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ORIGINAL ARTICLE

In vivo experimental approach to treatment against tabun poisoning

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Abstract

Organophosphorus compounds pose a potential threat to both military and civilian populations. Since post-exposure therapy has its limitations, our research was focused on the possibility of improving pretreatment in order to limit the toxic effects of tabun. We determined the protective index of various combinations of atropine, oximes (K074, K048, and TMB-4), and pyridostigmine given to mice before tabun intoxication. Although the tested oximes showed very good therapeutic efficacy in tabun-poisoned mice, the given pretreatments improved therapy against tabun poisoning. These regimens ensured survival of all animals up to 25.2 LD_{50} of tabun. Our results indicate that even pretreatment with atropine alone is sufficiently effective in enhancing the survival of mice poisoned by multiple doses of tabun, if oxime therapy follows. K048 is our oxime of choice for future research, as it shows better protective and reactivating potency.

Keywords: *Pretreatment; atropine; oxime; pyridostigmine; mice; tabun*

Introduction

The continued threat of military[1] or terrorist[2] use of nerve agents worldwide calls for effective therapeutic preparedness. Exposure to nerve agents, the most lethal organophosphorus compounds (OPs), leads to a progression of toxic signs (hypersecretions, muscle fasciculations, tremors, convulsions, and respiratory distress) due to the inhibition of acetylcholinesterase (AChE; EC 3.1.1.7) and subsequent increase in concentration of the neurotransmitter acetylcholine (ACh), which in turn hyperstimulates the cholinergic system at central and peripheral sites[3]. Current standard treatment against OPs combines anticholinergics (atropine) and AChE reactivators (oximes). Atropine blocks muscarinic acetylcholine-receptors and prevents them from further stimulation by ACh, while oximes reactivate inhibited AChE by displacing the phosphoryl residue from the active site and restoring enzyme activity. Atropine has been universally adopted, but in various countries the oximes of choice differ. For example, P2S (pralidoxime mesylate) is used in the UK, obidoxime is preferred in Germany, and TMB-4 in Israel[4].

One of the most hazardous OPs is the nerve agent tabun (ethyl-*N*,*N*-dimethyl phosphoramidocyanidate) because tabun-phosphorylated AChE is resistant to reactivation due to a low electrophilicity of the phosphoramidate conjugated to the AChE active site[5]. Although the oxime TMB-4 reactivates tabun-inhibited AChE showing beneficial effects in tabun-poisoned animals[6–8], it is the most toxic of the four most investigated oximes: 2-PAM (pralidoxime chloride), HI-6, obidoxime, and TMB-4[9]. Therefore, there is still a need to develop not only the most effective, but also the least toxic, organophosphate antidote. As post-exposure therapy has its limitations[10], our research was focused on improving pretreatment in order to limit the toxic effects of tabun. Pretreatment with oximes is possible, because oximes in addition to their reactivation potency also act as protectors of AChE against phosphorylation, by reversible inhibition of the enzyme[11,12]. Based on our promising results from previous studies with K-oximes[13–17], we decided to test the two most potent AChE reactivators, oximes K074 and K048, in combination with atropine in the pretreatment and therapy of tabun-poisoned mice. This study was conducted to determine whether oxime

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and atropine in pretreatment would improve the efficacy of a standard therapy regimen. Oxime TMB-4 was included for comparison.

Current protection against nerve agent poisoning consists of pretreatment with carbamate pyridostigmine[18]. Pyridostigmine carbamylates the active site serine of AChE and prevents phosphorylation by organophosphate. In contrast to very slow dephosphorylation, decarbamylation is faster. On the other hand, the pyridostigmine-induced increase in ACh levels can itself cause symptoms of poisoning, so it would be useful to counteract the effects of accumulated ACh with anticholinergic drugs[19]. A potent prophylaxis, which combines pyridostigmine with benactyzine and trihexyphenidyl (PANPAL), was developed for the Czech Army[20,21]. With this in mind, we examined the influence of pyridostigmine alone or in combination with atropine on the resistance to tabun exposure in mice and on the therapeutic efficacy of coadministered oximes and atropine.

Materials and methods

Chemicals

Figure 1 shows the structures of the studied oximes, pyridostigmine, and tabun.

Pyridostigmine bromide was purchased from Sigma-Aldrich (Steinheim, Germany). Oximes K074 (1,3-bis(4-hydroxyiminomethylpyridinium) butane dibromide) and K048 (1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium) butane dibromide) were prepared as described earlier[22,23], while TMB-4 (1,3-bis(4-hydroxyiminomethylpyridinium) propane dibromide) was obtained from Bosnalijek, Sarajevo, Bosnia and Herzegovina. Tabun (ethyl-*N*,*N*-dimethyl phosphoramidocyanidate) was purchased from NC Laboratory, Spiez, Switzerland. Atropine sulfate was purchased from Kemika, Zagreb, Croatia.

Animals

Male BALB-C mice (purchased from the Institute of Immunology, Zagreb, Croatia) were selected by body weight (18–25 g), fed on a standard diet (PLIVA, Zagreb, Croatia), and had free access to water. Mice were kept in Macrolone cages at 21°C maintained by a thermostat, with exchanging light and dark cycles every 12 h, and were randomly distributed into groups of four animals.

Experimental design

The acute toxicity (LD_{50}) of pyridostigmine was based on 24-h mortality rates, determined according to Thompson[24] and Weil[25]. LD_{50} value and the 95% confidence limits were evaluated from results obtained with 4–6 doses of pyridostigmine (dissolved in water); four animals were injected per dose. LD_{50} values for K074, K048, and TMB-4 were 21.4, 224.9, and 73.5 mg/kg body weight, respectively, and were determined in our earlier studies[13,16].

Tabun was diluted in isopropyl alcohol; further dilutions were made in water immediately before use. Mice received a subcutaneous (s.c.) dose of 1.0–50.4 multiples of the tabun LD_{50} (317.5 µg/kg body weight). Oximes and pyridostigmine were dissolved in water or atropine just before use and given intraperitoneally (i.p.) at doses of 5% or 25% of their LD_{50}. Atropine was dissolved in water (5.0 mg/mL) and also administered i.p. (10.0 mg/kg body weight).

Pretreatment was given 15 min before tabun poisoning, while therapy was given 1 min after tabun poisoning. The efficacy of the antidotal regimens is expressed as a protective index (PI) with 95% confidence limits and maximal dose of poison (MDP). The PI is the ratio of the LD_{50} of tabun with and without treatment. The MDP is the highest multiple of the LD_{50} of tabun that was fully counteracted (survival of all animals) by the administered treatment.

This study was performed with the approval of the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb, Croatia.

Results

We first administered an oxime and atropine as pretreatment or as therapy in order to test the efficiency of either

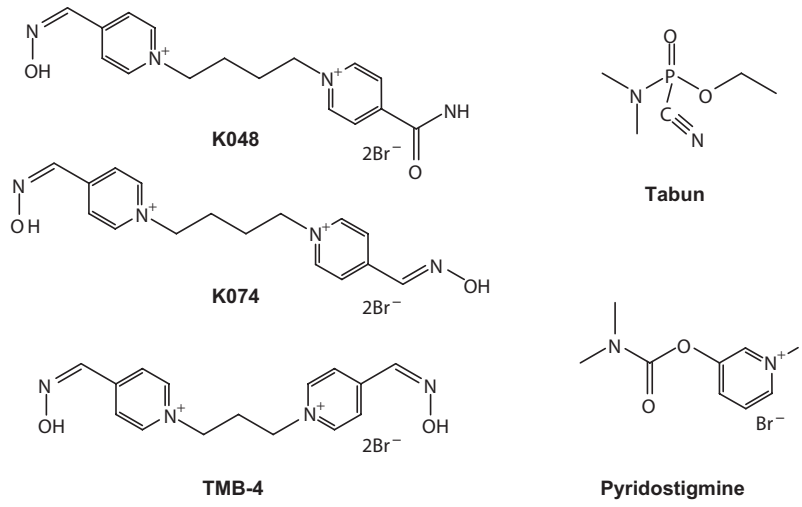

Figure 1. Structures of oximes, pyridostigmine, and tabun.

combination (Table 1). With the oxime dose of 5% of its LD_{50}, a higher therapeutic effect was obtained if the oxime was administered as therapy rather than as pretreatment. Between the three oximes at this dose, therapy with K048 ensured survival of all mice at as high a tabun dose as 10 LD_{50}. With a higher dose of oxime (25% of the respective LD_{50}), the highest therapeutic efficiency was achieved by pretreatment with TMB-4 and atropine. This antidotal regimen showed the lowest acute toxicity (5042.7 µg/kg) while the PI was 14.1 and the MDP was 7.9 LD_{50} of tabun.

Table 2 presents the therapeutic effects of atropine and atropine plus oximes administered as pretreatment and as therapy upon tabun poisoning in mice. Mice were pretreated with either atropine alone or a combination of atropine and one of the oximes, each at two different doses. The therapy, in turn, always combined an oxime and atropine. All these antidotal regimens were potent against tabun poisoning. Particularly effective were atropine pretreatment with therapy combining K048 (25% of its LD_{50}) and atropine, and the combination of TMB-4 (25% of its LD_{50}) plus atropine as both pretreatment and therapy. These regimens ensured survival of all animals at 25.2 LD_{50} of tabun. Furthermore, at the dose of 25% of LD_{50}, all oximes led to lower acute tabun toxicities than at the dose of 5% LD_{50}.

Table 3 shows the protective efficacy of pretreatment with pyridostigmine alone or in combination with atropine. Pyridostigmine alone, regardless of the dose (5% or 25% of its LD_{50}), did not decrease the tabun-induced acute toxicity in mice. However, the protective efficacy was improved when pyridostigmine was applied as pretreatment in combination with atropine.

Table 4 shows the therapeutic effect of pyridostigmine plus atropine pretreatment followed by therapy with oximes and atropine. Lower acute toxicities were obtained with higher antidote doses. K048 distinguished itself in therapeutic potency at both applied doses.

Discussion

Organophosphorus compounds pose a threat to both military and civilian populations, as evidenced by recent terrorist attacks[26]. As insecticides, they are an occupational hazard as well. As the first reaction of the OPs is interaction with cholinesterases, pretreatment is focused on protecting AChE against inhibition or on decreasing the concentrations of OPs. Pyridostigmine, an AChE carbamylating compound that has been used as prophylaxis, calls for a replacement that will provide better protection[21,27]. In addition to carbamylation,

Table 1. Therapeutic effect of oximes and atropine administered as pretreatment or therapy in tabun-poisoned mice.

Dose of oxime	Pretreatment	Therapy	LD_{50} (µg/kg)	95% Confidence limits (µg/kg)	PI	MDP
5% LD_{50}	K074 + atropine	None	898.5	678–1190	2.5	1.6
	K048 + atropine	None	1940.7	1479–2547	5.4	3.2
	TMB-4 + atropine	None	1425.1	1146–1772	4.0	2.0
	None[a]	K074 + atropine	2016.5	1764–2304	6.4	5.0
	None	K048 + atropine	3686.4	3326–4085	11.6	10.0
	None	TMB-4 + atropine	2828.8	1939–4126	8.9	4.0
25% LD_{50}	K074 + atropine	None	2264.1	1485–3453	6.4	3.2
	K048 + atropine	None	3595.0	1869–6913	10.1	3.2
	TMB-4 + atropine	None	5042.7	3457–7355	14.1	7.9
	None[a]	K074 + atropine	2853.0	1227–6636	8.0	5.0
	None[b]	K048 + atropine	2853.0	2428–3353	8.0	5.0
	None[b]	TMB-4 + atropine	2542.0	1759–3672	7.1	5.0

[a]Reference 16.
[b]Reference 13.

Table 2. Therapeutic effect of atropine and atropine plus oximes administered as pretreatment and therapy in tabun-poisoned mice.

Pretreatment	Therapy	LD_{50} (µg/kg)	95% Confidence limits (µg/kg)	PI	MDP
Atropine	None	485.3	381–617	1.4	1.0
Atropine	5% LD_{50} K074 + atropine	2137.0	1790–2548	6.0	4.0
Atropine	25% LD_{50} K074 + atropine	5661.0	4082–7850	15.9	15.9
Atropine	5% LD_{50} K048 + atropine	2853.0	2294–3547	8.0	4.0
Atropine	25% LD_{50} K048 + atropine	12,708.7	10,792–14,966	35.6	25.2
Atropine	5% LD_{50} TMB-4 + atropine	5996.5	5342–6731	16.8	12.6
Atropine	25% LD_{50} TMB-4 + atropine	9521.4	7980–11,360	26.7	20.0
5% LD_{50} K074 + atropine	5% LD_{50} K074 + atropine	1198.9	1004–1430	3.4	2.5
25% LD_{50} K074 + atropine	25% LD_{50} K074 + atropine	7131.8	5461–9313	20.0	16.0
5% LD_{50} K048 + atropine	5% LD_{50} K048 + atropine	4490.5	3115–6472	14.1	5.0
25% LD_{50} K048 + atropine	25% LD_{50} K048 + atropine	8002.0	6697–9560	25.2	20.0
5% LD_{50} TMB-4 + atropine	5% LD_{50} TMB-4 + atropine	5040.8	1999–12,708	16.0	10.0
25% LD_{50} TMB-4 + atropine	25% LD_{50} TMB-4 + atropine	12,702.8	8329–19,373	40.0	25.2

the AChE catalytic site can also be protected by ligands that reversibly bind to AChE, such as huperzine A, SAD, and decamethonium, due to direct competition between the ligand and the phosphorylating agent[28–31]. A new approach to reduce the *in vivo* toxicity of chemical warfare nerve agents is the use of bioscavengers—enzymes that react with a nerve agent before it inhibits AChE at physiologically important target sites (i.e. brain)[32,33]. Among the enzymes examined as potential stoichiometric or catalytic scavengers, significant progress has been made with human butyrylcholinesterase (BChE; EC 3.1.1.8) administered as prophylaxis[34,35]. Another approach to prevent the effects of OPs is based on the use of current antidotes (anticholinergics, reactivators, and others)[21]. Pyridinium compounds reversibly bind to the AChE active site and interfere with the binding of phosphorylating agents[11]. In the case of imminent threat of tabun exposure, pretreament should have an important role, because tabun-induced deleterious effects are extraordinarily difficult to counteract with currently used oximes[8,9,20,36].

In order to improve the efficacy of standard therapy, we determined the protective index of various combinations of atropine, oximes, and pyridostigmine given to mice before tabun poisoning. Despite pretreatment, the animals showed severe signs of toxicity. Muscle fasciculations and tremor generally occurred within 1–2 min after poisoning. Convulsions appeared with 3–4 min of latency. During the acute phase all animals exhibited cyanosis, dyspnea, and respiratory disorders. Within 24 h, animals that survived remained active, but tremor could be provoked. Although the intensity and duration of symptoms differed between pretreated and non-pretreated animals, the pattern described above was the same.

The first step was to evaluate the efficiency of the adopted regimen of therapy (oxime plus atropine) using two newer reactivators (K074 and K048) and conventional TMB-4 (Table 1). In previous studies, K048 and K074 showed very good therapeutic efficacy in tabun-poisoned mice[13,15]. Applied at the dose of 25% of their LD_{50} immediately after tabun poisoning, both K048 and K074 ensured survival of all animals at 5.0 LD_{50} of tabun[13,16]. Results in Table 1 show that both of these oximes are equally or even more effective at the lower dose applied in therapy. Combined atropine plus K048 therapy at 5% of K048 LD_{50} provided survival of all experimental animals after the administration of 10.0 LD_{50} of tabun. Thereby, oxime K048 appears to be superior to the currently used TMB-4, which applied in therapy regardless of the dose, did not show such a high antidotal potential. However, given as pretreatment at the dose of 25% its LD_{50}, TMB-4 plus atropine showed a high protective index. Although in our previous *in vitro* experiments TMB-4 showed a relatively weak protective potential[13,15], it is known that this oxime possesses curare- and atropine-like effects essential for the survival of tabun-poisoned mice[37]. This raises the question of how much atropine contributes to protection against tabun poisoning in mice. Therefore, for pretreatment, we used atropine alone or in combination with the tested oximes. Therapy that followed the poisoning combined one of the oximes and atropine (Table 2). In animal studies, the anticonvulsant effect of atropine has been documented to help against the side effects of nerve agents[38,39], and even though alternative agents have been investigated[40], atropine remains the most important drug against organophosphate poisoning[41]. In our experiments, pretreatment with atropine alone, followed by combined therapy with oximes (25% of their LD_{50}) and atropine (Table 2), ensured survival at 3–5 times higher doses of tabun than therapy alone (Table 1). Pretreatment combining atropine and the oximes did not achieve any higher survival. Our results show that the oximes exerted dose-dependent effects against tabun toxicity. This is in accordance with our previous results[14] and also with suggestions by other authors that the culprit for the ineffectiveness of oximes could be inadequate dosage[42]. When atropine alone was used in pretreatment and was followed by combined therapy with the lower dose of the oximes, the protective index (PI) ranged from 6.0 to 16.8, while with the higher dose it ranged from 15.9 to 35.6. Although 25% LD_{50} is a relatively high dose of antidote, it is applicable for counteracting the lethal effects of organophosphorus compounds.

Atropine was also effective in protecting the mice when it was applied with pyridostigmine 15 min before tabun

Table 3. Therapeutic effect of pyridostigmine or pyridostigmine plus atropine administered as pretreatment in tabun-poisoned mice.

Pretreatment	Therapy	LD_{50} (μg/kg)	95% Confidence limits (μg/kg)	PI	MDP
5% LD_{50} pyridostigmine	None	611.0	480–778	1.7	1.3
25% LD_{50} pyridostigmine	None	400.3	—	1.1	1.0
5% LD_{50} pyridostigmine +atropine	None	1600.3	1154–2219	5.0	4.0
25% LD_{50} pyridostigmine +atropine	None	3982.0	3202–4952	12.5	6.3

Table 4. Therapeutic effect of pretreatment with pyridostigmine and atropine followed by therapy with oximes and atropine in tabun-poisoned mice.

Pretreatment	Therapy	LD_{50} (μg/kg)	95% Confidence limits (μg/kg)	PI	MDP
5% LD_{50} pyridostigmine + atropine	5% LD_{50} K074 + atropine	3160.1	1643–6077	10.0	7.9
25% LD_{50} pyridostigmine + atropine	25% LD_{50} K074 + atropine	11,316.2	7852–16310	35.6	20.0
5% LD_{50} pyridostigmine + atropine	5% LD_{50} K048 + atropine	7564.8	6739–8492	23.8	20.0
25% LD_{50} pyridostigmine + atropine	25% LD_{50} K048 + atropine	11,989.5	10,049–14,305	37.8	25.2
5% LD_{50} pyridostigmine + atropine	5% LD_{50} TMB-4 + atropine	4490.6	3438–5864	14.1	7.9
25% LD_{50} pyridostigmine + atropine	25% LD_{50} TMB-4 + atropine	10,749.8	7049–16,395	33.9	20.0

poisoning (Table 3). Pyridostigmine is a "pretreatment adjunct"—a drug that must be taken before exposure to be effective, but only confers benefit if post-exposure therapy is given as well[43]. Therefore, we investigated the influence of pretreatment consisting of pyridostigmine and atropine on the potency of administered therapy in tabun-poisoned mice (Table 4). With that kind of treatment regimen, a good therapeutic efficacy of oxime K048 was confirmed once again. In combined therapy with atropine, K048 applied at either dose was highly effective against tabun poisoning thanks to combined pretreatment with pyridostigmine and atropine. On the other hand, pyridostigmine plus atropine as pretreatment only in the case of TMB-4 did not cause a striking improvement of therapy. Actually, better results were achieved by applying oxime TMB-4 plus atropine as pretreatment (Table 2). A reasonable explanation for that could lie in the high acute toxicities of pyridostigmine and TMB-4. Also, according to some authors, pyridostigmine may reduce the efficacy of oxime–atropine therapy and therefore should be used with caution[7,44].

Conclusion

Herein we showed that the combined pretreatment of atropine, oximes, and pyridostigmine in general improves the overall therapy. The main principle—protection of AChE against phosphorylation by tabun was reached using AChE inhibitors: oximes or pyridostigmine. In this way the enzyme is resistant to inhibition by tabun. Also, as atropine is required to compete with the excess of ACh at synapses, ample protection against intoxication by organophosphates and also elimination of side effects can be ensured by adding atropine to pretreatment regimens. Moreover, pretreatment with atropine alone is sufficient to enhance the survival of mice poisoned by multiple lethal doses of tabun if followed by oxime therapy. Between K074 and K048, K048 is our oxime of choice for future research, as it shows better protective and reactivating potency.

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Declaration of interest

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