Journal of Applied Biomedicine 15 (2017) 49–53

Contents lists available at ScienceDirect

Journal of Applied Biomedicine

journal homepage: www.elsevier.com/locate/jab

Original research article

A comparison of the reactivating and therapeutic efficacy of two novel bispyridinium oximes (K305, K307) with the oxime K203 and trimedoxime in tabun-poisoned rats and mice

Jiri Kassa*, Vendula Sepsova, Anna Horova, Kamil Musilek

Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, Trebesska 1575, 500 01 Hradec Kralove, Czechia

ARTICLE INFO

Article history:
Received 13 May 2016
Accepted 1 September 2016
Available online 8 October 2016

Keywords:
Tabun
Acetylcholinesterase
Oximes
Rats
Mice

ABSTRACT

The reactivating and therapeutic efficacy of two newly developed oximes (K305, K307) was compared with the oxime K203 and trimedoxime using *in vivo* methods The study determining percentage of reactivation of tabun-inhibited acetylcholinesterase in the peripheral as well as central nervous system (diaphragm, brain) in tabun-poisoned rats showed that the reactivating efficacy of both newly developed oximes is lower compared to the reactivating efficacy of the oxime K203 and trimedoxime. The therapeutic efficacy of all oximes studied roughly corresponds to their reactivating efficacy. While the ability of the oxime K305 to reduce acute toxicity of tabun in mice is approaching to the therapeutic efficacy of trimedoxime, the ability of another novel bispyridinium oxime K307 to reduce acute toxicity of tabun is significantly lower compared to trimedoxime and the oxime K203. Thus, the reactivating and therapeutic efficacy of both examined 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.

Introduction

Highly toxic organophosphorus compounds have been developed as chemical warfarre agents called nerve agents. They 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. The overstimulation of cholinergic receptors 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 centers (Bajgar, 2004; Delfino et al., 2009). The medical countermeasures of nerve agent poisonings include the administration of the antidotes that are able to counteract the main acute toxic effects of nerve agents. The standard antidotal treatment of nerve agent poisoning usually includes an anticholinergic agent to block the overstimulation of cholinergic receptors and an oxime to reactivate nerve agent-inhibited AChE (Dawson, 1994; Taylor, 1996). The compounds with nucleophilic oximate anion were discovered and considered to be able to reactivate nerve agent-inhibited AChE by dephosphonylating the enzyme active site and restoring its activity. While a lot of these reactivators are sufficiently effective to reactivate sarin or VX-inhibited AChE, their ability to reactivate soman, cyclosarin or tabun-inhibited AChE is generally low (Kassa, 2002; 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. It was found that commonly used antidotes are not able to sufficiently prevent tabun-induced acute toxic effects. The toxic effects of tabun are problematically antagonized due to the changes in hydrogen bonding and the conformational changes of AChE-tabun complex prior to an aging process in the AChE active site (Cabal and Bajgar, 1999; Ekström et al., 2006).

* Corresponding author at: Trebesska 1575 Faculty of Military Health Sciences, 500 01 Hradec Kralove, Czech Republic.
E-mail address: kassa@pmfhk.cz (J. Kassa).

http://dx.doi.org/10.1016/j.jab.2016.09.008
1214-021X/

While the anticholinergic drug such as atropine is able to counteract the effects of tabun at peripheral muscarinic cholinergic receptors (Bajgar, 2004), commonly used monopyridinium (*e.g.* pralidoxime) and bispyridinium oximes (*e.g.* obidoxime, trimedoxime, HI-6) are not able to sufficiently counteract the acute toxic effects of tabun because of their low ability 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).

Among recently developed oximes, the oxime K203 has been 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 not significant (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, K305 [1,5-bis(4-hydroxyiminomethyl-pyridinium)-pentane dibromide] and K307 (1-[2-(hydroxyimino-methyl) pyridinium-1-yl)-pent-1-yl]-4-(hydroxyiminomethyl) pyridinium 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 acute toxicity. They were developed based on the structure-activity relationship study and they were chosen based on the data obtained from *in vitro* evaluation of their ability to reactivate AChE inhibited by tabun (Winter et al., 2016**).** The aim of this study was to compare the reactivating and therapeutic efficacy of both newly developed oximes (K305, K307) with the oxime K203 and trimedoxime against tabun using *in vivo* methods.

Materials and methods

Animals

Male albino Wistar rats weighing 230–260 g and NMRI male mice weighing between 24 and 28 g were purchased from VELAZ, Czech Republic. They were kept in climate- and access-controlled rooms ($22 \pm 2\,^{\circ}C$ 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 94% 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 (K305, K307, 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 95% 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*

Prior to 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 occuring 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 160 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.,

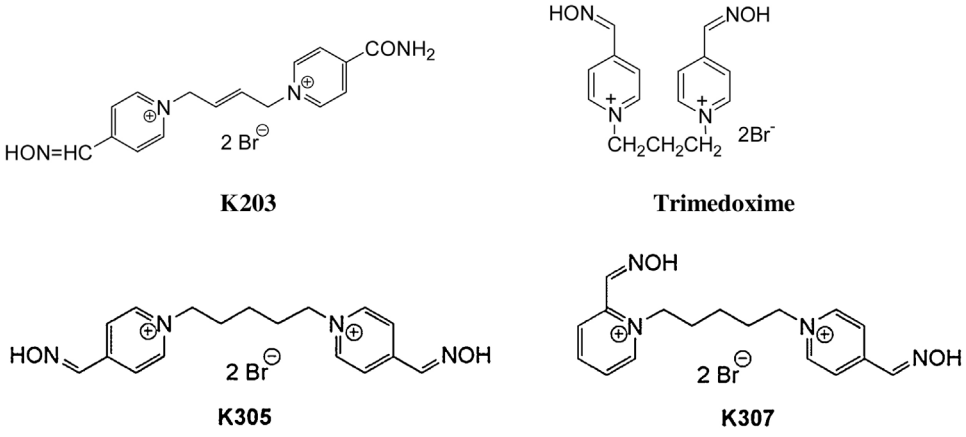

Fig. 1. Chemical structure of oximes.

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 of 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))]} x 100 (Clement et al., 1992). All experiments were perfomed 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 therapeutic efficacy of atropine alone and atropine in combination with one of tested oximes 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 (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 clearly demonstrate that the acute toxicity of both novel bispyridinium oximes K305 and K307 are similar and rather high. In addition, their acute toxicity is markedly higher than the acute toxicity of the oxime K203 and trimedoxime in rats as well as mice.

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. Both newly developed oximes (K305, K307) were able to reactivate tabun-inhibited AChE in the diaphragm although their reactivating efficacy was markedly lower compared to trimedoxime and the oxime K203. The ability of all bispyridinium oximes studied to reactivate tabun-inhibited AChE in the brain is generally much lower compared to the peripheral nervous system (diaphragm). The percengate of reactivation of tabun-inhibited AChE did not reach out for 10% regardless of the structure of bispyridiniumu oximes. In addition, the central reactivating efficacy of both newly developed oximes was lower compared to the oxime K203 and trimedoxime. To compare the reactivating efficacy of both newly developed oximes, the oxime K307 showed a slightly higher reactivating efficacy compared to the oxime K305 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 trimedoxime or the oxime K203 was used for the antidotal treatment of tabun poisoning.

A comparison of the therapeutic efficacy of newly developed oximes (K305, K307) 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). A wide spectrum of muscarinic (salivation) and niconitic (tonic-clonic convulsions) clinical signs was observed in tabun–poisoned mice within a few minutes regardless of type of antidotal treatment. They usually died within 30–50 min after poisoning with tabun. The therapeutic efficacy of atropine alone was negligible while the antidotal treatment involving atropine and one of the studied oxime brought a significant decrease in the acute toxicity of tabun. The therapeutic efficacy of both newly developed oximes was lower compared to the oxime K203 and trimedoxime. To compare the therapeutic efficacy of both newly developed oximes, the oxime K305 showed a higher therapeutic efficacy in comparison with the oxime K307. While the therapeutic efficacy of the oxime K305 was almost as high as the therapeutic

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)
K305	34.9 (29.3 – 41.6)	20.6 (17.3–25.5)
K307	21.0 (18.9 – 25.6)	19.2 (16.2–23.6)

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	15.56 ± 0.54	94.38 ± 3.42[a]
Atropine control	2.92 ± 1.03[c]	9.06 ± 2.64[c]
Atropine + K203	**7.58 ± 1.27**[c,d]	14.23 ± 5.67[c]
(% reactivation[b])	**(36.9)**	(6.1)
Atropine + trimedoxime	**6.47 ± 0.95**[c,d]	15.13 ± 4.19[c]
(% reactivation)	**(28.1)**	(7.1)
Atropine + K305	3.36 ± 0.78[c]	9.20 ± 1.25[c]
(% reactivation)	(3.5)	(0.2)
Atropine + K307	3.97 ± 1.20[c]	9.71 ± 1.19[c]
(% reactivation)	(8.3)	(0.8)

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

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
---	208.0 (176.7–229.6)	- - - -
Atropine	232.1 (210.3–256.2)	1.12
K203 + atropine	**395.8 (333.6–503.4)**[a,b]	**1.76**
Trimedoxime + atropine	**339.7 (313.4–368.1)**[a,b]	**1.63**
K305 + atropine	316.4 (282.4–363.0)[a]	1.52
K307 + atropine	279.8 (256.9–310.6)[a]	1.35

[a] Significantly different from the untreated group and the atropine group at the level of $2\alpha = 0.05$.
[b] Significantly different from the group treated by atropine in combination with K307 at the level of $2\alpha = 0.05$.

efficacy of trirmedoxime, the therapeutic efficacy of the oxime K307 was significantly lower compared to trimedoxime and the oxime K203. On the other hand, the difference between therapeutic efficacy of both newly developed oximes was not significant.

Discussion

Generally, oximes are not equally effective against all nerve agents. Their efficacy depends on many factors, especially on the chemical structure of nerve agents and the rate of aging of enzyme-inhibitor complex (Nurulain, 2011). It is known that nerve agent-inhibited AChE undergoes a proces of aging that makes the reactivation of nerve agent-inhibited AChE impossible. The aging kinetics of different nerve agents is different, ranging from a few minutes to many hours (Antonijevic and Stojiljkovic, 2007). Other factors that influence the effectiveness of oxime therapy represent the potency of nerve agents to inhibit AChE, their toxicokinetics, reactivating efficacy of oximes and their pharmacokinetics, correct dosing, evaluation for the persistent need of oxime therapy and correct timing (Antonijevic and Stojiljkovic, 2007; Nurulain, 2011).

According to the published results, currently used monopyridinium and bispyridinium oximes seem to be relatively poor reactivators of tabun-inhibited AChE. The values of their kinetic parameters 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 (Cabal et al., 2004; Kassa and Cabal, 1999a,b,c). Generally, bispyridinium oximes seem to be more effective to reactivate tabun-inhibited AChE and to counteract tabun-induced acute toxicity than monopyridinium oximes (Voicu et al., 2010). They have higher affinity towards both intact and tabun-inhibited AChE and, therefore, higher potency to reactivate tabun-inhibited AChE compared to monopyridinium oximes (Kuca et al., 2006b). On the other hand, bispyridinium oximes are less lipophilic than monopyridinium oximes and, therefore, their penetration across the blood-brain barrier is poor (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 (Kalasz et al., 2015; Lorke et al., 2008). In addition, the ability of compounds with the oxime group in position 4 to reactivate tabun-inhibited AChE is higher compared to reactivators with the oxime group at different positions (Cabal et al., 2004; Kuca et al., 2004), while the AChE reactivators with the oxime group in position 2 are the best reactivators of cyclosarin-inhibited AChE (Kuca et al., 2006a). On the other hand, the number of oxime groups is not so important. The oxime K203 has only one oxime group, but it is more effective in reactivation of tabun-inhibited AChE than bispyridinium oximes with two oxime groups, such as obidoxime (Kassa et al., 2008; Musilek et al., 2007). The chain linking two quaternary nitrogens in bispyridinium oximes also exerts a great influence on the reactivating efficacy, although this part of the oxime molecule does not play any role in the dephosphonylation process. The tri- or tetracarbon chain seems to be the most suitable for the sufficient ability of oximes to reactivate tabun-inhibited AChE (Kuca et al., 2006b; Worek et al., 1998).

To eliminate above mentioned limitations of the effects of AChE reactivators against tabun, new analogues of bispyridinium oximes were developed to extend their properties and increase their ability to reactivate tabun-inhibited AChE (Berend et al., 2008; Kim et al., 2005; Kovarik et al., 2013; Musilek et al., 2006). The design of both newly developed oximes (K305, K307) 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., 2006b; Musilek et al., 2011). The oximes K305 and K307 were designed as bis-oxime reactivators with aliphatic connecting linker that was formerly found to be beneficial for the reactivation if compared to aliphatically linked monooximes (Kassa et al., 2012). Their connecting linker was elongated to five methylene units and the oximes were positioned as 4,4 (K305) or 2,4-moieties (K307). Both compounds proved to be effective reactivators of tabun, cyclosarin or paraoxon-inhibited hAChE *in vitro* comparable with trimedoxime, obidoxime or asoxime within kinetic experiments (Winter et al., 2016).

Our results demonstrate that the ability of both newly developed bispyridinium oximes (K305 and K307) 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 reactivating and therapeutic efficacy of trimedoxime and the oxime K203. One reason for their weak effectiveness can be their relatively high toxicity that is caused by the presence of two oxime groups in their structure. Small safe dosage of both oximes can explain their lower reactivating and therapeutic efficacy compared to trimedoxime and the oxime K203. As the reactivating and therapeutic efficacy of both examined 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 development of new oxime structures realized according to the described requirements (Kuca et al., 2006b; Voicu et al., 2015) has not brought till now any significant progress in the potency of current antidotal treatment to sufficiently 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 create a new approach how to develop new 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.

Acknowledgements

The authors wish to thank to Mrs Jana Uhlirova for her skilful assistance. The study was funded by a grant of Ministry of Defense of the Czech Republic – Long-term organization development plan 1011“.

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