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A comparison of the reactivating and therapeutic efficacy of two novel bispyridinium oximes (K920, K923) with the oxime K203 and trimedoxime in tabun-poisoned rats and mice

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ABSTRACT

The potency of two novel oximes (K920, K923) to reactivate tabun-inhibited acetylcholinesterase and to reduce acute toxicity of tabun was compared with the oxime K203 and trimedoxime using *in vivo* methods. The study determining percentage of reactivation of tabun-inhibited peripheral acetylcholinesterase (diaphragm) and central acetylcholinesterase (brain) in tabun-poisoned rats showed that the reactivating efficacy of both newly developed oximes is lower than the reactivating potency of the oxime K203 and trimedoxime. The therapeutic efficacy of both newly developed oximes roughly corresponds to their weak reactivating efficacy. Their potency to reduce acute toxicity of tabun in mice was lower compared to the oxime K203 and trimedoxime. All differences in reactivating efficacy of oximes and different protective ratios were found for selected doses of oximes used in this study. Based on the results obtained, we can conclude that the reactivating and therapeutic potency of both newly developed oximes does not prevail the effectiveness of the oxime K203 and trimedoxime and, therefore, they are not suitable for their replacement of commonly used oximes for the treatment of acute tabun poisoning. The conclusion is only relevant for the experimental animals used in this study because of remarkable species differences in reactivating properties of oximes.

Introduction

Organophosphorus compounds have been used as pesticides and developed as chemical warfare agents called nerve agents. They are potent and persistent inhibitors of acetylcholinesterase (AChE, EC 3.1.1.7) in the central and peripheral nervous system. The inhibition of AChE after exposure to nerve agents leads to the accumulation of the neurotransmitter acetylcholine in synapses and to subsequent overstimulation of

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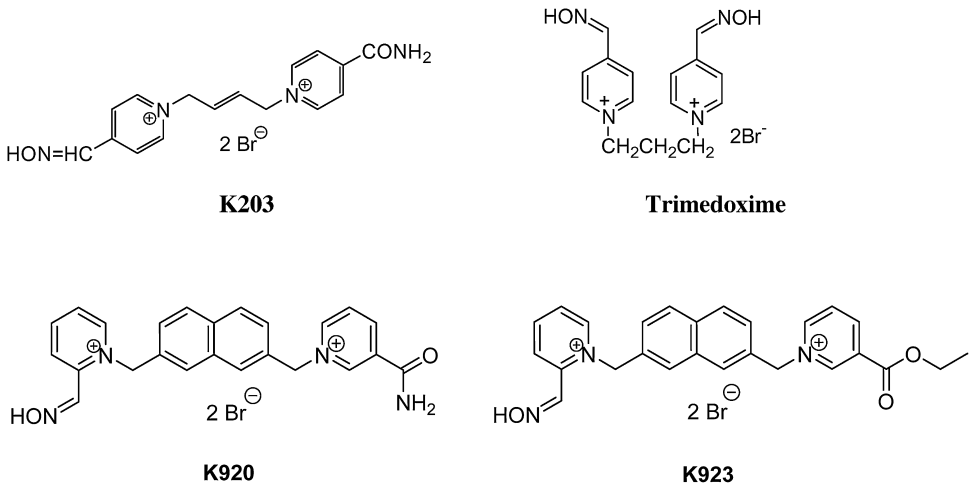

Fig. 1 – Chemical structure of oximes.

postsynaptic cholinergic receptors that results in muscarinic and nicotinic signs and symptoms including excitotoxicity, seizures and brain damage. The death is usually caused by respiratory failure resulting from bronchospasm, excessive bronchial secretion, paralysis of respiratory muscles, and depression of brain respiratory centres (Bajgar, 2004; Delfino et al., 2009). A current standard antidotal treatment of poisoning with nerve agents usually consists of a combined administration of an anticholinergic drug (preferably atropine) and an oxime (preferably pralidoxime or obidoxime). Generally, anticholinergics are used for relieving muscarinic signs and symptoms whereas oximes are used for reactivation of nerve agent-inhibited AChE (Bajgar, 2004).

Tabun (*O*-ethyl-*N*,*N*-dimethylphosphoramidocyanidate) is a well known nerve agent that presents a serious threat to military and civilian population. It differs from other highly toxic organophosphorus compounds 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 conformational changes of AChE-tabun complex in the AChE active site that make the nucleophilic attack of oximes very difficult (Cabal and Bajgar, 1999; Ekström et al., 2006).

Unfortunately, currently available antidotal treatment consisting of atropine and commonly used reactivator of inhibited AChE (pralidoxime, obidoxime, trimedoxime, HI-6) is not able to sufficiently counteract acute toxic effects of tabun because of low ability of oximes to reactivate tabun-inhibited AChE (Jokanovic and Prostran, 2009; Jokanovic, 2012; Wilhelm et al., 2014). Therefore, the antidotal treatment of acute poisoning with tabun still remains a serious problem and the development of new and more effective AChE reactivator is still very important (Sharma et al., 2015).

The oxime K203, developed at our Department of Toxicology and Military Pharmacy ten years ago, was considered to be promising reactivator of tabun-inhibited AChE. However, the differences between the reactivating and therapeutic efficacy of the oxime K203 and commonly used bispyridinium oximes (obidoxime, trimedoxime) are relatively small (Kassa et al., 2008). Therefore, we are still searching for a more efficacious oxime able to sufficiently reactivate tabun-inhibited AChE. For this purpose, two novel oximes, K920 [(naphtylene-2,7-diyl)-1-(4-carbamoylpyridinium)-1′-(2-hydroxyiminomethylpyridinium) dibromide] and K923 [(naphtylene-2,7-diyl)-1-(4-ethylcarboxylpyridinium)-1′-(2-hydroxyiminomethylpyridinium) dibromide] (Fig. 1), were synthesized at our Department of Toxicology and Military Pharmacy to improve the efficacy of antidotal treatment in reactivating tabun-inhibited AChE and eliminating tabun-induced lethal toxicity. They were developed based on the structure activity relationship study and they were chosen based on the data obtained from molecular docking and *in vitro* evaluation of their ability to reactivate acetylcholinesterase inhibited by organophosphorus compounds. Based on the evaluation of their potency to reactivate tabun-inhibited hAChE using *in vitro* methods, they proved to be relatively potent reactivators of tabun-inhibited hAChE *in vitro* comparable with trimedoxime and obidoxime. *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 two newly developed oximes (K920, K923) with the oxime K203 and trimedoxime against tabun using *in vivo* methods.

Materials and methods

Animals

Male albino Wistar rats weighing 200–230 g and NMRI male mice weighing between 20 and 24 g were purchased from VELAZ, Czech Republic. They were kept in climate- and access-controlled rooms (22 ± 2 °C and $50 \pm 10\%$ relative humidity) with the light from 07:00 h to 19:00 h and were allowed access to standard food and tap water *ad libitum*. The rats and mice were acclimatized in the laboratory vivarium for 14 days before starting the experiments, and they 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.

Chemicals

Tabun was obtained from the Technical Institute in Brno (Czech Republic) and was 92% pure. Its purity was assayed by acidimetric titration. The basic solution of tabun (1 mg/1 mL) was prepared in propyleneglycol three days before starting the experiments. Actual solution of tabun was prepared from its basic solution with the help of saline immediately before its administration. All oximes (K920, K923, K203, trimedoxime) were synthesized at our Department of Toxicology and Military Pharmacy of the Faculty of Military Health Sciences (Czech Republic). Their purity was analyzed using HPLC technique with UV detection (310 nm) and they were more than 96% pure (Jun et al., 2007). All other drugs and chemicals of analytical grade were obtained commercially (Sigma-Aldrich) and used without further purification. The saline solution (0.9% NaCl) was used as a vehicle. All substances were administered intramuscularly (i.m.) at a volume of 1 mL/kg body weight (b.w.) to rats and 10 mL/kg b.w. to mice.

In vivo *experiments*

Before starting the evaluation of reactivating and therapeutic efficacy of the oximes, the acute toxicity of tested oximes was determined in rats and mice by the assessment of their LD_{50} values and their 95% confidence limits using probit-logarithmical analysis of death occurring within 24 h after i.m. administration of each oxime at five different doses with eight animals per dose (Tallarida and Murray, 1987).

To evaluate the reactivating efficacy of the oximes, the rats were administered i.m. with either atropine alone or atropine in combination with one of the studied oximes. Atropine was administered at a dose of 10 mg/kg that is considered to be sufficiently effective but safe. It corresponds to 2% of its LD_{50} value. The oximes were administered at equitoxic doses corresponding to 5% of their LD_{50} values at 1 min after the rats received tabun i.m. at a dose of 390 μg/kg (LD_{50}). One-minute time interval was chosen by us to evaluate the maximal reactivating efficacy of all oximes studied. The rats were decapitated at 60 min after tabun administration, totally exsanguinated and the tissues (diaphragm and brain) were removed and immediately frozen at the temperature −70 °C. Within three days, they were homogenized in Tris–HCl buffer (0.02 mol/L, pH 7.6, 1:10) to determine AChE activity by standard spectrophotometric method (Ellman et al., 1961). Acetylthiocholine was used as a substrate (Tris–HCl buffer, N = 0.1 mol/L, pH 7.6). Helios Alpha, the spectrophotometer was used for determination of absorbancy at 436 nm. The AChE activity was expressed as μkat/kg (μmol substrate hydrolyzed/kg wet tissue within 1 s). The untreated control values for diaphragm and brain AChE activity were obtained from rats administered with saline buffer (physiological solution – 0.9% NaCl) instead of tabun and antidotes (saline control). The percentage of reactivation was calculated using the AChE activity values: {1 − [((saline control) − (oxime + atropine))/((saline control) − (atropine control))]} × 100 (Clement et al., 1999). All experiments were performed in the same part of the day (from 08:00 h to 10.00 h). The variability was statistically evaluated by the standard deviation (SD) calculated for each group. The differences between groups were calculated using means ± SD and the statistical significance was tested by one-way ANOVA test with Scheffe's *post hoc* test. The differences were considered significant when $2\alpha = 0.05$.

The potency of atropine alone and atropine in combination with one of tested oximes 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 occurring within 24 h after i.m. administration of tabun at five different doses with eight mice per dose (Tallarida and Murray, 1987). Then, tabun-poisoned mice were treated i.m. with atropine alone or with atropine in combination with one of tested oximes. Atropine was administered at a dose of 10 mg/kg that is considered to be sufficiently effective but safe. It corresponds to 5% of its LD_{50} value. The oximes were administered at equitoxic doses corresponding to 5% of their LD_{50} values at 1 min after i.m. challenge of tabun. One-minute time interval was chosen by us to evaluate the maximal therapeutic efficacy of all oximes studied. The LD_{50} values of tabun and their 95% confidence limit in tabun-poisoned mice treated with antidotes were assessed by the same method. The efficacy of tested antidotes 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 one-way ANOVA test with Scheffe's *post hoc* test and differences were considered significant when $2\alpha = 0.05$.

Results

The acute i.m. toxicity of tested oximes is summarized in Table 1. The results show that the acute toxicity of both novel bispyridinium oximes K920 and K923 is markedly higher than the acute toxicity of the oxime K203 and trimedoxime in rats as well as mice. The acute toxicity of both newly developed oximes is similar and rather high.

The ability of oximes at the selected doses to reactivate tabun-inhibited AChE in rat diaphragm and brain *in vivo* is shown in Table 2. Only one newly developed oxime K920 was able to reactivate tabun-inhibited AChE in the diaphragm although its reactivating efficacy was lower compared to trimedoxime and the oxime K203. The potency of another novel bispyiridinium oxime K923 to reactivate tabun-inhibited AChE in the diaphragm was negligible. The ability of both

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

OXIMES	LD_{50} (mg/kg) ± 95% confidence limit	
	Rats	Mice
K203	326.4 (285.4–373.2)	137.8 (116.2–163.3)
Trimedoxime	258.2 (220.4–267.2)	105.8 (93.3–112.2)
K920	5.38 (3.24–8.92)	3.26 (2.18–4.46)
K923	12.74 (8.72–16.60)	4.61 (3.11–7.42)

Table 2 – Percentage of reactivation of tabun-inhibited AChE by oximes in rat diaphragm and brain *in vivo*.

Treatment	AChE activity (μkat/kg)	
	Diaphragm	Brain
Saline control	7.68 ± 1.28	87.40 ± 6.90[a]
Atropine control	1.88 ± 0.69[*]	6.32 ± 2.28[*]
Atropine + **K203** (% reactivation[b])	**3.78 ± 1.04**[*,**] **(32.7)**	9.96 ± 2.29[*] (4.5)
Atropine + **trimedoxime** (% reactivation)	**3.02 ± 0.65**[*,**] **(19.5)**	9.04 ± 2.81[*] (3.4)
Atropine + **K920** (% reactivation)	2.34 ± 1.09[*] (7.8)	7.05 ± 2.08[*] (1.1)
Atropine + **K923** (% reactivation)	1.69 ± 0.75[*] (0)	6.43 ± 1.39[*] (0.2)

[a] Means ± SD, $N = 8$.
[b] % reactivation was determined using the AChE activity values: {1 − [((saline control) − (oxime + atropine))/((saline control) − (atropine control))]} × 100.
[*] Significantly different from the saline control group at the level of $2\alpha = 0.05$.
[**] Significantly different from the atropine control group at the level of $2\alpha = 0.05$.

newly developed bispyridinium oximes (K920, K923) to reactivate tabun-inhibited AChE in the brain is very low and it did not reach the reactivating efficacy of other oximes studied (trimedoxime, K203) although trimedoxime and the oxime K203 are weak reactivators of tabun-inhibited AChE in the brain, too. To compare the reactivating efficacy of both newly developed oximes, the oxime K920 showed a slightly higher reactivating efficacy compared to the oxime K923 in the diaphragm as well as in the brain. However, the difference between reactivating efficacy of both newly developed oximes was not significant. Based on the statistical evaluation of the obtained results, statistically significant differences between the activity of AChE in tabun-poisoned rats treated with atropine alone and rats treated with atropine in combination with one of tested oximes were only found in diaphragm when currently available oximes (trimedoxime, K203) were used for the antidotal treatment of tabun poisoning.

A comparison of the therapeutic efficacy of newly developed oximes (K920, K923) with the therapeutic efficacy of the oxime K203 and trimedoxime at the doses selected for this study roughly corresponds to the comparison of their reactivating efficacy (Table 3). 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 type of antidotal treatment. They died within 20–45 min after poisoning with tabun. The therapeutic potency of atropine alone was the lowest and the therapeutic efficacy of both newly developed oximes was similar and lower compared to the oxime K203 and trimedoxime. When the oxime K203 or trimedoxime was administered, tabun-induced muscarinic and nicotinic signs were attenuated. While both novel oximes were able to decrease acute toxicity of tabun approximately 1.2-fold, the oxime K203 and trimedoxime decreased the acute toxicity of tabun almost two times. Thus, the higher therapeutic efficacy of the oxime K203 and trimedoxime corresponds to their higher reactivating efficacy. All differences in reactivating efficacy of oximes and different protective ratios were found for the selected doses of oximes used in this study.

Table 3 – 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
–	317.1 (278.9–340.7)	–
Atropine	366.1 (349.1–391.6)[*]	1.15
K203 + atropine	**571.4 (502.2–649.2)**[*,**]	**1.80**
Trimedoxime + atropine	**581.8 (505.2–709.6)**[*,**]	**1.83**
K920 + atropine	400.2 (377.0–430.8)[*]	1.26
K923 + atropine	387.6 (363.3–418.5)[*]	1.20

[*] Significantly different from the untreated group at the level of $2\alpha = 0.05$.
[**] Significantly different from the atropine group and group treated by atropine in combination with K920 (K923) at the level of $2\alpha = 0.05$.

Discussion

Oximes are not equally effective against all organophosphorus compounds (OPC) including nerve agents and experimental as well as clinical evaluation of their reactivating and therapeutic efficacy is still a matter of controversy. The efficacy of oximes depends on many factors, especially on the chemical structure of OPC and the rate of ageing of enzyme-inhibitor complex. A particular oxime may be effective against a specific OPC and ineffective against others (Nurulain, 2011). In addition, AChE inhibited by OPC undergoes a process of ageing and an aged enzyme cannot be reactivated. The ageing kinetics of different OPC is different, ranging from a few minutes to many hours (Antonijevic and Stojiljkovic, 2007). Other factors that influence the effectiveness of oxime therapy include inhibition potency of OPC, their toxicokinetics, reactivating potency of oximes and their pharmacokinetics, correct dosing, evaluation for the persistent need of oxime therapy and correct timing (Antonijevic and Stojiljkovic, 2007; Nurulain, 2011). Clinical opinions on the value of oximes as adjunct in the treatment of human poisoning with OPC remain divided. While some authors reported disappointing experiences with oximes (Eddleston et al., 2002; Peter et al., 2006), according to other authors, oximes are beneficial if used properly (Jokanovic, 2012; Wilhelm et al., 2014).

In the case of tabun, bispyridinium oximes seem to be more effective to reactivate peripheral tabun-inhibited AChE and to

counteract tabun-induced acute toxicity than monopyridinium oximes (Voicu et al., 2010). On the other hand, bispyridinium oximes are less lipophilic than monopyridinium oximes and, therefore, they poorly penetrate across the blood–brain barrier, in maximum of 6% (Lorke et al., 2008; Zdarova Karasova et al., 2010). For this reason, their ability to reactivate tabun-inhibited AChE in the brain is lower compared to the peripheral compartment (Lorke et al., 2008; Kalasz et al., 2015). To eliminate above-mentioned limitation of the effects of AChE reactivators, new analogues of bispyridinium oximes were developed to extend their properties (Berend et al., 2008; Kovarik et al., 2013). Beside the essential oxime functional group, there is possibility to improve their potency to reactivate tabun-inhibited AChE and to counteract acute toxicity of tabun *via* change of its side chain or connecting linker (de Jong et al., 1981).

The design of both newly developed oximes (K920, K923) was based on the data obtained during the extensive work on oxime development and from structure activity relationship studies realized at our Department of Toxicology and Military Pharmacy (Cabal et al., 2004; Kuca et al., 2006; Musilek et al., 2011). The structure activity relationship studies demonstrate that there are four important structural factors influencing the affinity of AChE reactivators towards nerve agent-inhibited AChE and subsequent oxime reactivation: the presence of quaternary nitrogen in the molecule of reactivator, the structure of the chain linking two pyridinium rings, the presence of the oxime group and the position of the oxime group at the pyridinium ring (Kuca et al., 2006; Worek et al., 2012; Voicu et al., 2015). Both newly developed oximes (K920, K923) were designed as reactivators with aromatic connecting linker that was formerly found to be beneficial for the reactivation of organophosphorus compounds *in vitro* and *in vivo* (Musilek et al., 2007, 2010; Nurulain et al., 2009). In addition, the oxime K923 was designed with ethoxycarbonyl moiety as a representative of carboxylic derivatives that were formerly found to decrease toxicity of the reactivator (Kassa et al., 2009).

Our results demonstrate that the potency of both newly developed bispyridinium oximes (K920 and K923) administered at the selected doses to reactivate tabun-inhibited AChE and reduce tabun-induced acute toxicity is relatively low and it does not achieve the efficacy of trimedoxime and the oxime K203. One reason for their weak effectiveness is their high toxicity. Small safe dosage of both oximes can explain their markedly lower reactivating and therapeutic efficacy compared to trimedoxime and the oxime K203. As the reactivating and therapeutic potency of both newly developed oximes does not prevail the effectiveness of the oxime K203 and trimedoxime, they are not suitable for their replacement of commonly used oximes for the treatment of acute tabun poisoning. However, this fact is only relevant for the animal species used in this study (rats and mice) because of remarkable species differences in reactivating properties of oximes.

Conclusions

The changes in the structure of commonly used oximes realized according to the postulated requirements (Kuca et al., 2006; Voicu et al., 2015) are not enough to markedly increase the potency of current antidotal treatment to reactivate tabun-inhibited AChE and decrease tabun-induced acute lethal toxic effects, probably due to conformational changes of AChE-tabun complex in AChE active site that make the nucleophilic attack of oximes very difficult (Cabal and Bajgar, 1999; Ekström et al., 2006). Thus, it is necessary to find a new approach how to find new structures of AChE reactivators enable to better enter into the active site of AChE inhibited by tabun.

Conflict of interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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