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ORIGINAL ARTICLE

A comparison of reactivating and therapeutic efficacy of the oxime K203 and its fluorinated analog (KR-22836) with currently available oximes (obidoxime, trimedoxime, HI-6) against tabun in rats and mice

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Abstract

The potency of newly developed bispyridinium compound K203 and its fluorinated analog KR-22836 in reactivating tabun-inhibited acetylcholinesterase and reducing tabun-induced lethal toxic effects was compared with commonly used oximes (obidoxime, trimedoxime, the oxime HI-6) using *in vivo* methods. Studies determining the percentage of reactivation of tabun-inhibited blood and tissue acetylcholinesterase in rats showed that the reactivating efficacy of K203 is higher than the reactivating efficacy of its fluorinated analog KR-22836 as well as currently available oximes studied. The therapeutic efficacy of the oxime K203 and its fluorinated analog corresponds to their potency to reactivate tabun-inhibited acetylcholinesterase. According to the results, the oxime K203 is more suitable than KR-22836 for the replacement of commonly used oximes for the antidotal treatment of acute tabun poisoning due to its relatively high potency to counteract the acute toxicity of tabun.

Keywords: *Tabun; K oximes; HI-6; obidoxime; trimedoxime*

Introduction

Organophosphorus nerve agents are considered to be the most dangerous chemical warfare agents. The most important representatives of nerve agents are tabun, sarin, soman, cyclosarin, and VX. Their acute toxic effects are based on the phosphonylation of acetylcholinesterase (AChE, EC 3.1.1.7), leading to the irreversible inhibition of its active site and subsequent overstimulation of postsynaptic cholinergic receptors due to the accumulation of the neurotransmitter acetylcholine in synapses of the central and peripheral nervous systems[1,2].

The medical countermeasures of nerve agent poisonings include administration of antidotes that are able to counteract the main toxic effects of nerve agents. A current standard antidotal treatment of nerve agent poisoning usually consists of the combined administration of an anticholinergic drug (preferably atropine) and an oxime (preferably pralidoxime or obidoxime). The anticholinergic drug blocks the effects of overstimulation by acetylcholine accumulated at the muscarinic receptor sites, while the oxime (compound with nucleophilic oximate anion) repairs biochemical lesions by dephosphonylating nerve agent-inhibited AChE and restoring its activity[1,3]. While a lot of these reactivators are sufficiently effective to reactivate sarin- or VX-inhibited AChE, their potency to reactivate soman-, cyclosarin-, or tabun-inhibited AChE is generally low[3–5].

Tabun (*O*-ethyl-*N*,*N*-dimethylphosphoramidocyanidate) belongs to a highly toxic group of organophosphorus compounds misused as chemical warfare agents for

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military as well as for terroristic purposes. Tabun differs from other highly toxic organophosphates in its chemical structure and by the fact that commonly used antidotes are not able to sufficiently prevent tabun-induced acute toxic effects. Deleterious effects of tabun are extraordinarily difficult to antagonize due to the changes in hydrogen bonding and the conformational changes of the AChE–tabun complex prior aging process in the AChE active site that make the nucleophilic attack of oxime almost impossible[6,7].

While the anticholinergic drugs such as atropine are able to counteract the effects of tabun at the peripheral muscarinic cholinergic receptors[4], commonly used reactivators of phosphorylated AChE based on monopyridinium (e.g. pralidoxime) and bispyridinium oximes (e.g. obidoxime, trimedoxime) are not able to counteract the acute toxic effects of tabun because of their low reactivating efficacy[8]. In addition, the reactivating efficacy of the oxime HI-6, which is relatively efficacious against the adverse effects of soman[9], is not efficient for tabun-inhibited AChE[10,11]. Therefore, the replacement of commonly used oximes (pralidoxime, obidoxime) as well as H-oximes (the oxime HI-6) with a more effective oxime has been a long-standing goal for the treatment of tabun poisoning. For this reason, the new bispyridinium oxime K203 [1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide] (Figure 1) was synthesized in our department[12,13] and was found to be a promising oxime against tabun poisonings based on the evaluation of its reactivating and therapeutic efficacy against tabun[14]. Unfortunately, the oxime K203, as the other known oximes, especially bispyridinium oximes, has rather low potency to reactivate nerve agent-inhibited AChE in the brain due to its limited penetration across the blood–brain barrier (BBB)[14]. Therefore, the fluorinated analog of the oxime K203, KR-22836 [(*E*)-1-(4-carbamoylpyridinium)-4-(3-fluoro-4-hydroxyiminomethylpyridinium)-but-2-ene dibromide] was synthesized in the Medicinal Science Division of the Korea Research Institute of Chemical Technology to increase its penetration through the BBB[15,16].

The main aim of this study was to compare the reactivating and therapeutic efficacy of the newly developed oximes K203 and its fluorinated analog KR-22836 with the currently available oximes (obidoxime, trimedoxime, the oxime HI-6) against tabun using *in vivo* methods.

Materials and methods

Animals

Male albino Wistar rats weighing 200–220 g and NMRI male mice weighing between 18 and 22 g were purchased from Velaz (Prague, Czech Republic). They were kept in an air-conditioned room with light from 07:00 to 19:00 and were allowed access to standard food and tap water *ad libitum*. The rats were divided into groups of eight animals. Handling of the experimental animals was done under the supervision of the Ethics Committee of the Faculty of Military Health Sciences, Czech Republic.

Chemicals

Tabun was obtained from the Technical Institute in Brno (Czech Republic) and was 95% pure. All oximes with the exception of KR-22836 (obidoxime, trimedoxime, HI-6, K203) were synthesized at our Department of Toxicology of the Faculty of Military Health Sciences (Czech Republic). The oxime KR-22836 was synthesized in the Medicinal Science Division of the Korea Research Institute of Chemical Technology. The purity of oximes was analyzed using a high performance liquid chromatography (HPLC) technique. All other drugs and chemicals of analytical grade were obtained commercially and used without further purification. All substances were administered intramuscularly (i.m.) at a volume of 1 mL/kg body weight (b.w.) in rats and 10 mL/kg b.w. in mice.

Evaluation of acute toxicity of oximes

Before starting the evaluation of reactivating and therapeutic efficacy of oximes, the acute toxicity of tested oximes was evaluated in rats and mice by assessment of their LD_{50} values and their 95% confidence limits using probit-logarithmical analysis of death occurring within 24 h after

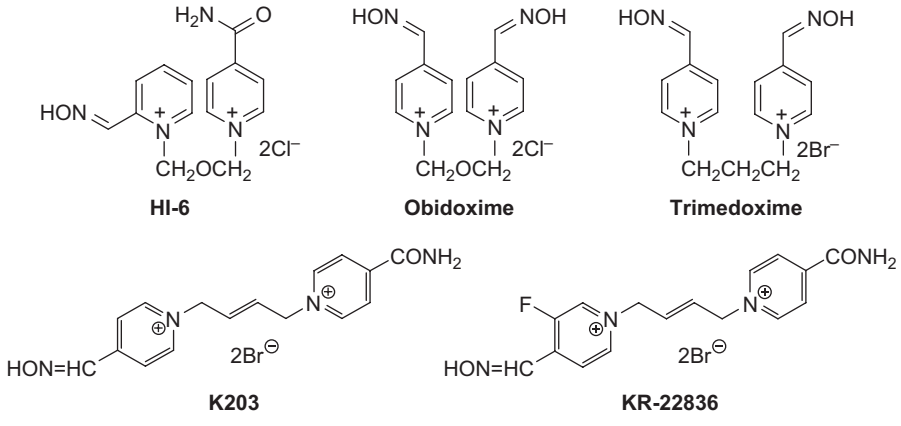

Figure 1. Chemical structures of oximes.

i.m. administration of each oxime at five different doses with eight animals per dose[17].

Evaluation of reactivating efficacy of oximes

To evaluate the reactivating efficacy of the oximes, the rats were injected i.m. with either atropine (21 mg/kg) alone or atropine (21 mg/kg) in combination with one of the oximes studied in equimolar doses (50 µmol/kg), 5 min before the rats received tabun i.m. at a dose of 180 µg/kg (LD_{50}). The prophylactic administration of antidotes was used because this procedure is suitable for a mechanistic study that compares the reactivating efficacy of various oximes. The technique should give better results than the treatment of animals after poisoning, and reduce the influence of aging of the nerve agent–AChE complex[18]. Moreover, some oximes are planned to be used prophylactically in certain chemical warfare scenarios[4]. The rats were decapitated and exsanguinated to obtain the blood 30 min subsequent to tabun poisoning. The blood was hemolyzed in Tris-HCl buffer (0.02 mol/L, pH 7.6, 1:20). The tissues, diaphragm and brain, were removed and homogenized in Tris-HCl buffer (0.02 mol/L, pH 7.6, 1:10) to determine AChE activity by the standard spectrophotometric method of Ellman *et al.*[19]. Acetylthiocholine was used as substrate (Tris-HCl buffer, 0.1 mol/L, pH 7.6). A Helios Alpha spectrophotometer was used for determination of absorbance at 436 nm. The AChE activity is expressed as µkat/kg or L (µmol substrate hydrolyzed/kg wet tissue or L blood within 1 s). The untreated control values for blood, diaphragm, and brain AChE activity were obtained from rats administered with saline instead of tabun and antidotes (saline control). The reactivation extent (%) was calculated using the AChE activity values: {1 – [((saline control) – (oxime + atropine))/((saline control) – (atropine control))]} × 100[18].

Evaluation of therapeutic efficacy of oximes

The potency of oximes in combination with atropine to eliminate tabun-induced lethal effects in mice was determined as follows. The LD_{50} value of tabun and its 95% confidence limits in non-treated tabun-poisoned mice was assessed using probit-logarithmical analysis of death occurring within 24 h after i.m. administration of tabun at five different doses with eight mice per dose[17]. Then, tabun-poisoned mice were treated i.m. with one of the tested oximes at equitoxic doses (5% LD_{50}) in combination with atropine (21 mg/kg), 1 min after i.m. challenge of tabun. The LD_{50} values of tabun and their 95% confidence limits in the treated tabun-poisoned mice were assessed by the same method. The efficacy of tested antidotal mixtures is expressed as a protective ratio (LD_{50} value of tabun in protected mice/LD_{50} value of tabun in unprotected mice). Statistical significance was determined by the use of Student's *t*-test and differences were considered significant when $p < 0.05$. Statistical evaluation was determined with the relevant computer programs[17].

Results

The acute i.m. toxicity of tested oximes is summarized in Table 1. The results show that the acute toxicity of the newly developed oxime K203 and its fluorinated analog KR-22836 is a little higher than the acute toxicity of obidoxime and trimedoxime in mice, but is significantly lower than the acute toxicity of obidoxime and trimedoxime in rats. Unfortunately, we were not able to calculate the LD_{50} value for KR-22836 in rats due to the limitation of its solubility. According to our results, the oxime HI-6 can be considered to be the least toxic for both animal species.

The ability of oximes to reactivate tabun-inhibited AChE in rat blood, diaphragm, and brain *in vivo* is shown in Table 2. Both newly developed oximes (K203 and its fluorinated analog KR-22836) seem to be effective reactivators of tabun-inhibited AChE in blood and diaphragm, but only the oxime K203 is able to significantly reactivate tabun-inhibited AChE in brain ($p < 0.05$). The reactivating efficacy of K203 is higher in comparison with the potency of obidoxime and trimedoxime to reactivate tabun-inhibited AChE in blood and brain and corresponds to the reactivating efficacy of trimedoxime in diaphragm. On the other hand, the potency of the oxime KR-22836 to reactivate tabun-inhibited AChE just corresponds to the reactivating efficacy of obidoxime and trimedoxime in blood and diaphragm. In addition, it is a significantly less effective reactivator of tabun-inhibited AChE than all other oximes tested in brain. Thus, the reactivating efficacy of KR-22836 corresponds to the potency of another newly developed oxime K203 to reactivate tabun-inhibited AChE in diaphragm only, and is significantly lower compared to K203 in blood and brain ($p < 0.05$) (Table 2).

These results correlate with the therapeutic potency of the oximes tested against lethal tabun poisoning in mice (Table 3). Tabun-poisoned mice showed a wide spectrum of clinical signs of poisoning including muscarinic (salivation) and nicotinic (tonic–clonic convulsions) signs within a few minutes, regardless of the type of antidote. They died within 20–30 min after poisoning with tabun. While the oxime KR-22836 was able to decrease the acute toxicity of tabun approximately 1.5-fold and, thus, its therapeutic efficacy was lower compared to the effectiveness of obidoxime and trimedoxime, the potency of the oxime K203 to reduce the acute lethal toxic effects of tabun in mice corresponded to the therapeutic efficacy

Table 1. LD_{50} values of oximes following intramuscular administration in rats and mice.

Oxime	LD_{50} (mg/kg) (95% confidence limits)	
	Rats	Mice
Obidoxime	211.1 (176.4–252.6)	188.4 (156.3–208.0)
HI-6	781.3 (738.4–826.6)	671.3 (627.4–718.3)
Trimedoxime	150.5 (142.1–159.4)	149.3 (124.1–184.5)
K203	326.4 (285.4–373.2)	95.0 (88.4–102.2)
KR-22836	>600	107.2 (86.0–134.0)

Table 2. Percent reactivation of tabun-inhibited AChE by oximes in rat blood, diaphragm, and brain *in vivo*.

	AChE activity (μkat/L or μkat/kg)		
Treatment	Blood	Diaphragm	Brain
Atropine	6.74 ± 0.02[a]	1.79 ± 0.80[a]	11.89 ± 2.68[a]
Atropine + obidoxime (% reactivation[b])	10.19 ± 0.64 (29.9[*†])	3.32 ± 0.57 (25.2[*])	15.47 ± 1.94 (2.9)
Atropine + HI-6 (% reactivation)	7.38 ± 0.58 (5.6)	2.50 ± 0.88 (8.9)	15.35 ± 3.43 (2.9)
Atropine + trimedoxime (% reactivation)	9.79 ± 0.79 (26.3[*†])	5.36 ± 1.47 (44.5[*†])	21.71 ± 4.66 (8.1[*])
Atropine + K203 (% reactivation)	11.07 ± 0.48 (37.5[*†])	5.28 ± 1.98 (43.5[*†])	29.34 ± 2.75 (14.6[*†])
Atropine + KR-22836 (% reactivation)	9.44 ± 0.36 (23.4[*†])	5.48 ± 0.62 (45.9[*†])	12.36 ± 1.71 (0.3)

Note. [*]Significantly different from the atropine group at a level of $p < 0.05$, [†]significantly different from the atropine + HI-6 group at a level of $p < 0.05$ as determined by Student's *t*-test.

[a]Mean ± SEM, $n = 8$. The untreated control value for rat blood AChE activity was 18.29 ± 1.33 μkat/L, for diaphragm AChE activity 9.83 ± 2.38 μkat/kg, and for brain AChE activity 131.4 ± 7.46 μkat/kg.

[b]Percent reactivation was determined using the AChE activity values: {1 – [((saline) – (oxime + atropine))/((saline) – (atropine control))]} × 100.

Table 3. Influence of type of oxime on the potency of antidotal treatment to reduce acute lethal effects of tabun in mice.

Treatment	LD_{50} (μg/kg) (95% confidence limits)	Protective ratio
—	331.5 (272.9–396.6)	—
Atropine	334.5 (272.0–381.1)	1.01
Obidoxime + atropine	565.6 (521.2–594.8)[*]	1.71
HI-6 + atropine	450.2 (394.2–543.6)	1.36
Trimedoxime + atropine	598.4 (495.5–681.5)[*]	1.81
K203 + atropine	564.2 (441.5–623.2)[*]	1.70
KR-22836 + atropine	504.7 (420.8–598.5)[*]	1.52

Note. [*]Significantly different from the untreated group at a level of $p < 0.05$.

of obidoxime and trimedoxime. In addition, the oxime HI-6 showed significantly lower potency to reduce the acute lethal toxic effects of tabun in mice in comparison with the other studied oximes. When atropine was used alone for the treatment of acute tabun poisonings, no therapeutic efficacy was found (Table 3).

Discussion

BBB penetration seems to be one of the key issues in development of the oxime type AChE reactivator, because all commonly used oximes have very low potency to reactivate nerve agent-inhibited AChE in the central nervous system (CNS) due to their limited penetration through the BBB. The potency of drugs to sufficiently penetrate across the BBB mainly depends on their lipophilicity. Enhanced lipophilicity should facilitate BBB penetration. One way to increase the lipophilicity of oximes is by their fluorination[15,16]. It was described that fluorine substitution can lead to an increase in BBB permeability due to changes in lipophilicity[20–22]. Therefore, the introduction of fluorine at heterocyclic ring positions of pyridinium oximes might represent a viable strategy for the enhancement of their lipophilicity, BBB permeability, and potency to reactivate nerve agent-inhibited AChE in the CNS. Fluorinated oximes were found to be more hydrophobic than non-fluorinated oximes[15,16].

Generally, commonly used monopyridinium and bispyridinium oximes seem to be relatively poor reactivators of tabun-inhibited AChE. The evaluation of their kinetic parameters characterizing *in vitro* reactivation of tabun-inhibited AChE showed that dissociation constants and rate constants are lower compared to the kinetic parameters describing the reactivation of sarin-, soman-, or cyclosarin-inhibited AChE by these oximes[23–25]. Therefore, several new structural analogs of currently available oximes have been developed to increase the potency of the oximes to reactivate tabun-inhibited AChE[26–28].

According to structure–activity analysis, the reactivating and therapeutic efficacy of oximes depends upon their chemical structure. The structure of a bridge connecting both pyridinium rings (in the case of bispyridinium oximes), the position of the oxime group, the chemical structure, and the position of a functional group situated on the second pyridinium ring are important factors influencing the potency of oximes to reactivate nerve agent-inhibited AChE[29]. To reach sufficient reactivating efficacy against tabun, both substituents are valuable when situated at position 4 of the pyridinium ring. The replacement of substituents to another usual position (position 2) decreases the reactivating efficacy of tested AChE reactivators against tabun[29]. This fact can explain the relatively low potency to reactivate tabun-inhibited AChE for the oxime HI-6, which is effective against fluorophosphonate-inhibited AChE[23–25], because the oxime HI-6 contains a dimethylether bridge and the oxime group at position 2. The chemical structure of the oxime HI-6 compared to other oximes studied is disadvantageous for the reactivation of tabun-inhibited AChE[30]. The oxime K203 was synthesized according to these structural requirements[12,13], and the results for evaluation of its reactivating, therapeutic, and neuroprotective efficacy show that it can be considered a very promising reactivator of tabun-inhibited AChE[14]. Nevertheless, the potency of the oxime K203 to reactivate tabun-inhibited AChE in the brain is rather low due to its limited penetration through the BBB. Therefore, its fluorinated derivative was designed and synthesized to be more effective in reactivating tabun-inhibited AChE in the CNS. Unfortunately, replacing hydrogen by fluorine at the heterocyclic ring position slightly decreased the potency of KR-22836 to reactivate tabun-inhibited AChE compared to K203, probably due to conformational changes making the entry of KR-22836 into the active center of AChE

more difficult. Our *in vivo* results correspond to the evaluation of the potency of K203 and KR-22836 to reactivate tabun-inhibited AChE *in vitro* using rat brain homogenate as a source of AChE (unpublished data).

According to our results, the newly developed oxime K203 seems to be significantly more efficacious to reactivate tabun-inhibited AChE in rats and reduce the lethal toxic effects of tabun in mice than its fluorinated analog KR-22836, as well as currently available oximes, and therefore it is suitable for the replacement of commonly used oximes for the treatment of acute tabun poisoning.

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Declaration of interest

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