Two Possibilities How to Increase the Efficacy of Antidotal Treatment of Nerve agent Poisonings

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Abstract: Highly toxic organophosphorus inhibitors of acetylcholinesterase referred as nerve agents are considered to be among the most dangerous chemical warfare agents. The oximes represent very important part of medical countermeasures of nerve agent poisonings. They are used to reactivate the nerve agent-inhibited acetylcholinesterase. Despite long-term research activities, there is no single, broad-spectrum oxime suitable for the antidotal treatment of poisoning with all organophosphorus agents. There are two approaches how to increase and broaden the effectiveness of antidotal treatment of poisoning with nerve agents - to develop new structural analogues of currently available oximes and/or to combine currently available or newly developed oximes. The review describes the evaluation of the potency of newly developed oximes (especially the oxime K203) or combinations of oximes to reactivate nerve agent-inhibited acetylcholinesterase and to counteract the acute toxicity of nerve agents in comparison with single commonly used oxime (obidoxime, trimedoxime or HI-6).

Keywords: Acetylcholinesterase, antidotal treatment, combination of oximes, nerve agents, oximes.

INTRODUCTION

Highly toxic organophosphorus compounds called nerve agents are considered to be the most dangerous chemical warfare agents. The most important representatives of nerve agents are tabun, sarin, soman, cyclosarin and VX. Despite of the entry into force in April 1997 of the Chemical Weapons Convention forbidding the development, production, stockpiling and the use of chemical warfare agents, they can be still misused in local wars or in terroristic attacks [1,2].

The acute toxic effects of nerve agents are based on phosphonylation of the enzyme 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 neurotransmitter acetylcholine (ACh) in synapses of the central and peripheral nervous systems As the enzyme AChE is critical for a normal control of nerve impulse transmission from cholinergic nerve endings to smooth and skeletal muscle cells, glandular cells, autonomic ganglia as well as within central cholinergic nervous system, all nerve agents produce autonomic signs of cholinergic overstimulation (shrinkage of the pupils, salivation, lacrymation), hypothermia, altered neuromuscular functions including tremor and convulsions as well as altered cognitive functions including the impairment of memory and learning [1,3,4].

As the basic mechanism of toxic effects of nerve agents is known, the medical countermeasures of nerve agent poisonings include the administration of the special medicaments called antidotes that are able to counteract the main toxic effects of nerve agents. The current standard antidotal treatment of poisoning with nerve agents usually involves a muscarinic cholinergic receptor antagonist to block the overstimulation of cholinergic receptors by ACh and an oxime to reactivate nerve agent-inhibited AChE [5,6]. In the past, the compounds with oximate anion that is bound on the pyridinium ring were discovered and considered as the compounds able to reactivate nerve agent-inhibited AChE by dephosphonylating the enzyme molecule and restore its activity. Their reactivating potency is based on the nucleophilic activity of the oxime group [7].

There are several currently available oximes for the antidotal treatment of nerve agent poisonings as reactivators of nerve agent-inhibited AChE. Their basic structure is very similar, they differ themselves by the number of the pyridinium rings (monopyridinium and bispyridinium oximes) and by the position of the oxime group on the pyridinium ring only. The most frequently used oximes are pralidoxime, obidoxime, trimedoxime and the oxime HI-6. Their reactivating potency is dependent on their chemical structure and concentration in the target tissue. Pralidoxime, obidoxime, and trimedoxime are sufficiently effective to reactivate sarin or VX-inhibited AChE, however, their potency to reactivate soman, cyclosarin or tabun-inhibited AChE is generally very low [1,5,8]. On the other hand, the oxime HI-6 is sufficiently effective not only against sarin and VX agent but also against cyclosarin and partly against soman. Nevertheless, it is a weak reactivator of tabun-inhibited AChE [8-10]. Therefore, the antidotal treatment of acute nerve agent poisonings is still considered to be a serious problem and its solution to achieve the satisfactorily effective antidotal treatment of acute poisonings with nerve

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agents regardless of their chemical structure still represents very important goal. Generally, there are two approaches how to improve the antidotal treatment of nerve agent poisonings:

- *the development of a new, more effective AChE reactivator, especially against tabun*
- *the combination of two oximes to cover the full spectrum of nerve agents*

THE DEVELOPMENT OF NEW AChE REACTIVATORS

To make the development of new oximes more effective, a special developmental process consisting of five steps, has been introduced at our Department of Toxicology of the Faculty of Military Health Sciences. The whole developmental process consists of several parts - prediction of new structures using artificial neural networks (ANN), molecular design, synthesis of new AChE reactivators, *in vitro* and *in vivo* testing. A short description of individual developmental steps is shown in Fig. (**1**) [11].

Prediction of New Structures Using Artificial Neural Networks

A biological activity of chemical compounds could be estimated using chemometric methods including Artificial Neural Network (ANN). On the basis of known biological activities of different substances, there is possibility to "learn" ANN without knowledge of exact interaction between a compound and an organism. Due to this fact, the described method can estimate biological activity of potential antidotes without necessity to synthesize them. ANN is used for the prediction of the appropriate structures of new AChE reactivators in this developmental process. Biological activity and structure of currently used AChE reactivators are used as input data set. Then, the model of relationships between chemical structure and biological activity is calculated. Afterwards, the prediction of new, more potent reactivators of AChE inhibited by nerve agents based on the definition of structural factors necessary for the successful reactivation of nerve agent-inhibited AChE is possible [12]. The main structural features which influence the ability of oximes to reactivate nerve agent-inhibited AChE are the oxime functional group (its position and amount), the connecting linker for bisquaternary reactivators and other substituent(s) on the second heteroaromatic ring [13-15].

Molecular Design

The method of molecular modelling is used for the study of AChE conformational changes caused by substances such as nerve agents. This study is performed by methods of molecular dynamics with possibility to calculate intramolecular energies of modified residues. Then, reconformational changes in AChE structure caused by reactivators are examined. The influence of these substances towards the enzyme is evaluated on the base of known structures and by docking method and subsequent molecular design simulations. The acquired description of interactions and their quantification got from interaction energies of model systems serve as proposition for new, more potent AChE reactivators [16].

Synthesis of new AChE Reactivators

All AChE reactivators predicted by ANN and molecular design are synthesized using methods prepared for synthesis of currently used AChE reactivators [14]. From the chemical view, all synthesized substances are mono or bis quaternary pyridinium rings connected mostly with three or four membered linkage chain. In all their structures, the oxime

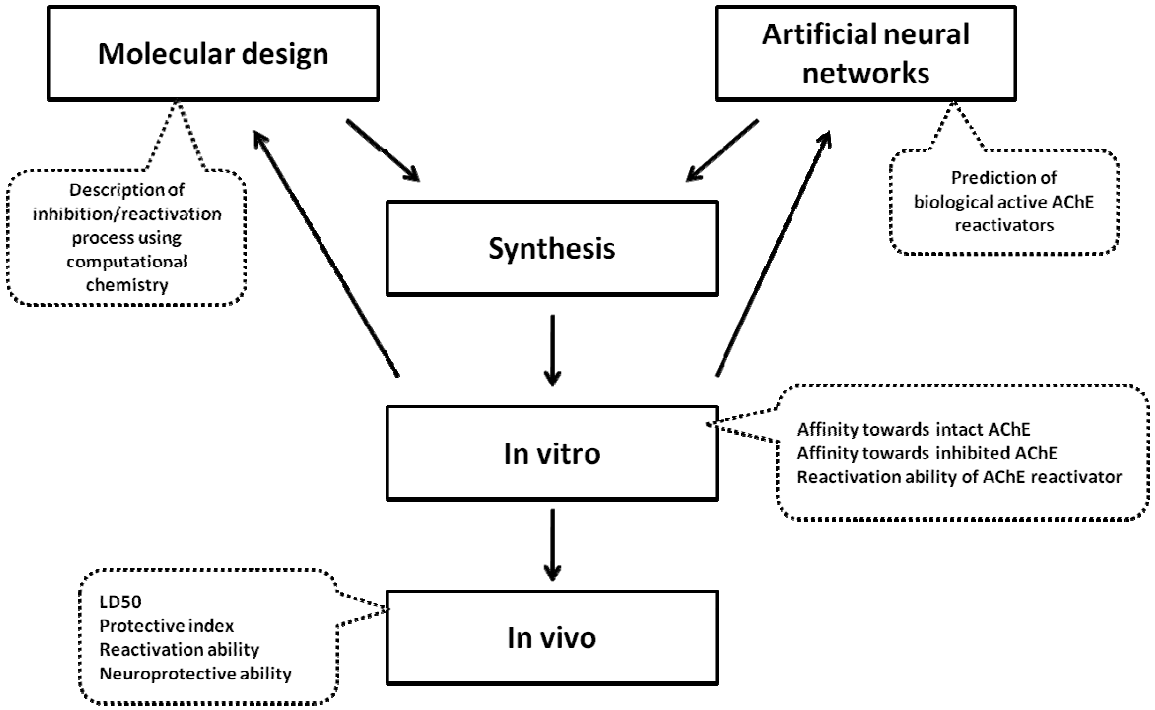

Fig. (1). Description of the developmental process.

group is functioning as nucleophile able to split the bond between enzyme and inhibitor. Generally, the oxime group is located in the position two or four on the pyridinium ring.

In Vitro Testing

The reactivation potency of newly synthesized AChE reactivators is firstly evaluated by *in vitro* experiments. The reactivation efficacy of the tested oximes has been assayed *in vitro* on a model of AChE inhibited by nerve agent using a standard reactivation test [17,18]. As a source of AChE, a homogenate of rat brains (rats of Wistar strain) without sex preference is commonly used, nevertheless, the homogenates from pig and human brains and rat and human erythrocytes as well as commercially available pure enzyme can be also used. The animals are killed in ether narcosis by cutting the carotides; the brains are removed, rinsed in physiological saline and homogenized in an Ultra-Turrax (Germany) homogenizer in distilled water to make a 10 % homogenate. The brain homogenate (0.5 mL) is mixed with isopropanol solution of nerve agent (20 μL) and distilled water (0.5 mL). The mixture is incubated at 25 °C for 30 min to achieve 95% inhibition of AChE. Then, the mixture is filled in assay vessel to the volume 23 mL with distilled water and sodium chloride (3 M, 2.5 mL) is added. Finally, 2 mL of 0.02 M ACh iodide (substrate for enzymatic reaction) is added and the enzyme activity (analyzed by potentiometric titration of decomposed ACh iodide) is measured at pH 7.6 and 25 °C using an autotitrator RTS 822 (Radiometer, Copenhagen, Denmark). The activities of intact (a_o) and nerve agent-inhibited (a_i) AChE are estimated. When nerve agent-inhibited AChE is incubated for 10 min with a solution of an oxime reactivator, the activity of reactivated AChE (a_r) is obtained. The activity values a_0, a_i and a_r are calculated from the slopes of initial parts of titration curves. Each value is the arithmetic mean of three independent measurements.

The kinetics of the reactivation process may be represented by the scheme:

$$EI + R \overset{K_R}{\rightleftharpoons} EIR \overset{k_R}{\rightarrow} E + P$$

where EI is the nerve agent-inhibited enzyme, R is the reactivator, E is the reactivated enzyme, EIR is the intermediate complex, and P is the product, usually unstable phosphonylated oxime. K_R is the dissociation constant and k_R is the rate constant for decomposition of the intermediate complex, respectively. For all oximes whose reactivation ability was screened, the percentage of reactivation (% R) is calculated from equation

$$\%R = \left[1 - \frac{a_0 - a_x}{a_0 - a_i}\right] \times 100$$

Thus, based on the activity values a_o, a_r and a_i, we are able to calculate the percentage of reactivation (%R) that characterizes the reactivating efficacy of tested oximes. In addition, the description of the reactivation process by the calculation of kinetic constants can help us to more thoroughly characterize *in vitro* ability of tested oximes to reactivate nerve agent-inhibited AChE. We usually calculate the dissociation constant of the oxime-reactivator complex with inactive, i.e. non-phosphonylated AChE (K_R), the first-order rate constant (k_R) and the second-order rate constant of reactivation (k_r) which represents overall reactivation ability.

Results of *in vitro* experiments are used as input data for ANN prediction. According to *in vitro* results, the most potent AChE reactivators are selected for *in vivo* experiments.

In Vivo Testing

Before starting *in vivo* evaluation of the reactivating and therapeutic efficacy of the chosen (and newly synthesized) AChE reactivators, their toxicity is evaluated by determining the medium lethal dose (LD_{50}) in mice and rats. The LD_{50} values and their 95% confidence limits are assessed using probit-logarithmical analysis of death occurring within 24 h after i.m. administration of AChE reactivators at five different doses with six or eight animals per dose [19]. After the LD_{50} is established, the therapeutic efficacy of AChE reactivators against nerve agents is assessed by the evaluation of their ability to prevent lethality of nerve agents. The efficacy of tested AChE reactivators is expressed as protective ratio (LD_{50} value of nerve agent in protected animals / LD_{50} value of nerve agent in unprotected animals).

The ability of oximes to reactivate nerve agent-inhibited AChE *in vivo* is determined as follows. To evaluate the reactivating efficacy of a newly developed oxime compared to commonly used oximes, the experimental animals are injected i.m. with either atropine alone or atropine in combination with one of the oximes studied in equitoxic dose corresponding to 5% of its LD_{50} value 5 min before the animals receive nerve agent i.m. at a dose of LD_{50}. The evaluation of the prophylactic reactivating efficacy of oximes is just used for the first testing of newly developed oximes such as an oxime K203. The reason is to reach the maximal reactivation of nerve agent-inhibited AChE without influence of aging of the complex enzyme-inhibitor and to decide if the newly developed oxime is promising or not. When the reactivating efficacy of chosen combinations of oximes compared to single oximes is evaluated, the antidotes are administered 1 minute after nerve agent poisoning. The animals are decapitated and exsaguinated to obtain the blood 60 min following nerve agent poisoning. The diaphragm and the brain are removed and homogenised in distilled water to determine AChE activity by a spectrophotometric method [20]. The reactivation % extent was calculated using the AChE activity values: {1- [((saline control) – (oxime + atropine))/((saline control) – (atropine control))]} x 100 [21].

THE OXIME K203 – THE MOST PROMISING NEWLY DEVELOPED OXIME FOR THE ANTIDOTAL TREATMENT OF ACUTE TABUN POISONINGS

As the antidotal treatment of acute poisoning with tabun is not satisfactorily solved till now, the development of new oxime structures is specially focused to find sufficiently effective reactivator of tabun-inhibited AChE. For tabun-inhibited AChE, at least one oxime in position four on the heteroaromatic ring is necessary for substantional reactivation whilst an oxime in position two has a low or no reactivation capability. Additionally, the optimal linker length suitable for tabun intoxication varies from 3 to 4

carbon bonds [22]. Especially, the (E)-but-2-ene-linker is suitable for the reactivator of tabun-inhbited AChE because it is slightly longer than 3 and slightly shorter than 4 carbon-carbon bonds due to the presence of double bond, which also restricts the conformational flexibility of (E)-but-2-ene linker. Moreover, the necessity of (E)- instead of (Z)-but-2-ene linker has been established [23].

The new bispyridinium oxime K203 [1-(4-carbamoyl-pyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene-dibromide] (Fig. **2**) was synthesized at our department several years ago [13] to improve the efficacy of the antidotal treatment in reactivating tabun-inhibited AChE and eliminating tabun-induced lethal toxicity. This compound has one oxime moiety present with the second oxime moiety replaced by a carbamoyl group. A four atom (E)-but-2-ene connecting linker was chosen because it provided better reactivation and slightly lower toxicity [16,17]. The purity of the oxime K203 was established by using NMR, MS, and elemental analysis [13].

The screening evaluation of the ability of newly developed oxime K203 in comparison with commonly used oximes (pralidoxime, obidoxime, trimedoxime, HI-6) to reactivate tabun-inhibited AChE *in vitro*, characterised by a percentage of reactivation, was provided using tabun-inhibited rat brain AChE and three concentrations of oximes tested (Table **1**) [13]. The reactivation potency *in vitro* should exceed 10% to warrant further investigation *in vivo* [24]. While pralidoxime and the oxime HI-6 showed very low reactivating efficacy, the other two currently available oximes (obidoxime, trimedoxime) improved over the potency of pralidoxime and the oxime HI-6 to reactivate tabun-inhibited AChE at all oxime concentrations. The oxime K203 showed the best reactivation ability among all oximes tested at 10^{-3} M and 10^{-4} M, that is the highest concentration attainable for human use [25]. Subsequently, obidoxime has the best reactivation ability at 10^{-5} M, followed by the oxime K203 with only minor differences between them. To more precisely characterize the *in vitro* reactivating efficacy of all tested oximes, the kinetic constants describing the reactivation process were calculated using the whole concentration scale from 10nM to 10mM (7 concentrations) (Table **2**) [13]. An affinity of the reactivators towards tabun-inhibited AChE is characterized by a K_R value. Obidoxime can be characterized as an oxime with the best affinity (the lowest K_R value) to the inhibited enzyme and the oxime HI-6 and newly developed oxime K203 just have a slightly lower affinity to the tabun-inhibited enzyme compared to obidoxime. On the other hand, pralidoxime and trimedoxime have substantially decreased affinity to the tabun-inhibited AChE. A rate constant k_R is the first-order rate constant that characterizes the ability of oxime to interrupt the covalent bond between enzyme and nerve agent. The oxime K203 showed the highest ability (the highest k_R value) among all tested oximes to break the bond between tabun and AChE, followed by trimedoxime while obidoxime and especially pralidoxime and the oxime HI-6 had very low capability to interrupt the covalent bond between tabun and AChE. Additionally, a rate constant k_r (the second-order rate constant) characterizes the velocity of the whole reactivating process. The newly developed oxime K203 has the highest velocity (the highest k_r value) for tabun-inhibited AChE while the other tested oximes (ecpecially pralidoxime) showed a substantial decrease in the reactivation velocity of the reactivation of tabun-inhibited AChE. Above mentioned results clearly demonstrate that the reactivation potency depends on the structure of the reactivator [14,15]. A hetero-aromatic ring with the quaternary nitrogen has been reported to increase reactivation ability [15], where a pyridinium ring gave better results compared to an imidazolium ring [14,26,27]. At least one hydroxyiminomethyl group in position 4 on the heteroaromatic ring is necessary for proper reactivation of tabun-inhibited AChE in contrast to the oximes with hydroxyiminomethyl group in position 2 (pralidoxime, HI-6). Two hydroxyiminomethyl groups at position 4 do not necessarily exhibit better reactivation properties than mono-oxime because the newly developed oxime K203 having one hydroxyiminomethyl group showed better potency to reactivate tabun-inhibited AChE than some AChE reactivators with two oxime groups, probably due to other structural factors (connecting linker and the second functional group).

Based on the promising results dealing with *in vitro* evaluation of the potency to reactivate tabun-inhibited AChE, the oxime K203 went to *in vivo* evaluation of its

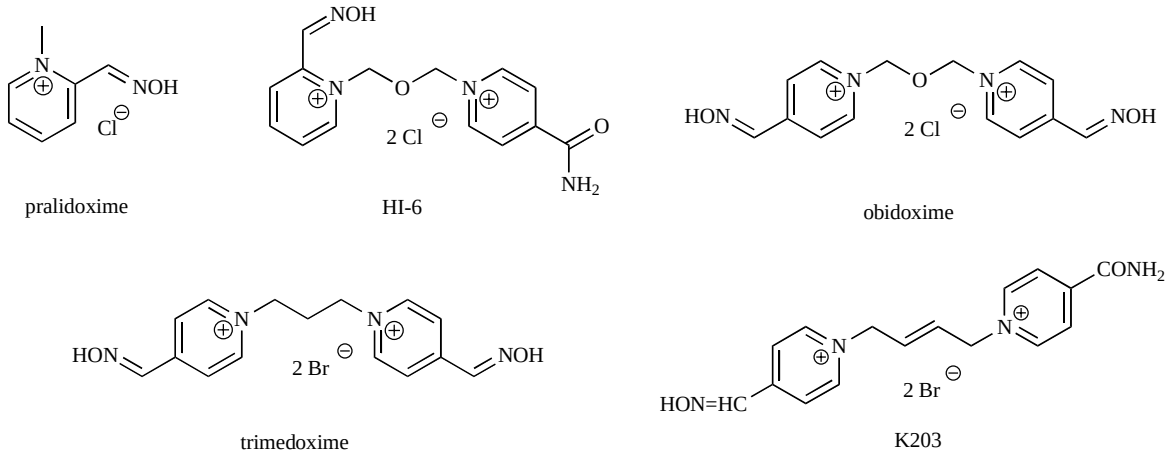

Fig. (2). Chemical structure of studied oximes.

Table 1. *In Vitro* Screening of the Potency of Commonly Used and Newly Synthesized Oximes to Reactivate Tabun-Inhibited AChE at the Three Concentrations by the Evaluation of % of Reactivation of Tabun-Inhibited Rat Brain AChE[a]

AChE reactivator	% reactivation ± SD(concentration)		
	10^{-3} M	10^{-4} M	10^{-5} M
Pralidoxime	4 ± 0	1 ± 0	0
Obidoxime	37 ± 1	34 ± 0	28 ± 2
Trimedoxime	41 ± 1	15 ± 0	6 ± 0
HI-6	2 ± 0	6 ± 0	4 ± 0
K203	55 ± 1	51 ± 0	14 ± 0

[a]Mean value of three independent determinations; time of inhibition – 30 min, time of reactivation by AChE reactivators – 10 min; pH 7.6; temperature 25 °C.

Table 2. Kinetic Constants of the Tested AChE Reactivators[a]

AChE reactivator	K_R ± SD [μM]	k_R ± SD [min^{-1}]	k_r [$min^{-1}M^{-1}$]
Pralidoxime	576 ± 43	0.006 ± 0.001	10
Obidoxime	3 ± 1	0.020 ± 0.002	6250
Trimedoxime	460 ± 41	0.079 ± 0.007	172
HI-6	6 ± 1	0.007 ± 0.002	1111
K203	6 ± 1	0.096 ± 0.003	16000

[a]K_R = dissociation constant of inhibited enzyme-reactivator complex; k_R = the first-order rate constant of reactivation; k_r = the second-order rate constant of reactivation (obtained as the ratio k_R/K_R).

ability to reactivate tabun-inhibited AChE in rats and to reduce acute toxicity of tabun in mice. Before starting the evaluation of reactivating and therapeutic efficacy of oximes, the i.m. acute toxicity of tested oximes was evaluated in rats and mice (Table **3**) [28]. The results show that the acute toxicity of the newly developed oxime K203 is higher than the acute toxicity of obidoxime and trimedoxime in mice but it is markedly lower than the acute toxicity of obidoxime and trimedoxime in rats while the oxime HI-6 can be considered to be the least toxic for both animal species. The prophylactic potency of oximes to reactivate tabun-inhibited AChE in rat blood, diaphragm and brain *in vivo* is shown in Table **4** [28]. The newly developed oxime K203 showed the best prophylactic reactivating efficacy in peripheral (diaphragm) as well as central (brain) compartment. Its reactivating efficacy is higher in comparison with the potency of obidoxime and trimedoxime to reactivate tabun-inhibited AChE in diaphragm and brain. On the other hand, the oxime HI-6 can be considered to be the worst reactivator of tabun-inhibited AChE among tested oximes. These results correlate with the therapeutic potency of the oximes tested against lethal tabun poisoning in mice (Table **5**) [28]. The potency of newly developed oxime K203 to eliminate lethal toxic effects of tabun in mice was higher in comparison with all commonly used oximes studied. It was able to decrease acute toxicity of tabun nearly two times. On the other hand, the oxime HI-6 showed significantly lower potency to eliminate lethal toxic effects of tabun in mice in comparison with other studied oximes.

Thus, the obtained *in vivo* results correlate with the results received by *in vitro* methods. The newly developed oxime K203 that was characterized by relatively high percentage of reactivation of tabun-inhibited AChE *in vitro*

Table 3. LD_{50} Values of Oximes Following i.m. Administration in Rats and Mice

AChE reactivators	LD_{50} (mg/kg) ± 95% confidence limit	
	Rats	Mice
Obidoxime	211.1 (176.4 – 252.6)	188.4 (156.3 – 208.0)
HI-6	781.3 (738.4 – 826.6)	671.3 (627.4 – 718.3)
Trimedoxime	*150.5 (142.1 – 159.4)*	149.3 (124.1 – 184.5)
K203	326.4 (285.4 – 373.2)	95.0 (88.4 – 102.2)

Table 4. Percentage of Reactivation of Tabun-Inhibited AChE by Oximes in Rat Blood, Diaphragm and Brain *In Vivo*

TREATMENT	AChE activity (μkat/L or μkat/kg)		
	Blood	**Diaphragm**	**Brain**
Atropine	3.55 ± 0.25[a]	3.76 ± 1.01[a]	11.1 ± 2.90[a]
Atropine + obidoxime	4.96 ± 0.14	7.45 ± 1.42	15.8 ± 4.06
(% reactivation[b])	(23.6[*x])	(34.8[*])	(4.8)
Atropine + HI-6	3.73 ± 0.29	5.25 ± 0.98	7.8 ± 2.41
(% reactivation)	(3.1)	(14.1)	(0)
Atropine + trimedoxime	4.59 ± 0.33	7.43 ± 1.10	13.8 ± 2.25
(% reactivation)	(17.5[*x])	(34.7[*x])	(2.8)
Atropine + K203	4.84 ± 0.18	8.43 ± 1.26	19.7 ± 3.67
(% reactivation)	(21.6[*x])	(44.1[*x])	(8.8[*x])

[a]Means ± S.E.M., N = 8. The untreated control value for rat blood AChE activity was 9.5 (μkat/L), for diaphragm AChE activity 14.4 μkat/kg and for brain AChE activity 109.1 μkat/kg.

[b]Percent reactivation was determined using the AChE activity values: {1 - [((saline) - (oxime+ atropine))/((saline) -(atropine control))]} x 100.

[*]Significantly different from the atropine group at a level of $P < 0.05$, [x] significantly different from the atropine + HI-6 group at a level of $P < 0.05$ as determined by one-way ANOVA test.

Table 5. The Influence of the Type of Oxime on the Potency of Antidotal Treatment to Eliminate Acute Lethal Effects of Tabun in Mice

Treatment	LD_{50} (μg/kg) ± 95% IS	Protective ratio
-----	295.2 (275.5 – 317.9)	----
Obidoxime + atropine	435.7 (410.8 – 460.5)[*x]	1.47
HI-6 + atropine	318.8 (302.7 – 336.5)	1.08
Trimedoxime + atropine	504.8 (460.3 – 553.0)[*x]	1.71
K203 + atropine	584.5 (549.3 – 626.7)[*x]	1.98

[*]significantly different from the untreated group at the level of $P < 0.05$, [x] significantly different from the group treated by atropine in combination with HI-6 at the level of $P < 0.05$.

[13,29,30] was found to be relatively efficacious reactivator of tabun-inhibited AChE in both (peripheral and central) compartments of tabun-poisoned rats and relatively effective to protect mice poisoned with the lethal doses of tabun [28,31]. It seems to be more efficacious to reactivate tabun-inhibited AChE in rats and to eliminate lethal toxic effects of tabun in mice than all currently available oximes and, therefore, it is suitable for the replacement of commonly used oximes for the treatment of acute tabun poisoning.

THE COMBINATION OF TWO OXIMES TO COVER THE FULL SPECTRUM OF NERVE AGENTS

The development of new structural analogues of known AChE reactivators brings from time to time a new promising oxime sufficiently effective against concrete nerve agent but not against all nerve agents regardless of their chemical structure because the sufficient effectiveness of AChE reactivators against concrete nerve agents requires specific structural features that are different for various nerve agents [15,32]. The literature data confirm that there is no single, broad-spectrum oxime suitable for the antidotal treatment of poisonings with all organophosphorus agents [8,20,33-35]. While trimedoxime and obidoxime are preferred for the treatment of acute poisoning with organophosphorus insecticides (OPI) because they are considered to be sufficiently effective reactivators of OPI-inhibited AChE [33,36,37], the oxime HI-6 appears to be a promising antidote against highly toxic fluorophosphonates, especially soman and cyclosarin, because it is able to protect experimental animals from adverse effects and improve survival of poisoned animals [38,39]. Nevertheless, the published results clearly demonstrate its low potency to reactivate tabun-inhibited AChE and protect tabun-poisoned animals from the lethal effects of this nerve agent [10,28]. Trimedoxime as well as obidoxime seem to be more effective oximes for the treatment of acute tabun poisonings than the oxime HI-6 but their potency to eliminate tabun-induced lethal effects is limited, when they are administered at low, human-relevant doses [20,34]. On te contrary, the newly developed oxime K203 has been considered to be promising reactivator of tabun-inhibited AChE but its reactivating and therapeutic efficacy against other nerve agents, especially against soman and cyclosarin, is relatively low [40,41].

Therefore, the combination of two oximes seems to be the best solution to cover the full spectrum of nerve agents. Surprisingly, only few studies are available in the open literature investigating the effects of combination of oximes against nerve agents in animal models [42-45]. 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 appears to be the most promising antidote against numerous nerve agents, especially soman and cyclosarin [10,46-48]. However, its potency to counteract the acute toxicity of tabun is rather low [10,20]. Therefore, the second oxime involving into the antidotal treatment of nerve agent poisonings should be the oxime sufficiently effective against tabun. Among currently available oximes, trimedoxime and obidoxime are considered to be relatively effective against tabun [20,49,50]. Additionally, a newly developed oxime K203 has been found to be a promising oxime against tabun [13,28,31].

The reactivating and therapeutic efficacy of chosen oxime mixtures was evaluated against nerve agents whose deleterious effects are extraordinarily difficult to antagonize (tabun, soman). The ability of single oximes and their combinations to reactivate nerve agent-inhibited AChE in rat blood, diaphragm and brain is shown in Tables **6-7** [41,51]. When the combination of HI-6 with obidoxime or K203 was used against tabun, the reactivating efficacy of antidotal treatment was markedly higher than the reactivating efficacy of single oximes in blood and diaphragm. In the brain, the reactivating efficacy of both mixtures of oximes (HI-6 + obidoxime, HI-6 + K203) corresponds to the reactivating efficacy of obidoxime or K203 alone (Table **6**). When the combination of HI-6 with trimedoxime or K203 was used against soman, the reactivating efficacy of antidotal treatment was slightly higher than the reactivating efficacy of the most effective single oxime (HI-6) in blood as well as in diaphragm but the difference among them was not significant (Table **7**). Above mentioned results correlate with the therapeutic efficacy of antidotal treatment involving a single oxime or combination of oximes in mice as it is shown in Tables **8-9** [41,51]. In the case of antidotal treatment of tabun-poisoned mice with atropine and combination of HI-6 with obidoxime or K203, the therapeutic efficacy of antidotes was increased compared to single oximes. Both mixtures of oximes were able to decrease acute toxicity of tabun more than two times, while single oxime treatment decreased acute toxicity of tabun less than two times (Table **8**). In the case of antidotal treatment of soman-poisoned mice with atropine and combination of HI-6 with trimedoxime or K203, the therapeutic efficacy of antidotes approximately corresponds to the therapeutic

Table 6. Percentage of Reactivation of Tabun-Inhibited AChE by Oximes and their Combinations in Rat Blood, Diaphragm and Brain *In Vivo*

TREATMENT	AChE activity (μkat/L or μkat/kg)		
	Blood	**Diaphragm**	**Brain**
Atropine	5.86 ± 0.91 (5.05 - 6.67)[a]	3.37 ± 0.62 (2.82 – 3.92)[a]	9.10 ± 3.27 (6.18 – 12.02)[a]
Atropine + obidoxime (% reactivation[b])	10.03 ± 0.81 (9.31 – 10.75) (34.2[*x])	4.52 ± 1.09 (3.55 – 5.49) (12.5)	14.71 ± 1.41 (13.43 – 15.96) (4.9[*x])
Atropine + HI-6 (% reactivation)	6.48 ± 0.83 (5.74 – 7.22) (5.1)	3.41 ± 1.36 (2.19 – 4.62) (0.5)	9.81 ± 1.08 (8.44 – 10.77) (0.6)
Atropine + K203 (% reactivation)	11.49 ± 0.80 (10.77 – 12.21) (46.2[*x])	5.07 ± 0.83 (4.33 – 5.81) (18.5[*])	19.64 ± 1.83 (18.00 – 21.28) (9.2[*x])
Atropine + obidoxime + HI-6 (% reactivation)	13.49 ± 1.65 (12.02 – 14.96) (62.6[*x])	6.33 ± 1.60 (4.90 – 7.76) (32.1[*])	13.86 ± 2.68 (11.46 – 16.25) (4.2)
Atropine + HI-6 + K203 (% reactivation)	14.04 ± 1.50 (12.70 – 15.38) (67.1[*x])	8.65 ± 1.99 (6.87 – 10.43) (57.2[*x])	18.45 ± 2.27 (16.42 – 20.48) (8.2[*x])

[a]Means ± S.D., N = 8, 95% confidence intervals. The untreated control value for rat blood AChE activity was 18.05 ± 1.81 (16.43 – 19.67) μkat/L, for diaphragm AChE activity 12.61 ± 2.91 (10.01 – 15.21) μkat/kg and for brain AChE activity 123.5 ± 13.83 (111.14 – 135.86) μkat/kg.

[b]Percent reactivation was determined using the AChE activity values: {1- [((saline) – (oxime + atropine))/((saline) – (atropine control))]} x 100.

[*]Significantly different from the atropine group at a level of $P < 0.05$, [x] significantly different from the atropine + HI-6 group at a level of $P < 0.05$ as determined by one-way ANOVA test.

Table 7. Percentage of Reactivation of Soman-Inhibited AChE by Oximes and their Combinations in Rat Blood and Diaphragm *In Vivo*

TREATMENT	AChE activity (μkat/L or μkat/kg)	
	Blood	Diaphragm
Atropine	5.93 ± 1.13[a]	1.88 ± 0.79[a]
Atropine + HI-6	6.97 ± 0.79	4.12 ± 1.89
(% reactivation[b])	(9.5)	(18.9[*x])
Atropine + trimedoxime	6.54 ± 0.43	1.28 ± 0.84
(% reactivation)	(5.6)	(0)
Atropine + K203	6.75 ± 0.68	1.33 ± 0.83
(% reactivation)	(7.5)	(0)
Atropine + HI-6 + trimedoxime	7.69 ± 0.51	6.61 ± 2.65
(% reactivation)	(16.1[*])	(40.1[*x])
Atropine + HI-6 + K203	7.07 ± 0.91	5.21 ± 1.05
(% reactivation)	(10.5)	(28.2[*x])

[a]Means ± S.D., N = 8. The untreated control value for rat blood AChE activity was 16.88 ± 1.88 μkat/L and diaphragm AChE activity 13.67 ± 2.38 μkat/kg.
[b]Percent reactivation was determined using the AChE activity values: {1- [((saline) – (oxime + atropine))/((saline) – (atropine control))]} x 100.
[*]Significantly different from the atropine group at a level of $P < 0.05$, [x] significantly different from atropine + trimedoxime and atropine + K203 group at the level $P < 0.05$ as determined by one-way ANOVA test.

Table 8. The Influence of the Type of Oxime and Combinations of Oximes on the Ability of Antidotal Treatment to Reduce Acute Lethal Effects of Tabun in Mice

Treatment	LD_{50} (μg/kg) ± 95% IS	Protective ratio
-----	250.3 (225.4 – 294.2)	----
Obidoxime + atropine	431.4 (382.8 – 478.7)[*]	1.72
HI-6 + atropine	368.1 (312.3 – 413.4)[*]	1.47
K203 + atropine	489.0 (431.6 – 543.4)[*x]	1.95
HI-6 + obidoxime + atropine	511.4 (376.6 – 635.4)[*]	2.05
HI-6 + K203 + atropine	539.4 (484.7 – 591.5)[*x]	2.16

[*]significantly different from the untreated group at the level of $P < 0.05$, [x] significantly different from the group treated by atropine in combination with HI-6 at the level of $P < 0.05$.

Table 9. The Influence of the Type of Oxime and Combinations of Oximes on the Ability of Antidotal Treatment to Reduce Acute Lethal Effects of Soman in Mice

Treatment	LD_{50} (μg/kg) ± 95% IS	Protective ratio
-----	77.6 (68.5 – 90.1)	----
Trimedoxime + atropine	102.2 (91.5 – 114.2)[*]	1.32
HI-6 + atropine	212.6 (119.1 – 319.3)[*x]	2.74
K203 + atropine	83.4 (54.3 – 110.3)	1.08
HI-6 + trimedoxime + atropine	211.3 (153.4 – 291.0)[*x]	2.72
HI-6 + K203 + atropine	197.2 (153.7 – 328.0)[*x]	2.54

[*]significantly different from the untreated group at the level of $P < 0.05$.
[x]significantly different from the group treated with trimedoxime + atropine and K203 + atropine at the level of $P < 0.05$.

efficacy of the most effective single oxime (HI-6) that was able to reduce acute toxicity of soman more than two times (Table **9**). These results are in agreement with *in vitro* evaluation of the ability of the combination of two oximes (HI-6 and obidoxime) to reactivate nerve agents (sarin, cyclosarin, VX, tabun) inhibited AChE showing that combining HI-6 and obidoxime had no negative effects on the reactivation of sarin-, cyclosarin-, VX- and tabun-inhibited human AChE and, in addition, it had a beneficial effects by broadening the spectrum of the individual oximes [52]. The beneficial effects of the combination of oximes compared to individual oxime treatment could be explained by an elevated plasma oxime level and synergetic effects of both oximes [52].

CONCLUSION

Generally, currently used monopyridinium and bispyridinium oximes seem to be relatively poor reactivators of AChE inhibited by some nerve agents (especially tabun, cyclosarin and soman). Their reactivating efficacy depends upon the chemical structure of bridge connecting both pyridinium rings (in the case of bispyridinium oximes), the position of oxime groups and the chemical structure of the substituent situated on the second pyridinium ring (in the case of bispyridinium oximes) [15,16]. To reach sufficient reactivating efficacy against tabun, for example, both substituents need to be situated on the position 4. The replacement of substituents to the other commonly used position (2) usually decreases the reactivating efficacy of tested AChE reactivators against tabun [15,32]. This fact can explain relatively low efficacy of the oxime HI-6, which is effective against fluorophosphonates including cyclosarin and soman [38,39], because the oxime HI-6 contains an ether bridge and the oxime group at position 2. Thus, the chemical structure of the oxime HI-6 compared to other oximes studied is disadvantageous for the reactivation of tabun-inhibited AChE [32]. On the other hand, the position of oxime group at position 2 is advantageous for the reactivation of cyclosarin-inhibited AChE. Therefore, the oxime HI-6 is so efficacious to reactivate cyclosarin-inhibited AChE [15,39]. The chemical structure of the oxime [(1) the number of pyridinium rings, (2) the bridge between pyridinium rings and (3) the number and position of oxime groups)] was shown to be important for the affinity of oximes for intact and phosphonylated AChE, and for potency and velocity of the reactivation of nerve agent-inhibited AChE [15,19].

This review clearly demonstrates that, unfortunately, no AChE reactivator is sufficiently effective against all known nerve agents. Therefore, many scientific institutions over the world are interested in the synthesis of new AChE reactivators [53-56]. The developmental process described in this review seems to be very useful for the development of new antidotes against chemical warfare agents. It permits us to select the most promising antidotes from many chemical substances very effectively and quickly. We are able to evaluate with the help of this developmental process tens of chemical substances per year to find promising antidotes for further pre-clinical and clinical examination. However, the development of new AChE reactivators, obviously structural analogues of monopyridinium or bispyridinium oximes, brings the potential increase in the reactivating and therapeutic effectiveness of antidotal treatment of poisoning with concrete nerve agent (such as the oxime K203 against tabun) but not with all nerve agents or, even, with organophosphorus insecticides. Therefore, the second approach how to improve the antidotal treatment of nerve agent poisonings, described in this review, was found to be very important and uselful. The combination of the oxime HI-6 (the oxime with the broadest spectrum among commonly used oximes) with an oxime sufficiently effective against tabun brings the medical countermeasure able to sufficiently protect the experimental animals exposed to nerve agents regardless of their chemical structure.

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DISCLOSURE

The authors disclose that part of information included in this review has been previously published in Benthan Science Publisher Current Organic Chemistry.

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