ORIGINAL ARTICLE

Evaluation of the neuroprotective efficacy of individual oxime (HI-6) and oxime mixtures (HI-6 + trimedoxime, HI-6 + K203) in tabun-poisoned rats

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Summary

The ability of the oxime HI-6 to reduce tabun-induced acute neurotoxic signs and symptoms was compared with the neuroprotective efficacy of two combinations of oximes (HI-6 + trimedoxime, HI-6 + K203) using a functional observational battery. Tabun-induced neurotoxicity and the neuroprotective effects of HI-6 alone, and HI-6 combined with trimedoxime or K203, in rats poisoned with tabun at a sublethal dose (200 µg/kg i.m.; 80% of LD₅₀ value), were monitored by the functional observational battery at 24 hours following tabun challenge. The results indicate that both oxime mixtures tested, combined with atropine are able to allow tabun-poisoned rats to survive for 24 hours following tabun challenge, while one non-treated tabun poisoned rat and one tabun-poisoned rat treated with the oxime HI-6 alone combined with atropine, died within 24 hours following tabun challenge. The oxime HI-6 alone, as well as both oxime mixtures combined with atropine, were able to decrease tabun-induced neurotoxicity in the case of sublethal poisonings, but they did not eliminate all tabun-induced acute neurotoxic signs and symptoms. Their ability to reduce tabun-induced acute neurotoxicity was almost the same, regardless of type of antidotal treatment. Thus, the tested combinations of oximes were not able to increase the neuroprotective effectiveness of the antidotal treatment of acute tabun poisonings compared to the individual oxime.

Key words: tabun; functional observational battery; neurotoxicity; mixture of oximes; rats

INTRODUCTION

Nerve agents are considered to be the most dangerous chemical warfare agents. These compounds pose potential neurotoxic threats to both military and civilian populations as evidenced by actual terrorist

attacks in Japan (Ohtomi et al. 1996). Their acute toxic effects are based on the phosphorylation 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 the synapses of the central and peripheral nervous systems (Lotti 2000, Marrs 1993).

The medical countermeasures after nerve agent poisonings include the administration of the antidotes that are able to counteract the main toxic effects of nerve agents. The standard antidotal treatment of nerve agent poisoning usually includes an anticholinergic agent to block the overstimulation of

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muscarinic cholinergic receptors, and an oxime to reactivate nerve agent-inhibited AChE (Dawson 1994, Taylor 1996).

Nerve agents can produce centrally-mediated seizure activity, that rapidly progresses to status epilepticus, and contributes to profound brain damage (Marrs 1993, Taylor 1996). The exposure of experimental animals to nerve agents in convulsion-inducing doses may result in irreversible lesions in the central nervous system (CNS), which can be manifested as behavioral effects in survivors that have convulsed (Jokanović 1993). Therefore, the ability of antidotes to block the acute neurotoxic effects of nerve agents and prevent development of irreversible lesions in CNS, is important for successful antidotal treatment. Generally, the oximes exert more potent effects in the peripheral compared to the central nervous system, due to their poor penetration into CNS, although some published results have described the penetration of oximes into CNS and subsequent reactivation of nerve agent-inhibited AChE in the brain (Cassel et al. 1997, Sakurada et al. 2003).

HI-6

Trimedoxime

K203

Fig. 1. Chemical structure of oximes studied.

The experience of stockpiling and use of a number of different nerve agents in Iraq and their use by terrorists in Japan (Ohtomi et al. 1996, Lawrence and Kirk 2007) emphasizes the necessity to introduce oximes with a broader spectrum. However, published results confirm that there is no single, broad-spectrum oxime suitable for the antidotal treatment of poisonings with all nerve agents (Bajgar 2005, Marrs et al. 2006, Kassa et al. 2007). Therefore, the replacement of commonly used oximes (pralidoxime, obidoxime) as well as H-oximes (the oxime HI-6) with a more effective oxime with a broader spectrum has been a long-standing goal for the treatment of nerve agent poisoning. During the past several decades, many new oximes have been synthesized. Although some of them can be considered to be promising oximes against some nerve agents, none of them is sufficiently effective against all nerve agents regardless of their chemical structure. (Musilek et al. 2007a, Maxwell et al. 2008). Two years ago, the oxime K203 [1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethyl pyridinium)-but-2-ene dibromide] (Fig. 1) was synthesized at our Department of Toxicology (Musilek et al. 2007b, 2008) and its pharmacokinetics was studied (Kalasz et al. 2008). Based on the *in vitro* and *in vivo* evaluation of its reactivating, therapeutic and neuroprotective efficacy, it was considered to be a promising oxime against tabun but not against cyclosarin (Musilek et al. 2007c, Kassa et al. 2008a,b, Kassa et al. 2009, Kovarik et al. 2009).

Another way to increase the potency of antidotes to counteract the acute neurotoxic effects of nerve agents and broaden their spectrum is to combine chosen oximes in the antidotal treatment. In this study, the combination of HI-6 with trimedoxime or K203 was used. The neuroprotective efficacy of the chosen mixtures of oximes in combination with atropine was evaluated against tabun (*O*-ethyl-*N,N*-dimethyl phosphoramidocyanidate) because its acute neurotoxicity is 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 (Cabal and Bajgar 1999, Ekström et al. 2006).

The main aim of this study was to compare, in tabun-poisoned rats, the neuroprotective efficacy of the oxime HI-6, which is considered to be the oxime with the broadest spectrum among commonly used oximes, with two mixtures of oximes containing the oxime HI-6 and trimedoxime, or the oxime K203 in combination with the anticholinergic drug atropine. The tabun-induced neurotoxic signs were determined

using a functional observational battery – a non-invasive and relatively sensitive type of neurological examination for a wide range of neurobiological functions including measurements of sensory, motor and autonomic nervous functions.

MATERIAL AND METHODS

Animals

Male albino Wistar rats weighing 220–250g were purchased from VELAZ (Prague, Czech Republic). They were kept in an air-conditioned room ($22 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity, with lights from 7.00 to 19.00 hr) and 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 performed in compliance with relevant laws and institutional guidelines and under the supervision of the Ethics Committee of the Faculty of Military Health Sciences in Hradec Králové (Czech Republic).

Chemicals and drugs

Tabun was obtained from the Military Technical Institute in Brno (Czech Republic) and was 98% pure as assayed by acidimetric titration. All oximes studied were of 98.5% purity and were synthesized at the Department of Toxicology of the Faculty of Military Health Sciences in Hradec Králové (Czech Republic) (Musílek et al. 2006, 2007b, 2008, Kuča et al. 2007). Their purity was analyzed using HPLC. 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.).

Procedure of experiments

Tabun was administered at a sublethal dose ($200 \mu\text{g/kg b.w.} - 80\% \text{LD}_{50}$). One minute following tabun challenge, the rats were treated with atropine (21 mg/kg b.w.) in combination with the oxime HI-6 alone or with the combination of HI-6 and trimedoxime or K203 at equitoxic doses corresponding to 5% of their LD_{50} values. The neurotoxicity of tabun was monitored using the functional observational battery at 24 hrs following tabun poisoning. The evaluated markers of tabun-induced neurotoxicity in the experimental animals were compared with the parameters obtained from the control rats given saline instead of tabun and antidotes at the same volume.

Functional observational battery

The functional observational battery consists of 44 measurements of sensory, motor and autonomic nervous functions. Some of them are scored (Table 1), the others are measured in absolute units (Frantik and Hornychová 1995, Hornychová et al. 1995). The first evaluation was obtained when tabun-poisoned rats were in the home cage. The observer evaluated each animal's posture, palpebral closure and involuntary motor movements. Then, each rat was removed from the home cage and briefly hand-held. The exploratory activity, piloerection and other skin abnormalities were noted. Salivation and nose secretion were also registered and scored. Then, the rats were placed on a flat surface which served as an open field. A timer was operated for 3 min during which the frequency of rearing responses was recorded. At the same time, gait characteristics were noted and ranked, and arousal, stereotypy and bizarre behaviours and abnormal posture were evaluated. At the end of the third min, the number of fecal boluses and urine pools on the adsorbent pad was registered. Reflex testing comprising recording each rat's response to the frontal approach of the blunt end of a pen, a touch of the pen to the posterior flank and an auditory clic stimulus was also used. The response to a pinch on the tail and the ability of pupils to constrict in response to light were then assessed. These measures were followed by a test for the aerial righting reflex and by the measurements of forelimb and hindlimb grip strength, body weight, body temperature and finally hindlimb landing foot splay. The whole battery of tests required approximately 6–8 min per rat. The observer of the behaviour did not know about the design of the experiments.

Data analysis

Data collected with the functional observational battery include categorical, ordinal and continuous values. Statistical analyses were performed on a PC with the special interactive programme NTX (Frantik and Hornychová 1995). The categorical and ordinal values were formulated as contingency tables and judged consecutively by the Chi-squared test of homogeneity, the Concordance-Discordance test and the Kruskal-Wallis test, respectively. The continual data were assessed by successive statistical tests: the CI for Delta, the Barlett test for Equality of Variance, the Williams test and the Test for Distribution Functions (Roth et al. 1962). The significance level $2\alpha'0.05$ was used.

Table 1. **Functional observational battery (FOB).**

Marker	Scored values only									
	−2	−1	0	1	2	3	4	5	6	7
Posture				sitting or standing	rearing	asleep	flattened	lying on side	crouched over	head bobbing
Catch difficulty				passive	normal	defense	flight	escape	aggression	
Ease of handling				very easy	easy	moderately difficult	difficult			
Muscular tonus	atonia	hypotonia	normal	hypertonia	rigidity	fasciculations				
Lacrimation			none	slight	severe	crusta	coloured crusta			
Palpebral closure				open	slightly drooping	half-way drooping	completely shut	ptosis		
Endo-exophthalmus		endo	normal	exo						
Piloerection			no	yes						
Skin abnormalities			normal	pale	erythema	cyanosis	pigmented	cold	injury	
Salivation			none	slight	severe					
Nose secretion			none	slight	severe	coloured				
Clonic movements			normal	repetitive	nonrhythmic	mild tremors	severe tremors	myoclonic	clonic	
Tonic movements			normal	contraction of extensors	opisthotonus	emprosthotonus	explosive jumps	tonic convulsions		
Gait			normal	ataxia	overcompensation of hindlimbs movements	feet point outwards from body	forelimbs are extended	walks on tiptoes	hunched body	body is flattened against surface

Gait score			normal	slightly impaired	somewhat impaired	totally impaired		
Mobility score			normal	slightly impaired	somewhat impaired	totally impaired		
Activity			very low	sporadic	reduced	normal	enhanced	permanent
Tension		none	partial (ears)	stupor				
Stereotypy		none	head weaving	body weaving	grooming	circling	others	
Bizarre behavior		none	head	body	self-mutilation	abnormal movements	others	
Approach response			no reaction	normal	slow reaction	energetic reaction	exaggerated reaction	
Touch response			no reaction	normal	slow reaction	energetic reaction	exaggerated reaction	
Click response			no reaction	normal	slow reaction	energetic reaction	exaggerated reaction	
Tail-pinch response			no reaction	normal	slow reaction	energetic reaction	exaggerated reaction	
Pupil size	miosis	normal	mydriasis					
Pupil response		no reaction	normal reaction					
Righting reflex			normal	slightly uncoordin.	lands on side	lands on back		

Table 2a. **The values of tabun-induced activity and neuromuscular neurotoxic markers measured at 24 hrs following tabun challenge by the functional observational battery** (No 1–2, 4–14 – scored values, No 3, 15–18 – values in absolute units); * statistically significant as compared with controls.

24 hours		Controls		Tabun + A + HI-6 + K203		Tabun + A + HI-6 + trimedoxime		Tabun + A + HI-6		Tabun	
No	Marker	x/M	± s	x/M	± s	x/M	± s	x/M	± s	x/M	± s
1	Posture	1.00		3.00*		1.00		3.00*		3.00*	
2	Muscular tonus	0.00		–1.00*		–1.00*		–2.00*		–2.00*	
3	Rearing	6.75	2.10	4.50	2.45	2.25*	1.83	1.13*	1.81	2.38*	2.76
4	Hyperkinesis	0.00		0.00		0.00		0.00		0.00	
5	Tremors	0.00		0.00		0.00		0.00		0.00	
6	Clonic movements	0.00		0.00		0.00		0.00		0.00	
7	Tonic movements	0.00		0.00		0.00		0.00		0.00	
8	Gait	0.00		1.00*		1.00*		0.00		1.00*	
9	Ataxia	0.00		1.00*		1.00*		0.00		1.00*	
10	Gait score	1.00		1.00		1.00		1.00		1.00	
11	Mobility score	1.00		1.00		1.00		1.00		1.00	
12	Activity	4.00		2.00*		2.00*		1.00*		1.00*	
13	RRF	1.00		1.00		1.00		1.00		1.00	
14	RRV	1.00		1.00		1.00		1.00		1.00	
15	Landing foot splay (mm)	88.56	16.68	71.38	20.33	91.13	14.79	59.88*	24.90	63.88*	17.96
16	Forelimb grip strenght (kg)	6.24	0.40	4.61*	0.96	5.20*	0.84	4.72*	2.00	4.34*	1.89
17	Hindlimb grip strenght (kg)	1.21	0.20	0.88*	0.20	0.93*	0.13	0.74*	0.40	0.64*	0.34
18	Grip strenght of all limbs (kg)	19.14	2.71	11.96*	2.91	14.85*	2.64	11.21*	5.54	10.16*	4.22
		n = 8		n = 8		n = 8		n = 7		n = 7	

Table 2b. **The values of tabun-induced sensorimotor and excitability neurotoxic markers measured at 24 hrs following tabun challenge by the functional observational battery (scored values);**
 * statistically significant as compared with controls.

24 hours		Controls		Tabun + A + HI-6 + K203		Tabun + A + HI-6 + trimedoxime		Tabun + A + HI-6		Tabun	
No	Marker	x/M	± s	x/M	± s	x/M	± s	x/M	± s	x/M	± s
1	Catch difficulty	2.00		1.00*		1.00*		1.00*		1.00*	
2	Ease handling	2.00		1.00*		1.00*		1.00*		1.00*	
3	Arousal (GSC)	1.00		2.00*		2.00*		1.00		2.00*	
4	Tension	0.00		0.00		0.00		0.00		0.00	
5	Vocalisation	0.00		0.00		0.00		0.00		0.00	
6	Stereotypy	0.00		0.00		0.00		0.00		0.00	
7	Bizarre behavior	0.00		0.00		0.00		0.00		0.00	
8	Approach response	2.00		1.00*		2.00		2.00		1.00*	
9	Touch response	2.00		2.00		2.00		2.00		2.00	
10	Click response	2.00		2.00		2.00		2.00		2.00	
11	Tail-pinch response	2.00		2.00		2.00		1.00*		1.00*	
		n = 8		n = 8		n = 8		n = 7		n = 7	

Table 2c. **The values of tabun-induced autonomic neurotoxic markers measured at 24 hrs following tabun challenge by the functional observational battery** (No 1–7, 10–11, 15 – scored values, No 8–9, 12–14 – values in absolute units); * statistically significant as compared with controls.

24 hours		Controls		Tabun + A + HI-6 + K203		Tabun + A + HI-6 + trimedoxime		Tabun + A + HI-6		Tabun	
No	Marker	x/M	± s	x/M	± s	x/M	± s	x/M	± s	x/M	± s
1	Lacrimation	0.00		0.00		0.00		0.00		0.00	
2	Palpebral closure	1.00		1.00		1.00		1.00		1.00	
3	Endo/exophthalmus	0.00		0.00		0.00		0.00		0.00	
4	Fur abnormalities	0.00		0.00		0.00		0.00		0.00	
5	Skin abnormalities	0.00		0.00		0.00		0.00		0.00	
6	Salivation	0.00		0.00		0.00		0.00		0.00	
7	Nose secretion	0.00		1.00*		0.00		3.00*		3.00*	
8	Urination	4.38	3.89	1.25	3.54	3.25	2.19	3.63	3.66	2.13	3.56
9	Defecation	0.00		0.00		0.00		0.00		0.00	
10	Pupil size	0.00		–1.00*		–2.00*		0.00		–2.00*	
11	Pupil response	1.00		0.00*		0.00*		1.00		0.00*	
12	Food receiving (%)	100.00	0.00	57.00*	14.97	60.00*	10.69	40.25*	16.26	26.00*	10.69
13	Body weight (g)	253.25	23.64	279.88	27.15	253.88	26.93	209.13*	28.82	212.38*	22.84
14	Body temperature (°C)	37.04	0.17	36.64*	0.31	36.55	0.71	35.95*	0.38	36.50*	0.31
15	Respiration	0.00		0.00		0.00		0.00		0.00	
		n = 8		n = 8		n = 8		n = 7		n = 7	

RESULTS

All tabun-poisoned rats who were given the antidotal treatment of a mixture of oximes in combination with atropine, survived till the end of the experiment (24 hrs following intoxication). On the other hand, one non-treated tabun-poisoned rat and one tabun-poisoned rat treated with the oxime HI-6 alone in combination with atropine died within 24 hr after tabun challenge.

The results of the experiments related to the measurement of tabun-induced neurotoxicity at 24 hr following tabun poisoning are divided into three parts [activity and neuromuscular measures, sensorimotor and excitability measures, and autonomic measures (Moser et al. 1997)] and summarized in Tables 2a–c. The observation of neurotoxic signs indicated that many functional disorders in tabun-poisoned rats lasted at least 24 hr. Tabun produced passive behaviour in the rats during handling and retention, miosis and a decrease in muscular tone at 24 hrs following tabun administration. The exploratory and rearing activity were significantly decreased and gait was somewhat impaired. Tabun-poisoned rats were not able to constrict their pupils in response to light due to tabun-induced miosis. The approach and tail-pinch response disappeared. A significant decrease in landing foot splay, limb grip strength, food receiving and body temperature were also observed at 24 hrs following tabun challenge (Table 2a–c). The potency of the oxime HI-6 alone as well as the oxime HI-6 combined with trimedoxime or K203 to eliminate tabun-induced acute neurotoxic effects was similar and relatively low. The oxime HI-6 was able to eliminate the impairment of gait, the absence of approach response and miosis. When the mixture of HI-6 with trimedoxime or K203 was administered, observations showed the elimination of the absence of the approach and tail-pinch response and a decrease in rearing activity, landing foot splay and body temperature (Table 2a–c).

DISCUSSION

Generally, the potency of currently available oximes to eliminate tabun-induced acute neurotoxic effects is low. Pralidoxime, a currently available oxime for the treatment of poisonings with highly toxic organophosphates (Dawson 1994), seems to be practically ineffective in preventing tabun-induced neurotoxicity (Kassa and Krejčová 2003) although its penetration into CNS is higher compared to the commonly used bispyridinium oximes such as obidoxime, trimedoxime or HI-6 (Cassel et al. 1997,

Sakurada et al. 2003, Lorke et al. 2008). Trimedoxime is able to partly eliminate tabun-induced acute neurotoxicity following i.m. administration of tabun at a sublethal dose, but its neuroprotective efficacy is limited (Kassa and Karasová 2007). The oxime HI-6 was developed and introduced by some countries for the antidotal treatment of severe acute soman poisonings because of its higher reactivation and therapeutic efficacy compared to currently used oximes such as pralidoxime, obidoxime and trimedoxime (Dawson 1994, Kassa and Cabal 1999). Nevertheless, it was demonstrated to be less efficacious in blocking tabun-induced acute neurotoxicity than trimedoxime and obidoxime (Kassa and Karasová 2007). The unsatisfactory efficacy of the above mentioned oximes in eliminating tabun-induced acute neurotoxicity can be explained by the low potency of oximes in reactivating tabun-inhibited AChE *in vitro* and *in vivo* (Puu et al. 1986, Jokanović et al. 1996, Worek et al. 1998). As no oxime has been developed to satisfactorily counteract the acute neurotoxicity of nerve agents regardless of their chemical structure, the combination of two oximes seems to be a rational means of ensuring the neuroprotective efficacy of the antidotal treatment of acute poisonings with nerve agents (including tabun) regardless of their chemical structure.

In combining oximes for the antidotal treatment of acute nerve agent poisonings, HI-6 should be considered to be the most important because it is the oxime with the broadest spectrum among the commonly used oximes. The HI-6 is sufficiently effective against many nerve agents but it is a weak reactivator of tabun-inhibited AChE (Worek et al. 1999, Lundy et al. 2006). Therefore, the second oxime included in the combination should be the oxime sufficiently effective against tabun. For our experiments, trimedoxime or the oxime K203 was chosen because trimedoxime has at least some effect against tabun-inhibited AChE (Kassa et al. 2007) and K203 has been found to be a promising oxime against tabun (Musilek et al. 2007c, Kassa et al. 2008a, Kovarik et al. 2009).

The *in vitro* evaluation of the potency of the combination of the two oximes in reactivating nerve agent inhibited AChE showed that combining two oximes had no negative effects on the reactivation of nerve agent-inhibited AChE and, in addition, it had a beneficial effect by broadening the spectrum of the individual oximes (Worek et al. 2007). These results correspond to *in vivo* results from mice, rats and guinea-pigs published by several authors (Clement et al. 1987, Kovačević et al. 1989, Maksimović and Kovačević 1989). They found that the mixture of the

oxime HI-6 and trimedoxime in the presence of atropine and diazepam ensures a higher degree of protection against poisoning by tabun, sarin and VX than the mixtures of HI-6 with pralidoxime or obidoxime (Kovačević et al. 1991). The beneficial effects of the combination of oximes compared to a single oxime treatment in reactivating nerve agent-inhibited AChE could be explained by an elevated plasma oxime level and the synergetic effect of both oximes (Worek et al. 2007). Nevertheless, our results demonstrate that the combination of the oxime HI-6 with trimedoxime or K203 did not bring any beneficial effect for the potency of HI-6 in reducing the acute neurotoxic effects of tabun. Due to the limited penetration of oximes through the blood-brain barrier (BBB), the elevated plasma oxime level was probably not enough to significantly increase the penetration of oximes across BBB.

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