxime. Unfortunately, these results were obtained on rat brain homogenate, which contains an AChE different from human AChE (Kuca *et al.*, 2005). The aim

of this work was to obtain reactivation data of both new oximes on human brain homogenate.

There exists a correlation for *in vivo* toxicity of nerve agents and their *in vitro* AChE potency. However, the correlation is more expressed within one species (Bajgar and Herink, 1995). Some attempts were made for the same correlation within different species (Bajgar, 2004). For reactivators used in treatment, this correlation (reactivation potency *in vitro vs* therapeutic efficacy *in vivo*) is more complicated and it can be applied within one species. Therefore, the studies should be focused to AChE from a human source (preferably brain).

In this work, we have tested reactivation potency of both newly developed oximes K074 and K075 to reactivate human brain AChE inhibited by tabun. Pralidoxime (gold standard of AChE reactivators), methoxime, obidoxime and currently the most promising oxime HI-6 - were taken as suitable compounds for comparison.

MATERIALS AND METHODS

Chemicals

AChE reactivators pralidoxime (2-PAM; 1-methyl-2-hydroxyiminomethylpyridinium chloride); obid-oxime (1,3-*bis*(4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride) and methoxime (MMB4; 1,1-*bis*(4-hydroxyiminomethylpyridinium)-methane dibromide) were purchased from Leciva (Czech Republic) and Merck (Germany), respectively. HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride) was purchased from Phoenix Chemicals Ltd (United Kingdom). Both novel AChE reactivators K074 (1,4-*bis*(4-hydroxyiminomethylpyridinium)butane dibromide) and K075 ((E)-1,4-*bis*(4-hydroxyiminomethylpyridinium)but-2-en dibromide) were synthesized at our department earlier, using modification of a synthetic method described by Patocka *et al.* (1970) and Poziomek *et al.* (1958) (Kuca *et al.*, 2005).

Purity of all tested AChE reactivators was tested using TLC (DC-Alufolien Cellulose F; mobile phase n-butanol : acetic acid : water - 5 : 1 : 2; detection by Dragendorff Reagent) and NMR (Varian Gemini 300, Palo Alto CA, USA) (Palecek *et al.*, 2005). Tabun (GA; *O*-ethyl-*N,N*-dimethyl phosphoramidocyanidate) was obtained from the Military Technical Institute (Brno, Czech Republic) in 97% purity. All other chemicals used in this experiment were of analytical grade and were purchased from Sigma Aldrich (Czech Republic).

Source of the ChEs

Human brains (nucleus caudatus) were obtained from the Faculty of Medicine in Hradec Kralove (Charles University in Prague, Czech Republic). Preparation of the homogenate was done as follows. The brain parts were washed with saline and homogenized in distilled water (10% w/v) by the use of an Ultra-Turrax instrument (Janke-Kunkel, Germany) at 20,000 rpm for 1 min. Aliquots (2 ml) of the homogenate were stored at -35°C in a freezer. They were thawed immediately prior to use.

Inhibition of ChEs

Brain homogenate (0.5 ml) was treated with a solution (0.5 ml) of tabun in appropriate concentrations for 30 min. Afterwards, the reaction solution was adjusted to 23 ml with 0.3 M sodium chloride. Then, 0.02 M solution of ACh iodide (2 ml) was added to the mixture. The liberated acetic acid was titrated with 0.01 M sodium hydroxide on an RTS 822 titrator (Radiometer, Denmark) in the pH-stat mode (pH 8.0) at room temperature (25°C). The slope of the linear part of the time dependence of the sodium hydroxide used represents the activity of the inhibited enzyme (in fact, the initial rate of the enzymatic reaction) (Kuca and Kassa, 2003).

Reactivation of ChE

The resulting inhibition of ChEs was set to 95%. Reactivation of the inhibited ChEs was performed immediately after its inhibition. A solution (1 ml) of the reactivator in an appropriate concentration was added to the inhibited enzyme. After 10 min reactivation at 25°C, the mixture was adjusted to 23 ml with 0.3 M sodium chloride solution. Then, 0.02 M ACh iodide (2 ml) was added, and immediately afterwards the activity of the reactivated enzymes was determined analogously as described in the previous text.

RESULTS AND DISCUSSION

All results obtained are summarized in Table I and in figure 2. We were not able to obtain appropriate constants for pralidoxime and HI-6 because of low reactivation potency of these oximes. Oxime K075

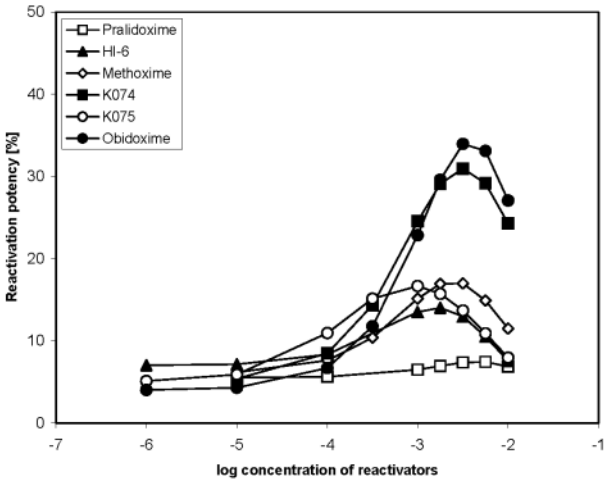

FIGURE 2 Dependence of the reactivation potency on the oximes concentration.

Table I Constants characterizing the reactivation process

Oxime	K_R [μM]	k_R [min^{-1}]	k_r [$min^{-1}. M^{-1}$]
Pralidoxime	-*	-*	-*
HI-6	-*	-*	-*
Methoxime	2512	0.05	20
Obidoxime	1412	0.06	42
K074	1995	0.08	40
K075	200	0.02	100

* We were unable to obtain appropriate constants because of low reactivation potency of tested oximes.

has the highest affinity (characterized by the dissociation constant K_R) towards the tabun-inhibited AChE (Table I). Methoxime, obidoxime and oxime K074 have almost similar affinities. Values of the first order rate constants characterizing splitting of the bond between inhibitor and enzyme (k_R) favour oximes K074 prior obidoxime, methoxime and K075. Constants k_r (the second order rate constant, characterizing the whole reactivation process), obtained as ratio k_R/K_R, shows that the most potent AChE reactivator tested in this work was K075 followed by obidoxime, K074 and methoxime.

As can be seen in figure 2, K074 and obidoxime seem to be the most potent tabun-inhibited AChE reactivators, with the maximum reactivation potency at the concentration $10^{-2.5}$ M. Unfortunately, this concentration is not attainable in human. Due to this, we have to focus our attention to the oximes able to sufficiently (5-10%; Bajgar, 2004) reactivate tabun-inhibited AChE at human relevant doses - 10^{-4} M and lower (Kuca and Kassa, 2003; Bajgar, 2004). From this point of view, oxime K075 seems to be the most potent reactivator of tabun-inhibited AChE. Its highest reactivation potency was achieved at the concentration 10^{-4} M, which is believed to be attainable in an organism.

Although there are already commercially available AChE reactivators (*e.g.*, pralidoxime, obidoxime, HI-6), their potency for reactivating AChE inhibited by all kinds of nerve agents and pesticides is unsatisfactory (Worek *et al.*, 1996; 1999; Kassa and Cabal, 1999a,b,c). Due to this fact, many laboratories throughout the world are still interested in the development of new more potent, so-called broad spectrum reactivators (Pang *et al.*, 2003; Kim *et al.*, 2005, Musilek *et al.*, 2005; 2006; Picha *et al.*, 2005). Unfortunately, such substances are still not developed today. For this reason, we focused on the development of AChE reactivators able to reactivate tabun-inhibited AChE. It is known, that this agent is very difficult to reactivate because of the lone electron pair, which hinders the access of the oximate anion splitting the bond between AChE and an inhibitor (Eto, 1976; Koplovitz *et al.*, 1995).

As shown by our former results, we found that tabun-inhibited AChE is best reactivated by bispyridinium AChE reactivators with oxime groups in position four (Cabal *et al.*, 2004). Our other data indicate that the most suitable length of the connecting chain between two quaternary nitrogens is three or four membered linker (Kuca *et al.*, 2003).

Oximes K074 (four membered connection chain) and K075 (thanks to the presence of double bond, shorter connection chain compared to K074) fulfil this criterion. In view of these structural requirement and former *in vitro* results obtained on rat models, these promising results presented in this work were expected.

As a result, both AChE reactivators (K074 and K075) achieved good reactivation potency on human homogenate. Compared to other oximes tested, both newly developed oximes seem to have the superiority of obidoxime. Our data confirm the need of substantial structural requirements, which should be involved in the reactivator structure (bispyridinium reactivator with oxime group in the

position four at the pyridinum rings connected with three or four membered links) (Kuca *et al.*, 2006). According to our *in vitro* results, both with rat and human brain homogenates, promising reactivators K074 and K075 should be recommended for further investigation of their adsorption, distribution, metabolism and excretion, using other more sophisticated approaches.

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