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ORIGINAL ARTICLE

A COMPARISON OF THE NEUROPROTECTIVE EFFICACY OF INDIVIDUAL OXIMES (HI-6, TRIMEDOXIME, K203) AND THEIR MIXTURES (HI-6 + TRIMEDOXIME, HI-6 + K203) IN CYCLOSARIN-POISONED RATS

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Summary
The ability of three oximes (HI-6, trimedoxime, K203) to reduce cyclosarin-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. Cyclosarin-induced neurotoxicity and the neuroprotective effects of HI-6, trimedoxime or K203 alone and HI-6 combined with trimedoxime or K203 in rats poisoned with cyclosarin at a sublethal dose (80 μg/kg i.m.; 70% of LD_{50} value) were monitored by the functional observational battery at 24 hours and 7 days following cyclosarin challenge. The results indicate that all types of antidotal treatment are able to survive cyclosarin-poisoned rats 7 days following cyclosarin poisoning while one non-treated cyclosarin-poisoned rats died within 24 hours following cyclosarin challenge. All three oximes alone as well as both oxime mixtures combined with atropine were able to slightly decrease cyclosarin-induced neurotoxicity in the case of sublethal poisoning but they did not eliminate all cyclosarin-induced acute neurotoxic signs and symptoms. Their ability to reduce cyclosarin-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 antidotal treatment of acute cyclosarin poisoning compared to the individual oximes.

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

INTRODUCTION

Highly toxic organophosphorus compounds called nerve agents are considered to be the most dangerous chemical warfare agents. Their acute toxic effects are based on phosphylation of acetylcholinesterase (AChE, EC 3.1.1.7) leading to the irreversible inhibition of this enzyme and subsequent overstimulation of postsynaptic cholinergic receptors due to the accumulation of the neurotransmitter acetylcholine in synapses of the central and peripheral nervous systems (1, 23, 26). The current standard antidotal treatment of poisoning with nerve agents usually includes a muscarinic cholinergic receptor antagonist (preferably atropine) to block the overstimulation of cholinergic receptors by acetylcholine and an oxime to reactivate nerve agent-inhibited AChE (9, 36).

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Nerve agents can produce centrally-mediated seizure activity that rapidly progresses to status epilepticus and contributes to profound brain damage (26, 36). Therefore, the ability of antidotes to block the acute neurotoxic effects of nerve agents and prevent the development of irreversible lesions in the central nervous system (CNS) is important for successful antidotal treatment. Generally, the oximes exert more potent effects in the peripheral compared to central system due to their poor penetration into CNS. Nevertheless, there are published results demonstrating the penetration of oximes into CNS and subsequent reactivation of nerve agent-inhibited AChE in the brain (3, 34). Although the rate of reactivation of nerve agent-inhibited AChE in the brain is lower compared to the peripheral system, the role of reactivation of nerve agent-inhibited AChE in the brain is important for survival from nerve agent exposure (1, 9).

Unfortunately, experimental results confirm that there is no currently available, broad-spectrum oxime suitable for the antidotal treatment of poisonings with all nerve agents (2, 13, 27). 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 agents poisoning. During the past several decades, a lot of new oximes were 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 (28, 32). Recently, the oxime K203 [1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide] was synthesized at our Department of Toxicology (30) and its pharmacokinetics was studied (7). Based on *in vitro* and *in vivo* evaluation of its reactivating, therapeutic and neuroprotective efficacy, it was considered to be the promising oxime against tabun but not against cyclosarin and soman (11, 12, 14, 15, 16, 19).

As no oxime able to suffciently reactivate AChE inhibited with all nerve agents has been found till now, to combine two oximes able to cover the whole spectrum of nerve agents seems to be the promising way how to reach the sufficiently effective antidotal treatment of acute poisonings with nerve agents regardless of their chemical structure. The mixture of two oximes could express the sufficient ability to reactivate nerve agent- inhibited AChE in the peripheral as well as central nervous system regardless of the chemical structure of nerve agent.

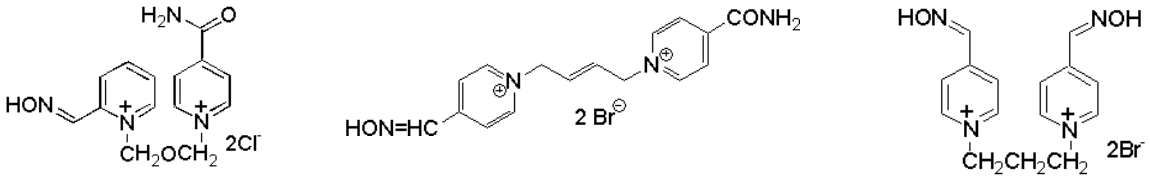
In this paper, the combination of HI-6 with trimedoxime or K203 was used. The neuroprotective efficacy of the chosen mixtures of oximes in combination with atropine was evaluated against cyclosarin (GF; cyclohexyl methylphosphonofluoridate) because its acute neurotoxicity is difficult to antagonize due to low potency of all commonly used oximes to reactivate cyclosarin-inhibited brain AChE (6, 27)

The main aim of this study was to compare the neuroprotective efficacy of three individual oximes (trimedoxime, HI-6, K203) with two mixtures of oximes containing the oxime HI-6 and trimedoxime or the oxime K203 in combination with an anticholinergic drug atropine in cyclosarin-poisoned rats. The cyclosarin-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

Male albino Wistar rats weighing 200-230g were purchased from VELAZ (Prague, Czech Republic). They were kept in an air-conditioned room (22 ± 2 oC and 50 ± 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 7 animals. Handling of the experimental animals was performed in compliance with relevant laws and institutional guidelines and done under the supervision of the Ethics Committee of the Faculty of Military Health Sciences in Hradec Kralove (Czech Republic).

Cyclosarin was obtained from Military Technical Institute in Brno (Czech Republic) and was 98.5 % pure. Its purity was assayed by acidimetric titration. Trimedoxime, the oxime HI-6 and a newly developed oxime K203 (Figure 1) of 97.0 % purity were synthesized earlier at the Department of Toxicology of the Faculty of Military Health Sciences in Hradec Kralove (Czech Republic) (31, 32). Their purity was analysed 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.)



HI-6 **K203** **Trimedoxine**

Figure 1. Chemical structure of oximes studied

Cyclosarin was administered at a sublethal dose (80 µg/kg b.w. - 70% LD_{50}). One minute following cyclosarin challenge, the rats were treated with atropine (21 mg/kg b.w.) in combination with trimedoxime, HI-6 or K203 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 cyclosarin was monitored using the functional observational battery at 24 hours and 7 days following cyclosarin poisoning. The evaluated markers of cyclosarin-induced neurotoxicity in experimental animals were compared with the parameters obtained from control rats given saline instead of cyclosarin and antidotes at the same volume (1 mL/kg b.w.).

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 (5). The first evaluation was obtained when cyclosarin-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 handheld. 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 started 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 behaviors 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 behavior did not know about the design of experiments.

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	aggrression	
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						
SKIN ABNORMALITIES			*normal*	pale	erythema	cyanosis	pigmented	cold	injury	
SALIVATION			*none*	sllight	severe					
NOSE SECRETION			*none*	slight	severe	coloured				

MARKER	Scored values only									
	-2	-1	0	1	2	3	4	5	6	7
CLONIC MOVEMENTS			*normal*	repetitive movements of mouth and jaws	nonrhythmic quivers	mild tremors	severe tremors	myoclonic jerks	clonic convulsions	
TONIC MOVEMENTS			*normal*	contraction of extensors	opisthotonus	emprostho-tonus	explosive jumps	tonic convulsions		
GAIT			*normal*	ataxia	overcompen-sation of hindlimbs movements	feet point outwards from body	forelimbs are extended	walks on tiptoes	hunched body	body is flattened against surface
AROUSAL (GSC)				*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					
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 SIZE	considerable miosis	miosis	*normal*	mydriasis	considerable mydriasis					
PUPIL RESPONSE			no reaction	*normal reaction*						
RIGHTING REFLEX (RRV)				*normal*	slightly uncoordin.	lands on side	lands on back			

Table 1. Functional Observational Battery

Data collected with the functional observational battery include categorial, ordinal and continuous values. Statistical analyses were performed on a PC with a special interactive programme NTX (5). The categorial and ordinal values were formulated as contingency tables and judged consecutively by Chi-squared test of homogeneity, Concordance-Discordance test and Kruskal-Wallis test, respectively. The continual data were assessed by successive statistical tests: CI for Delta, Barlett test for Equality of Variance, Williams test and Test for Distribution Functions (33). The differences were considered significant when $p < 0.05$.

RESULTS

All treated cyclosarin-poisoned rats survived till the end of experiment (7 days following the intoxication). On the other hand, one non-treated cyclosarin-poisoned rat died within 24 hr after cyclosarin challenge.

The evaluation of cyclosarin-induced neurotoxic signs at 24 hours following intoxication proved significant alteration of 12 observed parameters. Cyclosarin caused passive behavior of rats during handling and catching, miosis and a decrease in muscular tonus. The exploratory activity was significantly decreased. In addition, the rats poisoned with cyclosarin did not show any reaction during a reflex testing consisting of recording each rat`s response to the frontal approach of the blunt end of a pen. No ability of pupils to constrict in response to light was demonstrated either. Additionally, a significant decrease in hindlimb grip strength, body temperature and food receiving was also observed at 24 hours following cyclosarin challenge (Table 2).

The newly developed oxime K203 as well as currently available trimedoxime and HI-6 in combination with atropine showed a slight potency to reduce cyclosarin-induced neurotoxic signs but they were not

able to eliminate all cyclosarin-induced signs of acute neurotoxicity. Miosis, no ability of pupils to constrict in response to light, no reaction during a reflex testing consisting of recording each rat`s response to the frontal approach of the blunt end of a pen and a decrease in muscular tonus, hindlimb grip strength, body temperature and food receiving were observed at 24 hours following cyclosarin challenge and antidotal treatment with atropine in combination with K203, obidoxime or HI-6. In addition, cyclosarin poisoned rats treated with K203 in combination with atropine showed passive behavior of rats during catching and handling. In the case of treatment with combinations of oximes (HI-6 + trimedoxime, HI-6 + K203), the cyclosarin-poisoned rats showed practically the same neurotoxic sings and symptoms at 24 hr after cyclosarin poisoning with the exception of the loss of the reaction during a reflex testing consisting of recording each rat`s response to the frontal approach of the blunt end of a pen. In addition, cyclosarin poisoned rats treated with the oxime mixture consisting HI-6 + K203 in combination with atropine showed passive behavior of rats during catching and handling (Table 2).

24 hours:		**Controls**		**GF + A + HI-6 + K203**		**GF + A + HI-6 + trimedoxime**		**GF + A + K203**		**GF + A + trimedoxime**		**GF + A + HI-6**		**GF**	
No	***Marker***	***x/M***	***-/+s***	***x/M***	***-/+s***	***x/M***	***-/+s***	***x/M***	***-/+s***	***x/M***	***-/+s***	***x/M***	***-/+s***	***x/M***	***-/+s***
1	posture	1,00		**3,00***		1,00		1,00		**3,00***		1,00		**3,00***	
2	catch difficulty	2,00		**1,00***		2,00		**1,00***		2,00		2,00		**1,00***	
3	ease of handling	2,00		**1,00***		2,00		**1,00***		2,00		2,00		**1,00***	
4	muscular tonus	0,00		**-1,00***		**-1,00***		**-1,00***		**-1,00***		**-1,00***		**-1,00***	
5	lacrimation	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
6	palpebral closure	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
7	endo/exophtalmus	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
8	fur abnormalities	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
9	skin abnormalities	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
10	salivation	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
11	nose secretion	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
12	rearing	7,57	7,04	3,29	2,81	1,71	2,63	10,14	7,36	3,43	4,08	1,14	5,21	5,43	5,13
13	urination	0,86	2,27	2,29	3,68	1,14	1,86	2,29	3,73	3,57	7,74	1,86	2,48	3,14	6,39
14	defecation	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
15	hyperkinesis	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
16	tremors	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
17	clonic movements	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
18	tonic movements	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
19	gait	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
20	ataxia	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
21	gait score	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
22	mobility score	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
23	arousal (GSC)	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
24	activity	4,00		2,00		2,00		4,00		4,00		2,00		**1,00***	
25	tension	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
26	vocalisation	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
27	stereotypy	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
28	bizzare behavior	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
29	approach response	1,00		1,00		1,00		**2,00***		1,00		**2,00***		**2,00***	
30	touch response	2,00		2,00		2,00		2,00		2,00		2,00		2,00	
31	click response	2,00		2,00		2,00		2,00		2,00		2,00		2,00	
32	tail-pinch response	2,00		2,00		2,00		2,00		2,00		2,00		2,00	
33	pupil size	0,00		0,00		**-1,00***		**-1,00***		**-1,00***		**-1,00***		**-1,00***	
34	pupil response	1,00		**0,50***		**0,00***		**0,00***		**0,00***		**0,50***		**0,00***	
35	RRF	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
36	RRV	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
37	landing foot splay (mm)	69,71	9,53	71,14	18,58	68,00	22,02	76,86	19,17	73,43	17,44	77,86	14,55	72,29	38,26
38	forelimb grip strength (kg)	5,39	0,70	5,90	0,51	5,34	0,97	6,21	1,10	5,56	1,00	5,93	0,97	5,54	0,52
39	hindlimb grip strength (kg)	1,26	0,16	**0,83***	0,26	**0,76***	0,31	**0,79***	0,23	**0,80***	0,13	**0,91***	0,28	**1,13***	0,05
40	grip strength of all limbs (kg)	19,73	2,15	**16,49***	2,22	**14,01***	3,27	16,51	3,98	16,16	4,68	17,09	6,36	**16,60***	1,87
41	food receiving (%)	100,00	0,00	**75,00***	22,45	**45,86***	1,07	**84,14***	19,88	**74,29***	24,95	**76,43***	19,07	**85,71***	17,48
42	body weight (g)	271,71	25,32	280,86	24,64	279,57	43,63	302,57	14,71	263,17	17,60	248,29	26,74	282,50	21,24
43	body temperature (°C)	38,00	0,20	**37,53***	0,39	**37,51***	0,36	**37,64***	0,27	**36,97***	0,63	**37,61***	0,39	**37,64***	0,32
44	respiration	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
		n = 7		**n = 7**		**n = 7**		**n = 7**		**n = 7**		**n = 7**		**n = 6**	

Table 2. The values of cyclosarin-induced neurotoxic markers measured at 24 hours following cyclosarin challenge by the Functional observational battery (No 1-11, 15-36, 44 - scored values, No 12-14, 37-43 - values in absolute units). Statistical significance: * $p < 0.05$ - comparison with the control values (applied abbreviations: GF – cyclosarin, A - atropine; RRF - air righting reflex; RRV - air righting reflex from vertical position; x/M - average or modus value; ± s - standard deviation; n - number of surviving animals).

The 7 days neurotoxicity evaluation showed similar results. While the evaluation of cyclosarin-induced neurotoxic signs and symptoms at 7 days following intoxication showed almost the same clinical picture compared to 24 hours after cyclosarin challenge – a significant alteration of 7 observed parameters (passive behavior of rats during handling and catching, ataxia, miosis, no ability of pupils to constrict in response to light and a significant decrease in hindlimb grip strength), the antidotal treatment of cyclosarin poisoning brought similarly slight elimination of cyclosarin-induced neurotoxic signs and symptoms regardless of the type of antidotes used (Table3).

7 days		Controls		GF + A + HI-6 + K203		GF + A + HI-6 + trimedoxime		GF + A + K203		GF + A + trimedoxime		GF + A + HI-6		GF	
No	*Marker*	*x/M*	*-/+s*	*x/M*	*-/+s*	*x/M*	*-/+s*	*x/M*	*-/+s*	*x/M*	*-/+s*	*x/M*	*-/+s*	*x/M*	*-/+s*
1	posture	1,00		1,00		1,00		1,00		1,00		**3,00***		**3,00***	
2	catch difficulty	2,00		2,00		2,00		2,00		2,00		**1,00***		**1,00***	
3	ease of handling	2,00		2,00		2,00		2,00		2,00		**1,00***		**1,00***	
4	muscular tonus	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
5	lacrimation	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
6	palpebral closure	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
7	endo/exophtalmus	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
8	fur abnormalities	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
9	skin abnormalities	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
10	salivation	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
11	nose secretion	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
12	rearing	3,43	5,00	4,43	3,87	1,29	1,60	1,14	2,04	2,43	3,31	2,43	2,23	0,33	0,82
13	urination	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
14	defecation	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
15	hyperkinesis	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
16	tremors	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
17	clonic movements	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
18	tonic movements	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
19	gait	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
20	ataxia	0,00		0,00		0,00		0,00		0,00		**1,00***		**1,00***	
21	gait score	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
22	mobility score	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
23	arousal (GSC)	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
24	activity	4,00		3,00		4,00		4,00		4,00		4,00		4,00	
25	tension	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
26	vocalisation	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
27	stereotypy	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
28	bizzare behavior	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
29	approach response	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
30	touch response	2,00		2,00		2,00		2,00		2,00		2,00		2,00	
31	click response	2,00		2,00		2,00		2,00		2,00		2,00		2,00	
32	tail-pinch response	2,00		2,00		2,00		2,00		2,00		2,00		2,00	
33	pupil size	0,00		**-2,00***		**-1,00***		**-1,00***		**-2,00***		0,00		**-2,00***	
34	pupil response	1,00		**0,00***		**0,00***		**0,00***		**0,50***		**0,50***		**0,00***	
35	RRF	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
36	RRV	1,00		1,00		1,00		1,00		1,00		1,00		1,00	
37	landing foot splay (mm)	87,86	14,61	**70,57***	6,85	84,57	16,97	69,14	22,04	86,43	18,35	72,00	20,03	74,43	35,35
38	forelimb grip strength (kg)	6,33	1,34	7,00	0,91	6,53	0,66	6,54	2,03	6,70	1,27	6,24	2,45	6,78	1,31
39	hindlimb grip strength (kg)	1,31	0,11	**1,09***	0,21	**1,09***	0,23	1,20	0,46	**1,11***	0,21	1,04	0,36	**1,18***	0,04
40	grip strength of all limbs (kg)	19,59	1,85	18,19	2,76	19,21	2,74	19,93	7,57	21,71	3,02	18,36	6,94	20,83	2,51
41	food receiving (%)	100,00		100,00		100,00		100,00		100,00		100,00		100,00	
42	body weight (g)	295,57	26,88	321,29	8,69	295,14	25,79	287,71	74,10	**261,43***	19,16	251,43	52,78	304,00	32,86
43	body temperature (°C)	37,31	0,38	**36,80***	0,27	**36,80***	0,49	36,54	1,07	37,41	0,38	36,79	1,47	37,50	0,46
44	respiration	0,00		0,00		0,00		0,00		0,00		0,00		0,00	
		n = 7		n = 7		n = 7		n = 7		n = 7		n = 7		n = 6	

Table 3. The values of cyclosarin-induced neurotoxic markers measured at 7 days following cyclosarin challenge by the Functional observational battery (No 1-11, 15-36, 44 - scored values, No 12-14, 37-43 - values in absolute units). Statistical significance and abbreviations: see Table 2.

DISCUSSION

Nerve agents including cyclosarin appear to cause centrally mediated seizure activity that can rapidly progress to status epilepticus and contribute to profound brain damage. Nerve agent-induced hyperstimulation of cholinergic muscarinic receptors in the brain can induce the first phase of centrally

mediated seizures, whereas sustained seizures (status epilepticus) are probably associated with increased glutamatergic activity leading to excitotoxic damage predominantly in the hippocampus, amygdala, piriform and entorhinal cortices (29). Thus, the exposure of experimental animals to nerve agents in convulsions-inducing doses may result in irreversible lesions in CNS that can be manifested as neurobehavioral effects in convulsing survivors (1). Therefore, the ability of antidotes to counteract acute neurotoxic effects of nerve agents and prevent nerve agent-poisoned organisms from irreversible lesions in CNS is very important for the successful antidotal treatment of acute nerve agent poisonings.

Unfortunately, the neuroprotective efficacy of all currently available oximes as well as newly developed oximes is rather low, probably due to poor penetration through blood-brain barrier (BBB) and low reactivating efficacy in the CNS (8, 22, 37). Even the oxime HI-6 that is considered to be the best reactivator of cyclosarin-inhibited AChE (6, 9, 10, 20, 21) is not satisfactorily effective oxime for the elimination of cyclosarin-induced neurotoxic signs and symptoms due to low penetration through BBB and low reactivating efficacy in the CNS (6, 22). On the other hand, it is known that the oximes may also attentuate nerve agent-induced brain insult via different mechanisms other than AChE reactivation (35).

As no oxime has been developed to satisfactorily counteract the acute neurotoxicity of nerve agents regardless of their chemical structure till now, the combination of two oximes seems to be the rational solution how to ensure the neuroprotective efficacy of antidotal treatment of acute poisonings with nerve agents (including cyclosarin) regardless of their chemical structure. To combine the oximes for the antidotal treatment of acute nerve agent poisonigs, the oxime HI-6 should be considered to be the most important oxime because it is the oxime with the broadest spectrum among commonly used oximes. The oxime HI-6 is sufficiently effective against many nerve agents but it is weak reactivator of tabun-inhibited AChE (24, 39). Therefore, the second oxime, involved into 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 (13) and the oxime K203 was found to be a promising oxime against tabun (11, 19, 30).

In vitro evaluation of the potency of the combination of two oximes to reactivate nerve agents 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 effects by broadening the spectrum of the individual oximes (38). These results correspond to *in vivo* results from mice, rats and guinea-pigs published by several authors (4, 18, 25). It was found that the mixture of the oxime HI-6 and trimedoxime in the presence of atropine and diazepam ensures the higher degree of protection against poisoning by tabun, sarin and VX than the mixtures of HI-6 with pralidoxime or obidoxime (17). 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 synergetic effects of both oximes (38). 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 single oximes (HI-6, trimedoxime, K203) to reduce acute neurotoxic effects of cyclosarin. The absence of an increase in the neuroprotective efficacy of chosen oxime mixtures compared to tested single oximes could be explained by a limited penetration of oximes through BBB (22) and limited reactivation of nerve agent-inhibited AChE in the brain (6, 9).

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REFERENCES

1. BAJGAR, J. Organophosphate/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. Adv. Clin. Chem., 2004, vol. 38, p. 151-216.
2. BAJGAR, J. Complex view on poisoning with nerve agents and organophosphates. Acta Med. (Hradec Kralove), 2005, vol. 48, 3-21.
3. CASSEL, G. - KARLSSON, L. - WAARA, L., et al. Pharmacokinetics and effects of HI-6 in blood and brain of soman-intoxicated rats: a microdialysis study. Eur. J. Pharmacol., 1997, vol. 332, p. 43-52.
4. CLEMENT, J.G. – SHILOFF, J.D. – GENNINGS, C. Efficacy of a combination of acetylcholinesterase reactivators, HI-6 and obidoxime, against tabun and soman poisoning of mice. Arch. Toxicol., 1987, vol. 61, p. 70-75.

5. FRANTIK, E. – HORNYCHOVA, M. Clustering of neurobehavioral measures of toxicity. Homeostasis, 1995, vol. 36, p. 19-25.
6. JOKANOVIC, M. – PROSTRAN, M. Pyridinium oximes as cholinesterase reactivators. Structure-activity relationship and efficacy in the treatment of poisoning with organophosphorus compounds. Curr. Med. Chem., 2009, vol. 16, p. 2177-2188.
7. KALASZ, H. – LAUFER, R. – SZEGI, P., et al. HPLC study of the pharmacokinetics of K-203. Acta Chromatogr., 2008, vol. 20, p. 575-584.
8. KALASZ, H. – FÜRESZ, J. – TEKES, K. Monitoring the pharmacokinetics of pyridinium aldoximes in the body. Mini-Rev. Med. Chem., 2009, vol. 9, p. 448-462.
9. KASSA, J. Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. J. Toxicol. Clin. Toxicol., 2002, vol. 40, p. 803-816.
10. KASSA, J. – CABAL, J. A comparison of the efficacy of acetylcholinesterase reactivators against cyclohexyl methylphosphonofluoridate (GF agent) by in vitro and in vivo methods. Pharmacol. Toxicol., 1999, vol. 84, p. 41-45.
11. KASSA, J. – KARASOVA, J. – MUSILEK, K., et al. An evaluation of therapeutic and reactivating effects of newly developed oximes (K156, K203) and commonly used oximes (obidoxime, trimedoxime, HI-6) in tabun-poisoned rats and mice. Toxicology, 2008, vol. 243, p. 311-316.
12. KASSA, J. – KARASOVA, J. – VASINA, L., et al. A comparison of neuroprotective efficacy of newly developed oximes (K203, K206) and commonly used oximes (obidoxime, HI-6) in tabun-poisoned rats. Drug Chem. Toxicol., 2009, vol. 32, p. 128-138.
13. KASSA, J. – KUCA, K. - BARTOSOVA L., et al. The development of new structural analogues of oximes for the antidotal treatment of poisoning by nerve agents and the comparison of their reactivating and therapeutic efficacy with currently available oximes. Curr. Org. Chem., 2007, vol. 11, p. 267-283.
14. KASSA, J. - ZDAROVA KARASOVA, J. - CAISBERGER F., et al. The influence of combinations of oximes on the reactivating and therapeutic efficacy of antidotal treatment of soman poisoning in rats nad mice. Toxicol. Mech. Meth., 2009, vol. 19, p. 547-551.
15. KASSA, J. - ZDAROVA KARASOVA, J. – MUSILEK, K., et al. A comparison of reactivating and therapeutic efficacy of newly developed oximes (K156, K203) and commonly used oximes (obidoxime, HI-6) in cyclosarin-poisoned rats nad mice. Toxicol. Mech. Meth., 2009, vol. 19, p. 346-350.
16. KASSA, J. - ZDAROVA KARASOVA, J. – TESAROVA, S., et al. A comparison of the neuroprotective efficacy of newly developed oximes (K156, K203) and currently available oximes (obidoxime, HI-6) in cyclosarin-poisoned rats. Acta Med. (Hradec Kralove), 2008, vol. 51, p. 215-221.
17. KOVACEVIC, V. – MAKSIMOVIC, M. – DELJAC, V., et al. Protective effects of mixture of oximes in poisoning by nerve chemical warfare agents. Acta Pharm. Jugoslav., 1991, vol. 41, p. 75-78.
18. KOVACEVIC, V. – MAKSIMOVIC, M. – PANTELIC, D., et al. Protective and reactivating effects of HI-6 PAM-2 mixture in rats poisoned with nerve chemical warfare agents. Acta Pharm. Jugoslav., 1989, vol. 39, p. 161-165.
19. KOVARIK, Z. - LUCIC VRDOLJAK, A. – BEREND, S., et al. Evaluation of oxime K203 as antidote in tabun poisoning. Arh. Hig. Rada Toksikol., 2009, vol. 60, p. 19-26.
20. KUCA, K. – CABAL, J. - JUN D., et al. Potency of new structurally different oximes to reactivate cyclosarin-inhibited human brain acetylcholinesterase. J. Enz. Inhib. Med. Chem., 2006, vol. 21, p. 663-666.
21. KUCA, K. – CABAL, J. – JUN, D., et al. In vitro reactivation potency of some acetylcholinesterase reactivators against sarin- and cyclosarin-induced inhibitions. J. Appl. Toxicol., 2005, vol. 25, p. 296-300.
22. LORKE, DE. – KALASZ, H. – PETROIANU, GA., et al. Entry of oximes into the brain: A review. Curr. Med. Chem., 2008, vol. 15, p. 743-753.
23. LOTTI, M. Organophosphorus compounds. In SPENCER, PS. – SCHAUMBURG, HH. (eds). Experimental and Clinical Neurotoxicology, New York, Oxford University Press, 2000, p. 898-925.
24. LUNDY, PM. – RAVEH, L. – AMITAI, G. Development of the bisquaternary oxime HI-6 toward clinical use in the treatment of organophosphate nerve agent poisoning. Toxicol. Rev., 2006, vol. 25, p. 231-243.
25. MAKSIMOVIC, M. – KOVACEVIC, V. Protective and reactivating effects of HI-6-toxogonin mixture in rats and guinea-pigs poisoned by nerve agents. Acta Pharm. Jugoslav., 1989, vol. 39, p. 27-33.
26. MARRS, TC. Organophosphate poisoning. Pharmacol. Ther., 1993, vol. 58, p. 51-66.

27. MARRS, TC. – RICE, P. – VALE, JA. The role of oximes in the treatment of nerve agent poisoning in civilian casualties. Toxicol. Rev. 2006, vol. 25, p. 297-323.
28. MAXWELL, DM. – KOPLOVITZ, I. – WOREK, F., et al. A structure-activity analysis of the variation in oxime efficacy against nerve agents. Toxicol. Appl. Pharmacol., 2008, vol. 231, p. 157-164.
29. MCDONOUGH, JH. JR. – SHIH, T-M. Neuropharmacological modulation of nerve agent-induced seizure and neuropathology. Neurosci Biobehav. Rev., 1997, vol. 21, p. 559-579.
30. MUSILEK, K. – JUN, D. – CABAL, J., et al. Design of a potent reactivator of tabun-inhibited acetylcholinesterase - synthesis and evaluation of (E)-1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (K203). J. Med. Chem., 2007, vol. 50, p. 5514-5518.
31. MUSILEK, K. – KUCA, K. – JUN, D., et al. Synthesis of bispyridinium compounds bearing propane linker and evaluation of their reactivation activity against tabun- and paraoxon-inhibited acetylcholinesterase. Lett. Org. Chem., 2006, vol. 3, p. 831-835.
32. MUSILEK, K. – KUCA, K. – JUN, D., et al. Progress in synthesis of new acetylcholinesterase reactivators in period 1990-2004. Curr. Org. Chem., 2007, vol. 11, p. 229-238.
33. ROTH, Z. – JOSIFKO, M. – MALY, V., et al. Statistical Methods in Experimental Medicine (in Czech), SZN Praha 1962.
34. SAKURADA, K. – MATSUBARA, K. – SHIMIZU, K., et al. Pralidoxime iodide (2-PAM) penetrates across the blood-brain barrier. Neurochem. Res., 2003, vol. 28, p. 1401-1407.
35. SHROT, S. – MARKEL, G. – DUSHNITSKY, T., et al. The possible use of oximes as antidotal therapy in organophosphate-induced brain damage. NeuroToxicology, 2009, vol. 30, p. 167-173.
36. TAYLOR, P. Anticholinesterase agents. In HARDMAN, JG. – LIMBIRD, LE. (eds). The Pharmacological Basis of Therapeutics. New York, McGraw Hill, 1996, p.161-176.
37. VOICU, VA. – THIERMANN, H. – RÄDULESCU, FS., et al. The toxicokinetics and toxicodynamics of organophosphonates versus the pharmacokinetics and pharmacodynamics of oxime antidotes: biological consequences. Bas. Clin. Pharmacol. Toxicol. 2010, vol. 106, p. 73-85.
38. WOREK, F. – AURBEK, N. – THIERMANN, H. Reactivation of organophosphate-inhibited human AChE by combinations of obidoxime and HI 6 in vitro. J. Appl. Toxicol., 2007, vol. 27, p. 582-588.
39. WOREK, F. – DIEPOLD, C. – EYER, P. Dimethylphosphoryl-inhibited human cholinesterases: inhibition, reactivation, and aging kinetics. Arch. Toxicol., 1999, vol. 73, p. 7-14.