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A comparison of the therapeutic and reactivating efficacy of newly developed bispyridinium compounds (K206, K269) with currently available oximes against tabun in rats and mice

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Abstract

The potency of newly developed bispyridinium compounds (K206, K269) in reactivating tabun-inhibited acetylcholinesterase and eliminating tabun-induced lethal toxic effects was compared with commonly used oximes (obidoxime, trimedoxime, the oxime HI-6) using *in vivo* methods. Studies which determined percentage of reactivation of tabun-inhibited blood and tissue AChE in poisoned rats showed that the reactivating efficacy of both newly developed oximes is comparable with obidoxime and trimedoxime in blood but lower than the reactivating potency of trimedoxime and obidoxime in the diaphragm and brain. Nevertheless, the differences in reactivating efficacy of obidoxime, trimedoxime and K206 was not significant while the potency of K269 to reactivate tabun-inhibited acetylcholinesterase was significantly lower. Both newly developed oximes were also found to be relatively efficacious in elimination of the lethal toxic effects in tabun-poisoned mice. Their therapeutic efficacy corresponds to the therapeutic potency of obidoxime. The oxime HI-6, relatively efficacious against soman, did not seem to be an adequately effective oxime in reactivation of tabun-inhibited AChE and to counteract lethal effects of tabun. Both newly developed oximes (K206, K269) are significantly more efficacious in reactivating tabun-inhibited AChE in rats and to eliminate lethal toxic effects of tabun in mice than the oxime HI-6 but their reactivating and therapeutic potency does not prevail over the effectiveness of currently available obidoxime and trimedoxime and, therefore, they are not suitable for their replacement of commonly used oximes for the treatment of acute tabun poisoning.

Keywords: *tabun, acetylcholinesterase, reactivation, K206, K269, obidoxime, trimedoxime, HI-6, inhibition, acetylthiocholine*

Introduction

The current standard treatment for poisoning with organophosphorous compounds called nerve agents usually consists of the combined administration of anticholinergic drugs (preferably atropine) and oximes (preferably pralidoxime or obidoxime). Anticholinergic drugs block the effects of overstimulation by acetylcholine accumulated at muscarinic receptor sites while oximes, compounds with nucleophilic oximate anion, repair biochemical lesions by dephosphorylating tabun-inhibited acetylcholinesterase (AChE, EC 3.1.1.7) and restoring its activity [1,2].

Tabun (*O*-ethyl-*N,N*-dimethyl phosphoramidocyanidate) is an organophosphorus compound used as a chemical warfare agent for military as well as terrorist purposes. Its deleterious effects are extraordinarily difficult to antagonize because of the existence of a free electron pair located on the amidic nitrogen and conformational changes of AChE-tabun complex prior an aging process in AChE active site that make the nucleophilic attack of oximes almost impossible [3,4,5]. While anticholinergic drugs such as atropine are able to counteract the effects of tabun at peripheral cholinergic receptors [6], commonly used reactivators

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of phosphorylated AChE based on monopyridinium (e.g. pralidoxime) and bispyridinium oximes (e.g. obidoxime, methoxime) are not able to counteract the acute toxic effects of tabun because of their minimal reactivating efficacy [7]. In addition, the reactivating efficacy of the oxime HI-6, which is relatively efficacious against adverse effects of soman [8], is not as efficient for tabun-inhibited AChE [9,10]. 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. New bispyridinium compounds, K206 (*E*)-1-(3-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide and K269 (*E*)-1-[4-(1-aminohydroxyiminomethyl)-pyridinium]-4-(4-hydroxyiminomethyl-pyridinium)-but-2-ene dibromide (Figure 1) were synthesized at our Department of Toxicology [11,12] to improve the efficacy of antidotal treatment in reactivating tabun-inhibited AChE and eliminating tabun-induced lethal toxicity. The evaluation of their potency to reactivate tabun-inhibited AChE using *in vitro* methods showed that the reactivating efficacy of both newly developed oximes is similar to the effectiveness of obidoxime and better than the potency of HI-6 to reactivate tabun-inhibited AChE at both concentrations studied (10^{-3}, 10^{-5} M) [11,12]. *In vitro* assessment of reactivating efficacy of oximes is usually followed by the evaluation of their reactivating efficacy *in vivo* and their therapeutic efficacy against lethal nerve agent poisoning. The aim of this study was to compare the reactivating and therapeutic efficacy of newly developed oximes (K206, K269) with currently available oximes (obidoxime, trimedoxime, the oxime HI-6) against tabun using *in vivo* methods.

Material and methods

Male albino Wistar rats weighing 190–225 g and NMRI male mice weighing between 22 and 25 g were purchased from Konarovice, Czech Republic. They were kept in an air-conditioned room with the light from 07:00 to 19:00 hr and were allowed access to standard food and tap water *ad libitum*. The rats were divided into groups of 8 animals. Handling of the experimental animals was done under the supervision of the Ethics Committee of the Faculty of Military Health Sciences, Czech Republic.

Tabun was obtained from the Technical Institute in Brno (Czech Republic) and was 95% pure. All oximes (obidoxime, trimedoxime, the oxime HI-6, K206, K269) were synthesized at the Department of Toxicology of the Faculty of Military Health Sciences (Czech Republic). Their purities were analyzed using a 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.).

Before starting the evaluation of reactivating and therapeutic efficacy of oximes, the acute toxicity of tested oximes was evaluated in rats and mice by the assessment of their LD_{50} values and their 95% confidence limits using probit-logarithmical analysis of death occuring within 24 h after i.m. administration of each oxime at five different doses with eight animals per dose [13].

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 equitoxic, human relevant dose (5% LD_{50}) 5 min before the rats received tabun i.m. at a dose of 200 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 nerve agent-AChE complex [14]. Moreover, some oximes are planned to be used prophylactically in certain chemical warfare scenarios

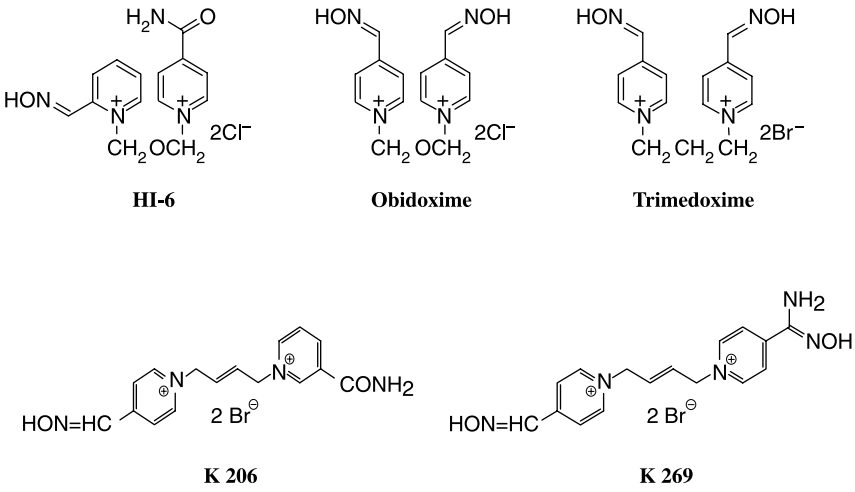

Figure 1. Chemical structure of oximes.

[6]. The rats were decapitated and exsanguinated to obtain the blood 30 min following tabun poisoning. The blood was hemolyzed in Tris-HCl buffer (M = 0.02 mol/L, pH 7.6, 1:20). The tissues, diaphragm and brain were removed and homogenized in Tris-HCl buffer (M = 0.02 mol/L, pH 7.6, 1:10) to determine AChE activity by standard spectrophotometric method of Ellman et al. [15]. Acetylthiocholine was used as substrate (Tris-HCl buffer, N = 0.1 mol/L, pH 7.6). Helios Alpha, the spectrophotometer was used for determination of absorbance at 436 nm. The AChE activity was 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 obtain from rats administered with saline instead of tabun and antidotes (saline control). The reactivation rate was calculated using the AChE activity values: {1- [((saline control) – (oxime + atropine))/((saline control) – (atropine control))]} × 100 [14].

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 limit in tabun-poisoned mice was assessed using probit-logarithmical analysis of death occuring within 24 h after i.m. administration of tabun at five different doses with eight mice per dose [13]. Then, tabun-poisoned mice were treated i.m. with one of tested oximes at equitoxic doses (5% LD_{50}) in combination with atropine (21 mg/kg) at 1 min after i. m. challenge of tabun. The LD_{50} values of tabun and their 95% confidence limit in treated, tabun-poisoned mice were assessed by the same method. The efficacy of tested antidotal mixtures was expressed as 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 [13].

Results

The acute i.m. toxicity of tested oximes is summarized in Table I.The results show that the acute toxicity of newly developed oxime K206 corresponds to the acute toxicity of obidoxime and trimedoxime in mice but it is lower than the acute toxicity of obidoxime and trimedoxime in rats. On the other hand, another newly developed oxime K269 is significantly more toxic than all other oximes studied in mice as well as in rats. 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 II. Both newly developed oximes seem to be effective reactivators of tabun-inhibited AChE in blood but only the oxime K206 is able to markedly reactivate tabun-inhibited AChE in diaphragm and brain. Its reactivating efficacy is lower in comparison with potency of obidoxime and trimedoxime to reactivate tabun-inhibited AChE in diaphragm and brain but the differences in their reactivating potency are not significant. On the other hand, the oxime K269 is significantly weeker reactivator of tabun-inhibited AChE than obidoxime and trimedoxime in diaphragma and brain. Its reactivating efficacy corresponds to the reactivating potency of the oxime HI-6 that is considered to be the worst reactivator of tabun-inhibited AChE among currently available oximes.

Table I. LD_{50} values of oximes following i.m. administration in rats and mice.

	LD_{50} (mg/kg) ± 95% confidence limit	
OXIMES	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)
K206	386.6 (332.2–449.9)	175.1 (164.1–186.9)
K269	113.5 (92.1–138.9)	43.9 (41.5–46.5)

These results correlate with the therapeutical potency of the oximes tested against lethal tabun poisoning in mice (Table III). Tabun – poisoned mice showed wide spectrum of clinical signs of poisoning including muscarinic (salivation) and niconitic (tonic-clonic convulsions) signs within a few minutes regardless of the type of antidotes. They died within 20–30 minutes after poisoning with tabun. Both newly developed oximes (K206, K269) were able to decrease the acute toxicity of tabun approximately 1.5-fold. Their therapeutic efficacy corresponds to the potency of obidoxime and trimedoxime in decreasing acute toxicity of tabun. On the other hand, the oxime HI-6 showed significantly lower potency to eliminate acute lethal toxic effects of tabun in mice.

Discussion

Generally, currently used monopyridinium and bispyridinium oximes seem to be relatively poor reactivators of tabun-inhibited AChE. Therefore, new structural analogues of currently available oximes have been developed to increase the potency of oximes to reactivate tabun-inhibited AChE [16,17]. The values of kinetic parameters of the oximes tested for the reactivation of tabun-inhibited AChE *in vitro* showed that dissociation constants and rate constants are lower compared to kinetic parameters describing the reactivation of sarin, soman or cyclosarin-inhibited AChE by these oximes [18,19,20,21]. Their reactivating efficacy depends upon the chemical structure of bridge connecting both pyridinium rings (in the case of

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

	AChE activity (μkat/L or μkat/kg) Blood	Diaphragm	Brain
Atropine	3.93 ± 0.21[a]	5.89 ± 1.25[a]	11.7 ± 4.32[a]
Atropine + obidoxime (% reactivation[b])	5.51 ± 0.34 (24.2*[x])	8.78 ± 0.58 (19.2*)	35.2 ± 6.97 (25.3*[x])
Atropine + HI-6 (% reactivation)	4.50 ± 0.53 (8.7)	6.99 ± 1.97 (7.3)	13.6 ± 4.79 (2.1)
Atropine + trimedoxime (% reactivation)	4.91 ± 0.21 (15.0*)	8.71 ± 1.01 (18.7*)	25.0 ± 8.07 (14.4*)
Atropine + K206 (% reactivation)	5.53 ± 0.27 (24.5*[x])	7.61 ± 0.85 (11.4)	19.0 ± 2.90 (7.8*)
Atropine + K269 (% reactivation)	5.73 ± 0.24 (27.6*[x])	6.49 ± 1.23 (4.0)	11.0 ± 2.7 (0)

[a] Means ± S.E.M., N = 8. The untreated control value (saline control) for rat blood AChE activity was 10.45 (μkat/L), for diaphragm AChE activity 20.99 μkat/kg and for brain AChE activity 104.7 μkat/kg; [b] Percent reactivation was determined using the AChE activity values: {1- [((saline control) – (oxime + atropine))/((saline control) – (atropine control))]} x 100; * Significantly different from the atropine group at a level of $P < 0.05$, [x] significantly different from the atropine + HI-6 group at a level of $P < 0.05$ as determined by the Student's test.

bispyridinium oximes), the position of oxime group, the chemical structure and the position of the substituent situated on the second pyridinium ring [10,22]. To reach sufficient reactivating efficacy, both substituents should be situated on the position 4. The replacement of substituents to another common position (position 2) usually decreases the potency of tested oximes to reactivate tabun-inhibited AChE [22]. This fact can explain relatively low efficacy of the oxime HI-6, which is effective against fluorophosphonates [19,20,21], 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 [18].

Previously published *in vitro* results correlate with our results obtained *in vivo*. Both newly developed oximes (K206, K269), that were characterized by moderate percentage of reactivation of tabun-inhibited AChE *in vitro* [11,12], were found to be relatively efficacious reactivators of tabun-inhibited AChE in blood of tabun-poisoned rats and relatively efficacious to protect mice poisoned with lethal doses of tabun. On the other hand, their potency to reactivate tabun-inhibited AChE in diaphragm and brain of tabun-poisoned rats is lower compared to obidoxime and trimedoxime. Nevertheless, the K206-induced % reactivation of tabun-inhibited AChE in blood, diaphragm and almost in brain reached 10% that is considered to be necessary for survival of nerve agent - poisoned animals [23]. The lower reactivating efficacy of both newly developed oximes (especially K269) in peripheral and central nervous system can be caused by the position 3 of the substituent (carbamoyl group) situated on the second pyridinium ring for K206 and by low dosage due to relatively high acute toxicity for K269. On the contrary of other oximes studied, the oxime HI-6, that seems to be a poor tabun-inhibited AChE reactivator *in vitro*, is not able to sufficiently reactivate tabun-inhibited AChE in tabun-poisoned rats nor protect mice poisoned with lethal doses of tabun when it is administered at human-relevant doses. Its therapeutic efficacy corresponds to the effectiveness of atropine alone in the case of the treatment of tabun-poisoned mice [24].

Our results confirm that there is no single, broad-spectrum oxime suitable for the antidotal treatment of poisonings with all organophosphorus agents [1,25]. While trimedoxime and obidoxime are preferred for the treatment of acute poisoning with organophosphorus insecticides (OPI) because they are considered to be sufficiently effective reactivators of OPI-inhibited AChE [26,27,28], the oxime HI-6 appears to be a promising antidote against highly toxic fluorophosphonates, especially soman and cyclosarin, because it is able to protect experimental animals from adverse effects and improve survival of poisoned animals [19,20]. Nevertheless, our results clearly demonstrate its low potency to reactivate tabun-inhibited AChE in rats and protect tabun-poisoned mice from its lethal toxic effects. Trimedoxime as well as obidoxime seem to be more effective oximes for the treatment of acute tabun poisonings than the oxime HI-6 but their potency to eliminate tabun-induced

Table III. The influence of the type of oxime on the potency of antidotal treatment to eliminate acute lethal effects of tabun in mice.

Treatment	LD_{50} (μg/kg) ± 95% confidence limit	Protective ratio
–	295.2 (275.5 – 317.9)	–
Obidoxime + atropine	435.7 (410.8 – 460.5)*[x]	1.47
HI-6 + atropine	318.8 (302.7 – 336.5)	1.08
Trimedoxime + atropine	504.8 (460.3 – 553.0)*[x]	1.71
K206 + atropine	436.9 (395.3 – 466.7)*[x]	1.48
K269 + atropine	431.0 (404.9 – 459.6)*[x]	1.46

* significantly different from the untreated group at the level of $P < 0.05$, [x] significantly different from the group treated by atropine in combination with HI-6 at the level of $P < 0.05$.

lethal effects is limited, when they are administered at low, human-relevant doses. They are not able to reach 70% reactivation of tabun-inhibited AChE that is necessary for non-toxic equilibrium state [23]. Both newly developed oximes (especially K206) are more efficacious to reactivate tabun-inhibited AChE in rats and to eliminate lethal toxic effects of tabun in mice than the oxime HI-6, but their reactivating and therapeutic potency does not prevail the effectiveness of currently available obidoxime and trimedoxime and, therefore, they are not suitable for the replacement of commonly used oximes for the treatment of acute tabun poisoning.

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