ORIGINAL ARTICLE

Therapeutic efficacy of a novel bispyridinium oxime K203 and commonly used oximes (HI-6, obidoxime, trimedoxime, methoxime) in soman-poisoned male rats and mice

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Summary
The potency of a novel oxime K203 in reactivating soman-inhibited acetylcholinesterase and reducing acute toxicity of soman was compared with commonly used oximes (HI-6, obidoxime, trimedoxime, methoxime) using in vivo methods. The study determining percentage of reactivation of soman-inhibited blood and tissue acetylcholinesterase in rats showed that the potency of the oxime K203 to reactivate soman-inhibited acetycholinesterase in the peripheral compartment is slightly higher than obidoxime and trimedoxime, especially in the diaphragm, slightly lower than methoxime and markedly lower compared to the oxime HI-6. The reactivating efficacy of the oximes studied in the peripheral compartment roughly corresponds to their potency to reduce acute toxicity of soman in mice. Based on the obtained data, we can conclude that the oxime K203 is not suitable for the replacement of the oxime HI-6 for the antidotal treatment of acute soman poisoning due to its relatively low potency to counteract acute toxicity of soman.

Key words: soman; acetylcholinesterase; K203; HI-6; obidoxime; trimedoxime; methoxime; rats; mice

INTRODUCTION
Organophosphorus nerve agents exert their toxic effects by the phosphorylation and subsequent inactivation of acetylcholinesterase (AChE, EC 3.1.1.7). The inactivation of AChE allows the accumulation of acetylcholine in the cholinergic synapses of the central and peripheral nervous systems. Subsequent widespread overstimulation of cholinergic receptors is manifested as salivation, lacrimation, sweating, diarrhea, urination, muscular twitching and fibrillation and ultimately tonic/clonic convulsions (Lotti 2000, Bajgar 2004). In addition, nerve agent-induced centrally mediated seizures can rapidly progress to status epilepticus and contribute to profound brain damage and cardiac pathology (Tryphonas and Clement 1995, McDonough and Shih 1997).

The medical countermeasures of poisoning with nerve agents is usually based on a combined administration of a muscarinic cholinergic receptor antagonist to block the overstimulation of cholinergic receptors by acetylcholine and an oxime to reactivate nerve agent-inhibited AChE. Generally, anticholinergics (mainly atropine) are used for relieving muscarinic signs and symptoms whereas AChE reactivators (called oximes) are used for reactivation
of nerve agent-inhibited AChE (Lotti 2000, Baigar 2004). 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 (Kassa 2002, Marrs et al. 2006). Therefore, the development of a sufficiently effective AChE reactivator against some nerve agents such as tabun or soman is still very important.

Soman (pinacolyl methyl fluorophosphonate) belongs to a highly toxic group of organophosphorus compounds misused as chemical warfare agents for military as well as for terrorist purposes. Deleterious effects of soman are extraordinarily difficult to antagonize due to the very rapid aging of soman-inhibited AChE. The dealkylation of soman bound on the active site of AChE makes the nucleophilic attack of oximes almost impossible (Puu et al. 1986, Jokanovic and Prostran 2009).

A novel bispyridinium asymmetric oxime K203 [1-(4-carbamoylpyridinium)-4-(4-hydroxyimino-methylpyridinium)-but-2-ene dibromide] (Fig. 1) was primarily synthesized at our department (Musilek et al. 2008) to increase the efficacy of antidotal treatment of acute poisoning with tabun that was found to be resistant to conventional oxime therapy due to the conformational changes of AChE-tabun complex prior aging process in AChE active site (Ekström et al. 2006). As the oxime K203 was found to be promising reactivator of tabun-inhibited AChE (Kassa et al. 2008, Kovarik et al. 2009), we decided to evaluate the reactivating and therapeutic efficacy of K203 against other nerve agents including soman, because we are still searching for a broad-spectrum oxime able to sufficiently counteract acute toxicity of all nerve agents regardless of their chemical structure.

The aim of this study was to determine the reactivating and therapeutic efficacy of a novel bispyridinium oxime K203 in comparison with commonly used oximes (HI-6, obidoxime, trimedoxime, methoxime) against soman in rats and mice.

MATERIAL AND METHODS

Animals
Male albino Wistar rats weighing 220–260 g and NMRI male mice weighing between 20 and 25 g were purchased from VELAZ (Prague, Czech Republic). They were kept in an air-conditioned room with the light from 07:00 to 19:00 h and were allowed access to standard food and tap water ad libitum. The rats and mice 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
Soman was obtained from the Technical Institute in Brno (Czech Republic) in compliance with permission for the handling of chemical warfare agents and it was 92% pure. Its purity was assayed by acidimetric titration. All oximes (obidoxime, trimedoxime, methoxime, HI-6, K203) were synthesized at our Department of Toxicology of the Faculty of Military Health Sciences (Czech Republic) and they were more than 98% pure. The purity of oximes was analyzed using a HPLC technique. All other drugs and chemicals of analytical grade were obtained commercially and used without further purification. All compounds 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 oximes, the acute toxicity of tested oximes was evaluated in rats and mice by the assessment of their LD50 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 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 doses corresponding to 5% of their LD50 5 min before receiving soman i.m. at a dose of 74 μg/kg (LD50). 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 soman-AChE complex (Clement et al. 1992). The rats were decapitated and exsanguinated to obtain the blood and tissues 30 min subsequent to soman poisoning. AChE activity was measured in hemolyzed blood and homogenized tissue (diaphragm and brain) by the standard spectrophotometric method (Ellman et al. 1961). 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 obtained from rats administered with saline instead of soman and antidotes (saline control). The reactivation
% extent was calculated using the AChE activity values: \( \{1 - \frac{((\text{saline control}) - (\text{oxime + atropine}))}{((\text{saline control}) - (\text{atropine control}))}\} \times 100 \) (Clement et al. 1992).

The potency of oximes in combination with atropine to eliminate soman-induced lethal effects in mice was determined as follows. The LD50 value of soman and its 95% confidence limit in soman-poisoned mice treated with atropine alone (21 mg/kg) at 1 min after i.m. administration of soman was assessed using probit-logarithmical analysis of death occurring within 24 h after i.m. administration of soman at five different doses with eight mice per dose (Tallarida and Murray 1987). Then, soman-poisoned mice were treated i.m. with one of tested oximes at equitoxic doses (5% LD50) in combination with atropine (21 mg/kg) at 1 min after i.m. challenge of soman. The LD50 values of soman and their 95% confidence limits in soman-poisoned mice treated with an oxime in combination with atropine were assessed by the same method. The efficacy of tested oximes was expressed as protective ratio (LD50 value of soman in mice protected by the combination of oxime and atropine/LD50 value of soman in mice protected by atropine alone).

**Statistical evaluation**

The differences between groups were calculated using means ± SD and differences were tested by one-way ANOVA test with Scheffe’s post hoc test at the significance level \( 2\alpha = 0.05 \).

**RESULTS**

The acute i.m. toxicity of tested oximes is summarized in Table 1. The results show that the acute toxicity of the newly developed oxime K203 is slightly lower than the acute toxicity of obidoxime and trimedoxime but it is markedly higher than the acute toxicity of methoxime and the oxime HI-6. 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 soman-inhibited AChE in rat blood, diaphragm and brain *in vivo* is shown in Table 2. The newly developed oxime K203 seems to be a week reactivator of soman-inhibited AChE in blood, diaphragm as well as in brain. It is able to somewhat reactivate soman-inhibited AChE in blood, diaphragm as well as in brain but the increase of AChE activity is relatively small and not significant compared to the activity of soman-inhibited AChE in poisoned rats treated with atropine alone. Its reactivating efficacy is slightly lower compared to methoxime in blood, diaphragm and brain, however, the difference between the reactivating efficacy of K203 and methoxime is very small. Obidoxime and trimedoxime are also weak reactivators of soman-inhibited AChE, especially in diaphragm and brain where both currently available oximes are completely ineffective. On the other hand, the oxime HI-6 is a relatively good reactivator of soman-inhibited AChE in the peripheral compartment.
Table 1. LD<sub>50</sub> values of oximes following i.m. administration in male rats and mice.

<table>
<thead>
<tr>
<th>Oximes</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg) ± 95% confidence limit</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Rats</td>
</tr>
<tr>
<td>K203</td>
<td>326.4 (285.4–373.2)</td>
</tr>
<tr>
<td>HI-6</td>
<td>781.3 (738.4–826.6)</td>
</tr>
<tr>
<td>Obidoxime</td>
<td>240.7 (180.5–267.2)</td>
</tr>
<tr>
<td>Trimedoxime</td>
<td>258.2 (220.4–267.2)</td>
</tr>
<tr>
<td>Methoxime</td>
<td>442.2 (421.2–477.8)</td>
</tr>
</tbody>
</table>

Table 2. Percentage of reactivation of soman-inhibited AChE by oximes in male rat blood, diaphragm and brain in vivo.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AChE activity (μkat/l or μkat/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td>Atropine</td>
<td>5.09±1.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Atropine + K203 (% reactivation)</td>
<td>5.98±1.20 (4.8)</td>
</tr>
<tr>
<td>Atropine + HI-6 (% reactivation)</td>
<td>9.71±0.78 (31.9**)</td>
</tr>
<tr>
<td>Atropine + obidoxime (% reactivation)</td>
<td>7.59±1.25 (17.3)</td>
</tr>
<tr>
<td>Atropine + trimedoxime (% reactivation)</td>
<td>5.10±1.03 (0.1)</td>
</tr>
<tr>
<td>Atropine + methoxime (% reactivation)</td>
<td>5.79±1.19 (6.2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> means ± SD, N = 8. The untreated control value (saline control) for rat blood AChE activity was 16.58 μkat/l, for diaphragm AChE activity 16.91 μkat/kg and for brain AChE activity 142.70 μkat/kg.

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

<sup>b</sup> significantly different from the atropine group
<sup>c</sup> significantly different from the atropine + obidoxime (trimedoxime, methoxime, K203) group

(blood, diaphragm) where its ability to reactivate soman-inhibited AChE is significantly higher compared to all other studied oximes. However, it is not able to reactivate soman-inhibited AChE in the brain as obidoxime and trimedoxime.

These results roughly correlate with the therapeutic potency of the oximes tested against lethal soman poisoning in mice (Table 3). Soman-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 40–60 minutes after poisoning with soman. The oxime K203 was able to decrease the acute toxicity of soman approximately 1.4-fold and, thus, its therapeutic efficacy corresponds to the effectiveness of obidoxime and trimedoxime. On the other hand, its potency to reduce acute lethal toxic effects of soman in mice is slightly lower than the therapeutic efficacy of methoxime and significantly lower than the therapeutic efficacy of the oxime HI-6 that is able to decrease the acute toxicity of soman 2.8-fold. Thus, the oxime HI-6 showed significantly higher potency to reduce acute lethal toxic effects of soman in mice in comparison with other studied oximes.
Table 3. The influence of the type of oxime on the potency of antidotal treatment to reduce acute toxicity of soman in male mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LD₅₀ (μg/kg) ± 95% confidence limit</th>
<th>Protective ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>74.3 (62.1–88.9)</td>
<td>–</td>
</tr>
<tr>
<td>K203 + atropine</td>
<td>109.4 (98.9–125.5)*</td>
<td>1.47</td>
</tr>
<tr>
<td>HI-6 + atropine</td>
<td>211.4 (158.1–282.8)*</td>
<td>2.85</td>
</tr>
<tr>
<td>Obidoxime + atropine</td>
<td>111.0 (99.1–127.4)*</td>
<td>1.49</td>
</tr>
<tr>
<td>Trimedoxime + atropine</td>
<td>110.5 (102.5–128.6)*</td>
<td>1.49</td>
</tr>
<tr>
<td>Methoxime + atropine</td>
<td>133.4 (109.9–181.2)*</td>
<td>1.80</td>
</tr>
</tbody>
</table>

* significantly different from the group treated by atropine alone  
* significantly different from the group treated by atropine in combination with obidoxime (trimedoxime, K203)

DISCUSSION

Based on the previously published results, the novel oxime K203 seems to be effective reactivator of tabun-inhibited AChE and it is considered to be suitable oxime for the antidotal treatment of acute tabun poisonings (Kassa et al. 2008, Kovarik et al. 2009). However, to reach the satisfactorily effective antidotal treatment of nerve agent poisoning, the broad-spectrum oxime, sufficiently effective against all nerve agents regardless of their chemical structure, should be found. As no broad spectrum oxime has been developed till now (Marrs et al. 2006, Kassa et al. 2007, Szinicz et al. 2007), it is important to know if some of newly developed oximes is able to sufficiently protect organisms against all nerve agents. Therefore, the evaluation of the ability of novel oximes to reactivate nerve agent-inhibited AChE and protect against acute signs and symptoms of nerve agents regardless of their chemical structure is necessary.

In this paper, the potency of the novel oxime K203 to reactivate soman-inhibited AChE and protect against acute toxicity of soman was evaluated in comparison with chosen currently available oximes including the oxime HI-6 that is considered to be the best oxime against soman (Kassa and Cabal 1999, Lundy et al. 2006). Our results demonstrate that the reactivating and therapeutic efficacy of the oxime K203 roughly corresponds to the efficacy of obidoxime and trimedoxime but it is slightly less effective than methoxime and significantly less effective than the oxime HI-6. These results might not be explained solely by significant differences in the reactivating efficacy of the oximes studied in the peripheral compartment (Shih 1993, Worek et al. 1998) but also by other antidotal mechanisms observed after administration of the oxime HI-6, such as direct antimuscarinic action and restoration of neuromuscular transmission (van Helden et al. 1996). There are several studies demonstrating that the ability of HI-6 to antagonize soman-induced toxic effects is significantly higher compared to other commonly used oximes (Kassa and Cabal 1999, Kassa 2002, Lundy et al. 2006, Marrs et al. 2006, Jokanovic and Prostran 2009, Kuča et al. 2009). The relatively high therapeutic potency of the oxime HI-6 may be due to various antidotal mechanisms based on reactivation of phosphorylated AChE, direct antimuscarinic and ganglion blocking actions, restoration of neuromuscular transmission, retardation of the formation of the aged inhibitor-enzyme complex and inhibition of acetylcholine release (van Helden et al. 1996). Thus, only the oxime HI-6 appears to be able to sufficiently protect experimental animals from soman-induced adverse effects and improve survival of soman-poisoned animals (Kassa and Cabal 1999, Lundy et al. 2006).

On the other hand, the oxime HI-6 as well as the other oximes studied including the oxime K203 are not able to sufficiently reactivate soman-inhibited AChE in the central nervous system due to difficult penetration through the blood-brain barrier. The lack of penetration of the oximes through the blood-brain barrier is caused by the quaternary structure of oximes (Lundy et al. 1990, Lorke et al. 2008, Zdarova Karasova et al. 2011).

The above described data confirm that HI-6 is a significantly more efficacious oxime than other currently available oximes in the case of the antidotal
treatment of severe soman poisoning although its therapeutic efficacy is also rather limited probably because of the lack of its central reactivation efficacy. On the contrary, the newly developed oxime K203 seems to be significantly less efficacious to reactivate soman-inhibited AChE in rats and reduce lethal toxic effects of soman in mice than the oxime HI-6 and, therefore, it is not suitable for the replacement of the oxime HI-6 for the treatment of acute soman poisonings.

DECLARATION OF INTERESTS

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

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