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ORIGINAL ARTICLE

In vitro reactivation potency of bispyridinium (*E*)-but-2-ene linked acetylcholinesterase reactivators against tabun-inhibited acetylcholinesterase

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Summary
Six potential bispyridinium (*E*)-but-2-ene linked AChE reactivators were tested on tabun-inhibited acetylcholinesterase *in vitro*. Their reactivation efficacy was compared to currently available AChE reactivators used in the case of organophosphorus intoxications – pralidoxime, HI-6 and obidoxime. According to the results obtained, (*E*)-1,4'-bis(4-hydroxyiminomethylpyridinium)but-2-ene dibromide (K075) seems to be the most potent reactivator of tabun – inhibited AChE. From the data, the reactivation potency of these compounds depends on structural factors such as the constitution of the linking chain between both pyridinium rings and the position of the oxime moiety at the pyridinium rings.

Keywords: organophosphate – tabun – acetylcholinesterase – reactivation – oxime

INTRODUCTION

Organophosphorus compounds (OC) are commonly known as nerve agents (soman, sarin, tabun, VX, etc.), pesticides (chlorpyrifos, paraoxon, diazinon, etc.), substances used for industrial purposes (tributylphosphate) or as potential therapeutics (metrifonate) (Eto 1976, Marrs 1993, Ringmann et al. 1999, Bajgar 2004, Marklund et al. 2005). Their chemical structure is derived from phosphonic and phosphoric acid or their thio-analogues, respectively (Marrs 1993). They are able to irreversibly inhibit enzyme acetylcholinesterase (AChE, EC 3.1.1.7) by phosphonylation or phosphorylation of serine hydroxyl in enzyme's active site (Scheme 1) (Bajgar 2004).

Therefore, the enzyme can not fulfil its physiological function – cleavage of the neuromediator acetylcholine – necessary to terminate cholinergic transmission. Afterwards, the redundant acetylcholine causes cholinergic overstimulation and subsequent cholinergic crisis which could end by a blockage in the breathing centre and death (Bajgar 2004). The threat of intoxications by these compounds rapidly increases with growing agricultural production and with the

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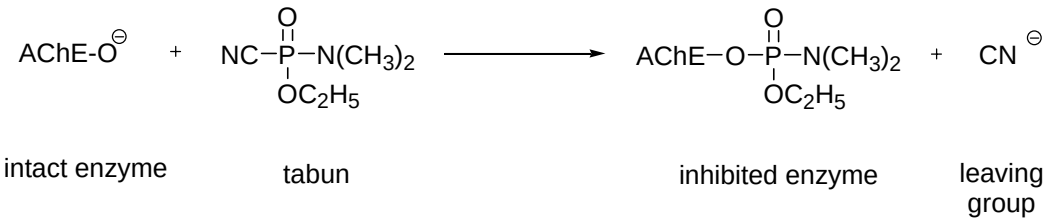
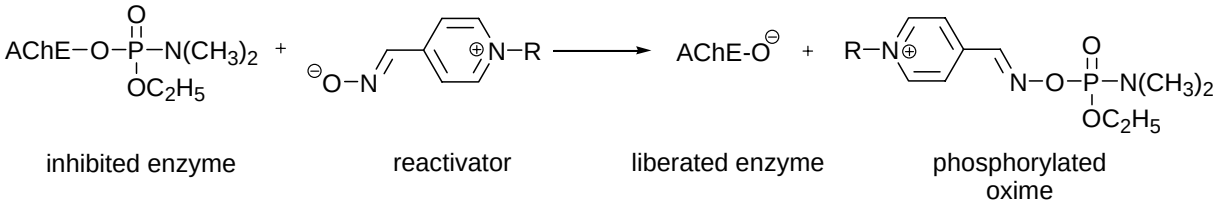
menace of terrorist attacks (Satoh et al. 2000, Krivoy et al. 2005).

Anticholinergic drugs such as atropine are used to counteract the effects of OC at the peripheral cholinergic receptor (Kassa 2002). On the other hand, they are not able to treat the inhibited enzyme. For this reason, AChE reactivators are used to cleave the covalent bond OC-enzyme by the nucleophilic reactive group and thereby restore the activity of AChE (Scheme 2) (Bajgar 2004).



Scheme 1. **Tabun phosphorylation of AChE**



Scheme 2. **Oxime induced reactivation of tabun-phosphorylated AChE**

The commonly used reactivators are monoquaternary or bisquaternary substances carrying the hydroxyiminomethyl group (oxime) as a nucleophilic agent, e.g. pralidoxime – "the golden standard of AChE reactivators" (**1**, 2-hydroxyiminomethyl-1-methylpyridinium chloride), oxime HI-6 (**2**, 1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride), obidoxime (**3**, Toxogonine®, 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride) (Fig. 1) (Kassa et al. 1997, Rousseaux et al. 1989, Petroianu et al. 2005).

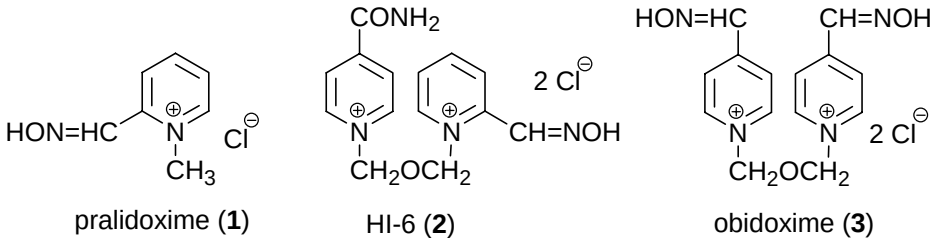
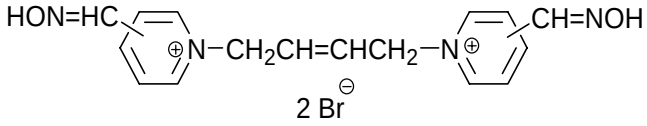


Fig. 1. **Examples of oxime reactivators currently used against OC intoxications**

Nevertheless, every type of OC needs a specific structure of AChE reactivator and no broad spectrum reactivator has been found in more than fifty years of investigations (Kuča et al. 2004, 2006). Therefore, the development and selection of new effective reactivators of AChE like antidotes for OC are very important.

Tabun (GA; *O*-ethyl-*N*,*N*-dimethylphosphoramidocyanidate) is one of the most dangerous compounds among nerve agents. It is extraordinarily difficult to counteract the AChE inhibition by GA due to the existence of a lone electron pair located on the amidic group that makes a nucleophilic attack almost impossible (Cabal et al. 2004). Moreover, GA is responsible for larger changes in the enzyme's cavity and therefore commonly used reactivators of phosphorylated AChE are not able to counteract its toxic effects (Patočka 1977, Koplovitz 1995, Ekstrom et al. 2006).

In 2006, we have developed six structurally relative AChE reactivators (K053, K068, K069, K075, K104, K105) (Fig. 2) (Musílek et al. 2006). These AChE reactivators differ from those currently used, in the structure and shape (rigidity) of the connecting chain between two pyridinium rings. Firstly, we tested their potency against the pesticide chlorpyrifos. According to these results, two of them were potent reactivators of the pesticide-inhibited AChE. Their reactivation potency reached 48% or 38% at concentration 10^5 M which is believed to be attainable for humans (K053, K075). Because of our previous results, we were interested in the difference in the reactivation potency of newly developed reactivators in comparison with those currently used, especially pralidoxime, oxime HI-6 and obidoxime, against a real nerve agent (GA).



Compound	Oxime position
K053	2,4'-CH=NOH
K068	2,2'-CH=NOH
K069	2,3'-CH=NOH
K075	4,4'-CH=NOH
K104	3,3'-CH=NOH
K105	3,4'-CH=NOH

Fig. 2. **Six potential oxime reactivators tested against tabun-inhibited AChE**

MATERIAL AND METHODS

Chemicals

Newly tested compounds were prepared earlier in our department using new synthetic approaches (Musílek et al. 2006). Obidoxime, oxime HI-6 and pralidoxime were purchased from commercial sources (Merck, Germany; Phoenix, Chemicals Ltd., United Kingdom; Zentiva, Czech Republic). The purity of the tested AChE reactivators was estimated using ^{1}H-NMR spectra (Varian Gemini 300, ^{1}H 300 MHz, ^{13}C 75 MHz, Palo Alto, CA, USA) and TLC techniques [DC-Alufolien Cellulose F, Merck, Germany, mobile phase BuOH-CH_3COOH-H_2O 5:1:2, detection by Dragendorff reagent - European Pharmacopoeia 5 (5 ml of standard solution additionally dissolved in 10 ml of acetic acid and 50 ml of water]. Tabun was obtained from the Military facility Brno (95 % purity). All other chemicals used were of reagent grade (Sigma Aldrich).

Source of enzyme

Rat (Wistar rats; Biotest Konárovice, Czech Republic) brain homogenate was chosen as 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 experiments

The reactivation efficacy of the synthesized oximes was tested *in vitro* on the model of rat brain homogenate inhibited by tabun using standard reactivation test with electrometric instrumentation (Kuča and Cabal 2005). The homogenate (0.5 ml) was mixed with a solution of tabun in dry isopropanol (0.02 ml) and incubated for 30 min at 25 °C, to obtain 95% inhibition. Then 3M NaCl (2.5 ml) was added and supplemented by distilled water to a volume of 23 ml. After that, 0.02M acetylcholine iodide (2 ml) 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_0) and GA-inhibited (a_i) AChE were determined. After tabun inhibition of AChE, the inhibited enzyme was incubated for 10 min with a solution of the reactivator and then the activity of the reactivated AChE (a_r) was measured as previously. The activity values a_0, a_i and a_r were calculated from the slopes of the initial part of the titration curves. Each value represents an arithmetic mean from three independent measurements. The percentage of reactivation was calculated from the following equation:

$$x = \left(1 - \frac{a_0 - a_r}{a_0 - a_i}\right) \cdot 100 \quad [\%]$$

RESULTS AND DISCUSSION

All results obtained are summarized in Table 1. The tested oximes showed reactivation potency at both chosen concentrations. Futhermore, the reactivation potency able to effectively cause the intoxication should overlap 10 % (Bajgar 2004). According to this, only obidoxime (**3**) and new oxime K075 are able to efficiently reactivate tabun-inhibited AChE at concentration 10^{-3}M. The substance K075 has already been described earlier also with very promising results in the reactivation of GA-inhibited AChE (Kuča et al. 2005). It surpasses commonly used reactivators against tabun poisonings *in vitro*. Other newly prepared compounds showed no reactivation potency at concentration 10^{-3}M, but they had some activity at lower concentration. This fact could be caused by a concurrent inhibition of the enzyme by the reactivator itself, because of the generally bell-shaped curve of reactivation dependence (Kuča et al. 2004).

Table 1. **Reactivation potencies** (%, mean value of three independent determinations) **of oximes tested** (time of inhibition by tabun – 30 min; time of reactivation by AChE reactivators – 10 min; pH 8; temperature 25 °C).

	Reactivation (%)	
Reactivator/Concentration	10^{-3} M	10^{-5} M
pralidoxime (1)	4 ± 1	0 ± 0
HI-6 (2)	2 ± 1	4 ± 1
obidoxime (3)	11 ± 0	0 ± 0
K053	0 ± 0	9 ± 2
K068	0 ± 0	10 ± 2
K069	0 ± 0	15 ± 2
K075	16 ± 1	23 ± 1
K104	0 ± 0	4 ± 0
K105	0 ± 0	16 ± 1

It is not possible to achieve this relatively high concentration (10^{-3}M) *in vivo* (Tattersall 1993). Therefore a concentration of 10^{-5}M is more suitable. Subsequently, the new tested compounds, especially oximes K069, K075 and K105, showed promising results at this concentration. These compounds also exceeded pralidoxime, HI-6 and obidoxime, which were at a concentration of 10^{-5}M practically ineffective.

As it can be seen, the reactivation potency depends on the concentration and chemical structure of the reactivator used. Furthermore, we can discuss the structural factors appropriate for reactivation of tabun-inhibited AChE. The hydroxyiminomethyl (oxime) group in position four on pyridinium ring showed the best results for tabun-inhibited AChE (Kuča et al. 2003a, 2003b). Newly prepared compounds do fulfil this criterion only partially. The compounds bearing at least one oxime group placed in position four show satisfactory reactivation (K053, K075, K105) while oxime with both oxime groups in position three is almost ineffective (K104). On the other hand, there are also two compounds with oxime group in position two and three respectively with promising results (K068, K069). This could be caused by another important criterion – the length and rigidity of the linking chain which also influence the reactivation potency (Kuča et al. 2003a, Musílek et al. 2005) The optimal length of the connecting bridge for GA-inhibited AChE lies between three and four atoms (Pang et al. 2003). New compounds suit this purpose thanks to the double bond which is shorter than single and so but-2-ene lies exactly between the propane and butane connecting chain (Carey et al. 2000). The double bond also counteracts the free rotation in the connecting chain, which is usual for currently used reactivators (HI-6, obidoxime) and influences the affinity to the enzyme. It gives to the molecule of the reactivator

enhanced rigidity (Musílek et al. 2005, 2006; Kuča et al. 2005). Moreover, π-electrons of the double bond in the connecting chain could interact with the amino acids in the cavity of the enzyme using cation – π interaction.

Due to all of the above mentioned facts the connecting chain could also highly influence the reactivator's potency. However, this presumption should be investigated further using appropriate methods like molecular design (Pang et al. 2003).

In conclusion, six novel bispyridinium compounds bearing (*E*)-but-2-ene linker were tested on tabun-inhibited AChE. Their reactivation efficacy was compared to currently used AChE reactivators – pralidoxime, HI-6 and obidoxime. The standard oximes did not show good reactivation efficacy for tabun-inhibited AChE with the exception of obidoxime (**3**), unfortunately not for a concentration applicable *in vivo*. The best result for the reactivation of tabun-inhibited AChE were obtained with (*E*)-1,4'-bis(4-hydroxyiminomethylpyridinium)but-2-ene dibromide (K075).

Results obtained in this work should further help chemists who are interested in the synthesis of new AChE reactivators to predict and prepare new more potent substances.

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