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Comparison of the neuroprotective effects of a novel bispyridinium oxime KR-22934 with the oxime K203 and obidoxime in tabun-poisoned male rats☆

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SUMMARY

The neuroprotective effects of a novel oxime KR-22934, the oxime K203 and obidoxime in combination with atropine in rats poisoned with tabun at a sublethal dose (200 μg/kg i.m.; 80% LD_{50}) were studied. The tabun-induced neurotoxicity was monitored at 24 h following tabun challenge using a functional observational battery and an automatic measurement of motor activity. The results indicate that all tabun-poisoned rats treated with oximes in combination with atropine were able to survive within 24 h following tabun poisoning. One tabun-poisoned rat without antidotal treatment died within 24 h. The oximes KR-22934 and K203 combined with atropine showed a similar potency to decrease tabun-induced neurotoxicity at 24 h after tabun administration while the neuroprotective efficacy of obidoxime was slightly higher. However, no oxime was able to eliminate tabun-induced neurotoxicity completely. When atropine was administered alone, negligible neuroprotective efficacy was observed. Based on the results, a novel oxime KR-22934 did not bring any improvement of the neuroprotective efficacy of antidotal treatment of acute tabun poisonings.

Introduction

Nerve agents are considered to be the most dangerous chemical warfare agents because of their high acute toxicity, rapid onset of clinical signs and symptoms and the rapid progression of acute intoxication. Severe poisonings with nerve agents can progress to death due to respiratory failure within tens of minutes. Their acute toxic effects are based on the irreversible inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) and subsequent overstimulation of postsynaptic cholinergic receptors due to the accumulation of the neurotransmitter acetylcholine in synapses of the central and peripheral nervous systems (Lotti et al., 2000; Bajgar, 2004; Delfino et al., 2009). Although antidotes against nerve agents have been developed based on the knowledge of above

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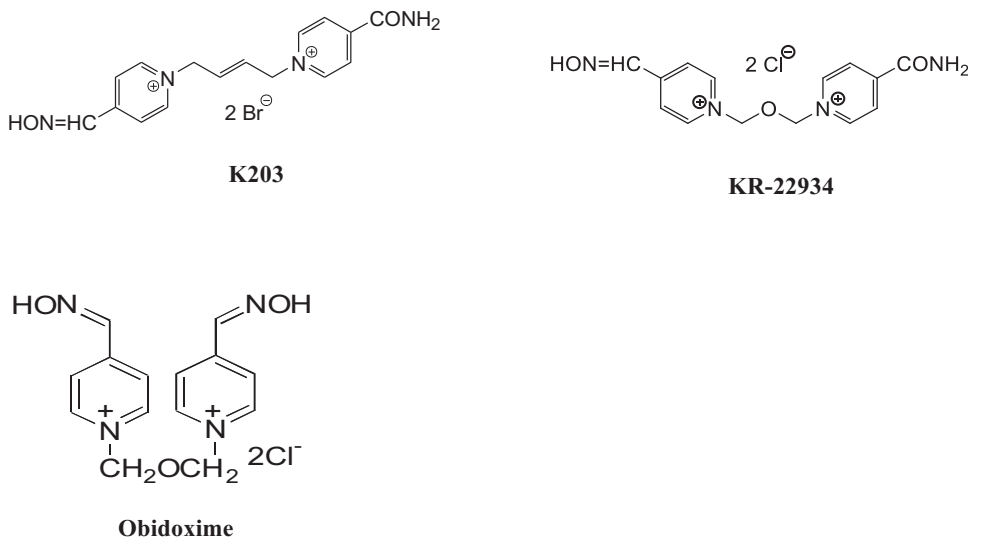

Fig. 1 – Chemical structure of oximes studied.

mentioned basic mechanism of acute toxicity of nerve agents, their efficacy is limited (Jokanovic and Prostran, 2009; Kassa et al., 2012).

In the case of severe intoxication, some nerve agents (especially soman, tabun and sarin) can cause centrally mediated seizure activity that can rapidly progress to status epilepticus and contribute to brain damage. The exposure of experimental animals to nerve agents in convulsions-inducing doses may result in irreversible lesions in the central nervous system (CNS) that can be manifested as behavioural effects in convulsing survivors (Taylor et al., 2001; Bajgar, 2004). Therefore, the ability of antidotes to counteract acute neurotoxic effects of nerve agents and prevent nerve agent-poisoned organisms from irreversible lesions in the CNS is very important for the successful antidotal treatment of acute nerve agent poisonings. Generally, the oximes exert more potent effects in the peripheral nervous system compared to the central nervous system due to their low penetration across the blood-brain barrier (BBB). Nevertheless, results demonstrating the penetration of oximes into the CNS and subsequent reactivation of nerve agent-inhibited AChE in the brain have previously been published (Cassel et al., 1997; Sakurada et al., 2003; Lorke et al., 2008). Although the percentage of reactivation of nerve agent-inhibited AChE in the brain is lower compared to the peripheral compartment, the role of reactivation of nerve agent-inhibited AChE in the brain is important for survival from nerve agent exposure (Bajgar, 2004).

One of the most important nerve agents is tabun (*O*-ethyl-*N*, *N*-dimethylphosphoramido-cyanidate). It differs from other highly toxic nerve agents in its chemical structure and by the fact that commonly used antidotes are not able to sufficiently prevent tabun-induced acute toxic effects. The deleterious effects of tabun are extraordinarily difficult to antagonize because of the existence of a free electron pair located on amidic nitrogen and 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).

As the ability of currently used monopyridinium (*e.g.* pralidoxime) and bispyridinium oximes (*e.g.* obidoxime, HI-6) to counteract the adverse effects of tabun is generally low (Kassa et al., 2005), the replacement of commonly used oximes (pralidoxime, obidoxime) as well as H oximes (the oxime HI-6) with a more effective oxime has been a long-standing goal for the treatment of tabun poisoning (Dohnal et al., 2005). The oxime K203, developed in our Department of Toxicology seven years ago, was considered to be a promising reactivator of tabun-inhibited AChE. However, the differences between the reactivating and therapeutic efficacy of K203 and some commonly used bispyridinium oximes (obidoxime, trimedoxime) are relatively small (Kassa et al., 2008). Therefore, we are still searching for a more efficacious oxime able to sufficiently reactivate tabun-inhibited AChE. Recently, the bispyridinium oxime, KR-22934 [1-(4-carbamoylpyridinium)-3-(4-hydroxyiminomethylpyridinium)-2-oxapropan-dichloride] (Fig. 1) was synthesized in Korea to improve the reactivating and therapeutic efficacy of antidotal treatment of tabun poisoning.

The aim of this study was to compare in tabun-poisoned rats the neuroprotective effects of the oxime KR-22934 with the oxime K203 and obidoxime in combination with an 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.

Materials and methods

Animals

Male albino Wistar rats weighing 200–220 g were purchased from VELAZ (Prague, Czech Republic). They were kept in an air-conditioned room (22 ± 2 °C and 50 ± 10% relative humidity, with lights from 7.00 to 19.00 h) 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 done under the supervision of the Ethics Committee of the Faculty of Military Health Sciences in Hradec Králové (Czech Republic).

Chemicals

Tabun was obtained from the Military Technical Institute in Brno (Czech Republic) and was 96.5% pure as assayed by acidimetric titration. The oximes with the exception of KR-22934 (obidoxime, K203) of 97.5% purity were synthesized at the Department of Toxicology of the Faculty of Military Health Sciences (Czech Republic). The oxime KR-22934 of 98% purity was synthesized in the Medicinal Science Division of the Korean Research Institute of Chemical Technology. The purity of the oximes was analyzed using an HPLC technique. 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 i.m. at a sublethal dose (200 μg/kg b.w. – 80% LD_{50}). One minute following tabun challenge, the rats were treated with atropine (21 mg/kg b.w.) alone or in combination with an oxime. The oximes were administered at equimolar doses corresponding to 50 μmol/kg. The neurotoxicity of tabun was monitored using the functional observational battery (FOB) at 24 h following tabun poisoning. FOB consists of 47 measurements of sensory, motor and autonomic nervous functions. Some of them are scored (Table 1), the others are measured in absolute units as described previously (Kassa and Kunešová, 2006). The evaluated markers of tabun-induced neurotoxicity in experimental animals were compared with the parameters obtained from the control rats to whom saline was administered instead of tabun and antidotes at the same volume (1 ml/kg b.w.).

Statistics

Data collected with the FOB and motor activity assessment include categorial, ordinal and continuous values. Their statistical analyses were performed on a PC with a special interactive programme NTX (Frantik and Hornychova, 1995). The categorial and ordinal values were formulated as contingency tables and judged consecutively by the 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 (Roth et al., 1962). The results were evaluated at the significance level $2\alpha = 0.05$.

Results

One tabun-poisoned rat without antidotal treatment died within 24 h following tabun administration. When tabun-poisoned rats were treated with atropine alone or with atropine in combination with an oxime, all animals survived till the end of experiment (24 h following tabun intoxication).

The results of the experiments related to the measurement of tabun-induced neurotoxicity at 24 h following tabun poisoning were divided into three parts (activity and neuromuscular measures, sensorimotor and excitability measures and autonomic measures – Moser et al., 1997) and summarized in Tables 2a–2c. The evaluation of tabun-induced neurotoxic signs at 24 h following intoxication proved the significant alteration in 22 observed parameters. Tabun produced passive behaviour of rats during handling and catching, a decrease in muscular tonus and an increase in the value for miosis and nose secretion. The level of rearing and unprovoked activity was reduced. In addition, no reaction was found during a reflex testing consisting of recording each rat's response to the frontal approach of the blunt end of a pen, to a touch of the pen to the posterior flank and to auditory click stimulus. No ability of pupils to constrict in response to light was demonstrated. A significant decrease in landing foot splay, forelimb and hindlimb grip strength, food receiving, body weight, body temperature and spontaneous horizontal as well as vertical motor activity were also observed at 24 h following tabun challenge (Tables 2a–2c).

While atropine alone was almost ineffective, obidoxime in combination with atropine was able to prevent several tabun-induced signs of neurotoxicity observed at 24 h following tabun challenge with the exception of a decrease in the level of rearing and unprovoked activity, a loss of rat's response to the frontal approach of the blunt end of a pen, a decrease in landing foot splay, grip strength of all limbs, body temperature, food receiving and spontaneous motor activity. The oximes KR-22934 and K203 were slightly less efficacious than obidoxime because they were not able to eliminate miosis and a loss of ability of the pupils to constrict in response to light, a decrease in muscular tone, passive behaviour of rats during handling and catching, a loss of rat's response to auditory click stimulus (KR-22934) and a decrease in hindlimb grip strength. However, in contrast to obidoxime, they were able to eliminate a decrease in landing foot splay, body temperature (K203) and a loss of rat's response to the frontal approach of the blunt end of a pen (KR-22934) (Tables 2a–2c).

Discussion

Nerve agents including tabun can cause centrally mediated seizure activity that can rapidly progress to status epilepticus and contribute to profound brain damage. Nerve agent-induced hyperstimulation of the cholinergic muscarinic receptors in the brain induces 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 (McDonough, 1997). Therefore, the ability of antidotes to counteract the acute neurotoxic effects of tabun and prevent tabun-poisoned organisms from irreversible lesions in CNS is very important for the successful antidotal treatment of acute tabun poisonings.

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 movements of mouth and jaws	Nonrhythmic quivers	Mild tremors	Severe tremors	Myoclonic jerks	Clonic convulsions	
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 behaviour			*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 h following tabun challenge by the functional observational battery (Nos. 1–2, 4–14, scored values; Nos. 3, 15–21, values in absolute units).

24 h		Controls		Tabun + A + KR-22934		Tabun + A + K203		Tabun + A + Obidoxime		Tabun + Atropine		Tabun	
No.	Marker	x/M	±s	x/M	±s	x/M	±s	x/M	±s	x/M	±s	x/M	±s
1	Posture	3.00		3.00		3.00		3.00		3.00		3.00	
2	Muscular tonus	0.00		−2.00*		−1.00*		0.00		−2.00*		−2.00*	
3	Rearing	9.13	4.45	2.25*	1.75	3.38*	1.60	3.50*	2.88	2.13*	2.47	1.50*	1.69
4	Hyperkinesis	0.00		0.00		0.00		0.00		0.00		0.00	
5	Tremors	0.00		0.00		0.00		0.00		0.00		0.00	
6	Clonic movements	0.00		0.00		0.00		0.00		0.00		0.00	
7	Tonic movements	0.00		0.00		0.00		0.00		0.00		0.00	
8	Gait	0.00		0.00		0.00		0.00		0.00		0.00	
9	Ataxia	0.00		0.00		0.00		0.00		0.00		0.00	
10	Gait score	1.00		1.00		1.00		1.00		1.00		1.00	
11	Mobility score	1.00		1.00		1.00		1.00		1.00		1.00	
12	Activity	4.00		1.00*		1.00*		1.00*		1.00*		1.00*	
13	RRF	1.00		1.00		1.00		1.00		1.00		1.00	
14	RRV	1.00		1.00		1.00		1.00		1.00		1.00	
15	Landing foot splay (mm)	78.19	12.05	67.88	13.46	71.75	13.97	65.00*	12.54	65.13*	10.48	52.00*	24.12
16	Forelimb grip strength (kg)	6.21	1.04	5.34	0.86	4.90*	0.89	5.88	0.46	5.00*	0.56	5.23*	0.40
17	Hindlimb grip strength (kg)	1.13	0.14	0.76*	0.26	0.79*	0.16	0.91	0.30	0.66*	0.27	0.73*	0.23
18	Grip strength of all limbs (kg)	19.50	2.00	14.13*	2.60	15.24*	4.52	16.66*	2.70	13.59*	4.12	14.00*	3.89
19	Vertical activity	167.50	93.54	17.63*	19.29	10.25*	12.37	54.13*	59.08	44.38*	46.91	48.13*	48.01
20	Horizontal activity	14.13	16.30	0.38*	0.74	0.25*	0.71	2.63*	4.34	0.75*	1.75	1.13*	2.10
21	Total motor activity	181.63	96.83	18.11*	19.29	10.50*	12.64	56.75*	63.09	45.13*	48.01	49.25*	48.41
		$n = 8$		$n = 8$		$n = 8$		$n = 8$		$n = 8$		$n = 7$	

Abbreviations: RRF, air righting reflex; RRV, air righting reflex from vertical position; A, atropine; *n*, number of surviving animals; x/M, mean or median; ±s, standard deviation.
* Statistically significant as compared with controls.

Obidoxime was able to partly reduce tabun-induced acute neurotoxicity, but its neuroprotective efficacy is not satisfactory (Kassa and Krejcova, 2003). The low efficacy of obidoxime and other currently available oximes in eliminating tabun-induced acute neurotoxicity is possible to explain by the low potency of oximes in reactivating tabun-inhibited AChE *in vitro* and *in vivo* (Puu et al., 1986; Jokanovic et al., 1996; Worek et al., 1998) and by limited penetration across BBB (Bajgar, 2004). The evaluation of the neuroprotective efficacy of the oxime K203 in tabun-poisoned rats brought relatively promising results but the difference between the neuroprotective efficacy of the oxime K203 and commonly used oximes is not so high (Kassa et al., 2009). Therefore, the fluorinated derivative of the oxime K203 (KR-22836) was designed and synthesized to be more effective in eliminating tabun-induced acute neurotoxic signs and symptoms due to its higher penetration through the BBB.

Table 2b – The values of tabun-induced sensorimotor and excitability neurotoxic markers measured at 24 h following tabun challenge by the functional observational battery (scored values).

24 h		Controls		Tabun + A + KR-22934		Tabun + A + K203		Tabun + A + Obidoxime		Tabun + Atropine		Tabun	
No.	Marker	x/M	±s	x/M	±s	x/M	±s	x/M	±s	x/M	±s	x/M	±s
1	Catch difficulty	2.00		1.00*		1.00*		2.00		1.00*		1.00*	
2	Ease of handling	2.00		1.00*		1.00*		2.00		1.00*		1.00*	
3	Arousal (GSC)	4.00		4.00		4.00		4.00		4.00		2.00*	
4	Tension	0.00		0.00		0.00		0.00		0.00		0.00	
5	Vocalization	0.00		0.00		0.00		0.00		0.00		0.00	
6	Stereotypy	0.00		0.00		0.00		0.00		0.00		0.00	
7	Bizzare behaviour	0.00		0.00		0.00		0.00		0.00		0.00	
8	Approach response	2.00		2.00		1.00*		1.00*		1.00*		1.00*	
9	Touch response	2.00		2.00		2.00		2.00		1.00*		1.00*	
10	Click response	2.00		1.00*		2.00		2.00		1.00*		1.00*	
11	Tail-pinch response	2.00		2.00		2.00		2.00		2.00		2.00	
		$n = 8$		$n = 8$		$n = 8$		$n = 8$		$n = 8$		$n = 7$	

* Statistically significant as compared with controls. Abbreviations: A, atropine; *n*, number of surviving animals; x/M, mean or median; ±s, standard deviation.

Table 2c – The values of tabun-induced autonomic neurotoxic markers measured at 24 h following tabun challenge by the functional observational battery (Nos. 1–7, 10–11, 15, scored values, Nos. 8–9, 12–14, values in absolute units).

24 h		Controls		Tabun + A + KR-22934		Tabun + A + K203		Tabun + A + Obidoxime		Tabun + Atropine		Tabun	
No.	Marker	x/M	±s	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		0.00	
2	Palpebral closure	1.00		1.00		1.00		1.00		1.00		1.00	
3	Endo/exophthalmus	0.00		0.00		0.00		0.00		0.00		0.00	
4	Fur abnormalities	0.00		0.00		0.00		0.00		0.00		0.00	
5	Skin abnormalities	0.00		0.00		0.00		0.00		0.00		0.00	
6	Salivation	0.00		0.00		0.00		0.00		0.00		0.00	
7	Nose secretion	0.00		0.00		0.00		0.00		0.00		3.00*	
8	Urination	2.50	5.55	5.00	3.82	3.50	6.93	1.00	2.45	2.13	4.16	1.13	2.10
9	Defecation	0.00		0.00		0.00		0.00		0.00		0.00	
10	Pupil size	0.00		−1.00*		−1.00*		0.00		−1.00*		−1.00*	
11	Pupil response	1.00		0.00*		0.00*		1.00		1.00		0.00*	
12	Food receiving (%)	100.00	0.00	35.50*	8.02	33.50*	9.09	45.00*	7.48	54.50*	21.92	37.25*	16.37
13	Body weight (g)	265.00	14.75	273.13	22.46	287.50	17.06	263.63	22.73	257.75	10.08	245.14*	10.98
14	Body temperature (°C)	37.03	0.17	36.61*	0.36	36.86	0.29	36.55*	0.41	36.55*	0.50	36.44*	0.50
15	Respiration	0.00		0.00		0.00		0.00		0.00		0.00	
		$n = 8$		$n = 8$		$n = 8$		$n = 8$		$n = 8$		$n = 7$	

* Statistically significant as compared with controls. Abbreviations as in Table 2b.

It is known that fluorine substitution can lead to an increase in BBB permeability because of changes in lipophilicity (Wildman and Crippen, 1999; Kirk, 2006; Mueller et al., 2007). Our previously published results demonstrate the slightly higher potency of KR-22836 to reduce tabun-induced acute neurotoxic signs and symptoms in comparison with the oxime K203 and obidoxime (Kassa et al., 2010a). On the other hand, the *in vivo* evaluation of reactivating efficacy of oximes in tabun-poisoned rats confirmed that the potency of the fluorinated analogue of K203 (KR-22836) to reactivate tabun-inhibited AChE in brain is not higher than the efficacy of K203 and obidoxime, probably due to conformational changes making the entry of KR-22836 into the active centre of AChE more difficult (Kassa et al., 2010b).

As the oxime K203 and its fluorinated analogue KR-22836 did not bring any marked improvement of the neuroprotective efficacy of antidotal treatment of acute tabun poisoning, other oximes were developed to increase the reactivating and neuroprotective efficacy of antidotal treatment of acute tabun poisonings. The structure-activity relationship study allowed us to postulate the requirements for the structural parameters of new reactivators of tabun-inhibited AChE (Kuca et al., 2006). Some new bispyridinium AChE reactivators with the potential ability to reactivate tabun-inhibited AChE and counteract tabun-induced acute neurotoxicity were developed but without extraordinary effects (Kuca et al., 2003; Cabal et al., 2004; Musilek et al., 2007). Therefore, we have decided to evaluate the neuroprotective efficacy of a novel oxime KR-22934 that was developed and synthesized in Korea.

Our results demonstrate that the potency of the studied oxime (KR-22934) to eliminate tabun-induced acute signs of neurotoxicity is not higher than the neuroprotective efficacy of currently available obidoxime and, therefore, it is not able to improve the neuroprotective efficacy of antidotal treatment of acute tabun poisonings.

We can conclude that the changes in the structure of commonly used oximes realized according to the postulated requirements (Kuca et al., 2006) are not able to markedly increase the potency of current antidotal treatment to eliminate tabun-induced acute neurotoxicity, probably due to the low penetration of these oximes across the BBB (Zdarova Karasova et al., 2010a,b). Thus, it is necessary to find a new approach in changing the known structures of AChE reactivators to reach better penetration through the BBB. A higher brain concentration of the AChE reactivator should bring a higher reactivation of nerve agent-inhibited brain AChE and more effective elimination of acute neurotoxic signs and symptoms of nerve agents including tabun.

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