

Comparative determination of the efficacy of bispyridinium oximes in paraoxon poisoning

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The inability of standard therapy to provide adequate protection against poisoning by organophosphorus compounds (pesticides and nerve agents) motivated us to search for new, more effective oximes. We investigated the pharmacotoxicological properties of six experimental K-oximes (K027, K033, K048, K074, K075, and K203) *in vivo*. The therapeutic efficacy of K-oximes (at doses of 5 or 25 % of their LD₅₀) combined with atropine was assessed in paraoxon-poisoned mice and compared with conventionally used oximes HI-6 and TMB-4. The bisoxime K074 was the most toxic (LD₅₀=21.4 mg kg⁻¹) to mice, while monoxime K027 was the least toxic (LD₅₀=672.8 mg kg⁻¹). With the exception of K033, all of the tested K-oximes showed better therapeutic efficiency than HI-6 and TMB-4. K027 and K048 stood out by demonstrating low acute toxicities and ensuring protective indices ranging from 60.0 to 100.0 LD₅₀ of paraoxon. Taking into account that these two oximes showed a similar therapeutic efficacy regardless of the applied doses, our results suggest that K027 and K048 could be antidotes for paraoxon intoxication.

KEY WORDS: *acute toxicity; mice; therapeutic efficacy*

Organophosphorus (OP) compounds are a heterogeneous group of organic compounds that represent a serious toxicological problem and therapeutic challenge (1). The introduction of synthetic OP compounds half a century ago has resulted in the repeated misuse of nerve agents during military conflicts and terrorist attacks as well as 3 million cases of poisoning by pesticides, all leading to nearly 260,000 human fatalities per year (2-4). The main toxic mechanism of OP compounds involves the inhibition of esterase enzymes; acetylcholinesterase (AChE) in synapses and red blood cell membranes, and butyrylcholinesterase (BChE) in plasma (5). Although acute BChE inhibition does not seem to cause clinical manifestations, AChE inhibition results in the accumulation of the neurotransmitter acetylcholine (ACh) at cholinergic synapses, with an overstimulation of cholinergic receptors of the muscarinic and nicotinic type (5). As these receptors are localised in most organs, a "cholinergic syndrome" may ensue increased sweating and salivation, profound bronchial secretion, bronchoconstriction, miosis, increased gastrointestinal motility, diarrhoea, tremors, muscular twitching, and various negative effects on the central nervous system. Death is believed to be due to respiratory failure (5).

Standard post-exposure antidotal treatment of OP poisoning includes administering an anticholinergic drug

such as atropine and AChE reactivators called oximes in accordance with the functional oxime group. Atropine decreases the effects of excess ACh primarily by blocking peripheral muscarinic receptor sites, resulting in reduced secretion and reversing the constriction of smooth muscles. Since it has little effect on nicotinic sites, skeletal muscle fasciculation continues, resulting in the paralysis of respiratory muscles, i.e., peripheral respiratory failure (6). Oximes break the OP-AChE bond and restore the activity of inhibited AChE (7). Thus far, only four oximes of a pyridinium aldoxime structure (2-PAM, TMB-4, HI-6, and obidoxime) have found clinical application, but none is sufficiently effective against all of the known OP compounds (7). Due to this fact, laboratories worldwide are searching for a broad spectrum AChE reactivator. K-oximes appear to be among the most promising compounds developed (8-10).

Therefore, the present study was undertaken to assess and compare the therapeutic efficacy of six experimental K-oximes (K027, K033, K048, K074, K075, and K203) combined with atropine in OP pesticide paraoxon poisoned mice.

MATERIALS AND METHODS

Chemicals

K-series oximes K027, K033, K048, K074, K075 and K203 were prepared as described earlier (11-15). TMB-4

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[1,3-bis(4-hydroxyiminomethylpyridinium) propane dichloride] was synthesised in Bosnalijek, Sarajevo, Bosnia and Herzegovina, while HI-6 [(1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride)] was synthesised at the University of Defence, Hradec Kralove, Czech Republic (16). Oximes were kept at room temperature and dissolved in distilled water or atropine immediately before use. Paraoxon (diethyl *p*-nitrophenyl phosphate) was purchased from Sigma-Aldrich, Steinheim, Germany. The stock solution of 50 mg mL⁻¹ of paraoxon was prepared in isopropanol. Further dilutions were made in saline, shortly before use. Atropine sulphate was purchased from Kemika, Zagreb, Croatia. The solution of 5 mg mL⁻¹ of atropine was prepared in distilled water.

Animals

Male NIH/Ola Hsd mice were purchased from the Institute of Immunology, Inc., Department of Experimental Animals and Antisera, Zagreb, Croatia. The mice were kept in Macrolone cages at 21 °C maintained by a thermostat with exchanging light and dark cycles every 12 h. The animals were fed a standard diet (4RF21, Mucedola, Milano, Italy) with free access to water. Selection was made by body weight (18-25 g) following random distribution into groups of four animals. This study was performed with the approval of the Ethics Committee of the Institute for Medical Research and Occupational Health in Zagreb, Croatia.

Acute toxicity

Acute toxicity (LD₅₀) was based upon 24 h-mortality rates calculated according to Thompson and Weil (17-18). Each LD₅₀ was evaluated from the results obtained with four to six doses of a given compound and four animals were injected per dose. Whenever the results of the experiment allowed, the 95 % confidence limits were estimated from tables described elsewhere (17-18).

Therapeutic efficacy

The therapeutic effect against paraoxon poisoning was tested by administering the studied oximes (5 or 25 % of their LD₅₀) together with atropine sulphate (10 mg kg⁻¹), immediately after paraoxon. The OP compound was given subcutaneously (*s.c.*) while therapy was administered intraperitoneally (*i.p.*). Mice were observed for 24 h and the antidotal efficacy of the tested oximes was expressed as protective index (PI) and maximal dose of poison (MDP). PI was the ratio of LD₅₀ between OP with antidote and OP given alone. MDP was the highest multiple of the LD₅₀ of OP, which was fully counteracted (survival of all animals) by the antidote.

RESULTS AND DISCUSSION

The inadequacy of standard therapy to provide protection against OP poisoning is a matter of continuous public and scientific concern. Within the last few years, a new generation of oximes has been developed in the Czech Republic (8). Structurally, they are bispyridinium oximes that differ in the length of the connection chain between two pyridinium rings, the position of the oxime group, and the number of oxime groups in a molecule. Promising results using several of the previously mentioned oximes were obtained for poisoning by the nerve agent tabun, as well as in the case of pesticide poisoning (8, 14, 19-29). Although the use of OP pesticides is under restriction in most parts of the world, especially in developed countries, some, such as parathion, are still widely (mis)used (30). The active metabolite of the insecticide parathion, paraoxon, is one of the most potent acetylcholinesterase-inhibiting compounds available (30). Therefore, it is important to determine the antidotal potency of new oximes in paraoxon poisoning. Previous studies have shown good potency of several K-oximes *in vitro* to reactivate AChE inhibited by paraoxon (22-23, 25, 27, 29). In the present study, we used mice as an experimental model to broaden our knowledge on the pharmacotoxicological properties of six K-oximes (K027, K033, K048, K074, K075, and K203). The conventional oximes HI-6 and TMB-4 were included for comparison.

The acute toxicity (LD₅₀; *i.p.*) of the tested oximes is shown in Table 1. Bisoximes K033, K074, and K075 were more toxic than monoximes K027 and K048, which is not surprising considering that oximes with two oxime groups have a higher affinity for native AChE (31). For example, bisoxime TMB-4 is the most toxic of the four mentioned conventional oximes (7) and the novel oximes K027 and K048 had nearly an eight and three-times lower acute toxicity, respectively, than TMB-4. Moreover, with an LD₅₀ of 672.8 mg kg⁻¹ body weight, K027 was the least toxic. Our result agrees with *in vitro* studies performed by Petroianu and Lorke (26) and Lorke et al. (32), which singled out K027 as the least toxic in comparison with the other oximes tested. An exception was noticed in the case of monoxime K203. Although K203 has one oxime and one carbamoyl moiety just like K027 and K048, its different stereoelectronic profile is probably why its LD₅₀ was so low (33).

Tables 2 and 3 show the therapeutic effects of the tested oximes combined with atropine on paraoxon toxicity in male mice. Exposure to paraoxon led to classical signs of cholinergic toxicity, but severe signs of toxicity were also observed in all of the animals despite antidotal treatment. Muscle fasciculation and tremor generally occurred within 1-2 min after poisoning. Convulsions appeared with a latency of 3-4 min. During the acute phase, all of the animals exhibited dyspnoea and cyanosis. The animals that survived remained active for 24 h.

Table 1 Chemical name, structure, and LD₅₀ of the tested oximes in male mice

Compound	Code	Structure	LD ₅₀ (mg kg ⁻¹) (95 % confidence limits)
1-(4-carbamoylpyridinium)-3-(4-hydroxyiminomethylpyridinium)-propane dibromide	K027		672.8 ^a (599.0-755.3)
bis-1,3-(2-hydroxyiminomethylpyridinium)-butane dibromide	K033		33.4 ^a (29.7-37.5)
1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-butane dibromide	K048		224.9 ^a (154.2-328.0)
1,4-bis(4-hydroxyiminomethylpyridinium)-butane dibromide	K074		21.4 ^b (19.0-24.0)
(E)-1,4-bis(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide	K075		37.5 (31.4-44.7)
(E)-1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide	K203		89.1 ^c (75.7-104.9)
1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride	HI-6		635.2 (555.3-725.2)
1,3-bis(4-hydroxyiminomethylpyridinium)propane dichloride	TMB-4		89.8 (-)

^aFrom ref. 19

^bFrom ref. 20

^cFrom ref. 21

Table 2 Therapeutic effect of the tested oximes (5 % of their LD_{50}) combined with atropine upon (s.c.) paraoxon toxicity* in male mice

Treatment	LD_{50} ($\mu\text{g kg}^{-1}$)	95 % confidence limits ($\mu\text{g kg}^{-1}$)	PI	MDP
atropine	4191.8	3563.3-4931.2	5.9	5.0
K027 + atropine	64032.4	50321.0-81479.8	74.1	50.4
K033 + atropine	13721.4	10356.2-18180.3	15.9	12.6
K048 + atropine	86457.0	56689.2-131856.0	100.0	63.0
K074 + atropine	23528.8	18491.0-29939.2	27.2	20.0
K075 + atropine	29648.3	23302.4-37722.5	34.3	25.2
K203 + atropine	24451.2	16966.8-35237.1	28.3	15.8
HI-6 + atropine	17290.2	8991.5-33248.3	20.0	12.6
TMB-4 + atropine	14282.4	10936.3-18652.2	20.0	20.0

* LD_{50} (paraoxon)=864.3 $\mu\text{g kg}^{-1}$

PI-protective index

MDP-maximal dose of poison

The administration of a single atropine dose of 10 mg kg^{-1} one minute after paraoxon resulted in a protective index (PI) of 5.9, although in most cases of nerve agent poisoning, therapy with atropine alone results in a PI below 2 (34). Even though it seemed that atropine reduced the signs of cholinergic toxicity elicited by paraoxon more efficiently than that caused by nerve agents, survival was improved and symptoms reduced only following the coadministration of oximes. A marked improvement in the therapy of paraoxon poisoning was noticed with all of the tested oximes compared to atropine alone (Tables 2 and 3). When they were applied at a dose of 5 % of their LD_{50} , conventional oximes HI-6 and TMB-4 ensured a PI of 20.0.

The PI of the equitoxic dose of K-oximes ranged from 15.9 to 100.0 LD_{50} of paraoxon (Table 2). The best results were obtained with oximes K027 and K048. The PI of the therapy composed of K027 or K048 and atropine was about 15 times better than that obtained by atropine alone. Moreover, these combinations ensured the survival of all animals at up to 63.0 LD_{50} of paraoxon. A dose-response relationship was observed for K074, K075, and K203, as an increase of the therapeutic dose from 5 to 25 % of their LD_{50} resulted in a 2 to 5 times higher PI (max. was 171.5). The highest PI was obtained using 25 % of K075 and K203 LD_{50} together with atropine, where all animals survived up to 100.0 LD_{50} of paraoxon (Table 3). These results confirmed the hypothesis

Table 3 Therapeutic effect of the tested oximes (25 % of their LD_{50}) combined with atropine upon (s.c.) paraoxon toxicity* in male mice

Treatment	LD_{50} ($\mu\text{g kg}^{-1}$)	95 % confidence limits ($\mu\text{g kg}^{-1}$)	PI	MDP
atropine	4191.8	3563.3-4931.2	5.9	5.0
K027 + atropine	54992.1	44222.2-68384.9	63.6	50.4
K033 + atropine	21782.1	14282.4-33220.0	25.2	15.8
K048 + atropine	51903.4	43503.0-61927.0	60.0	40.0
K074 + atropine	47141.1	37051.0-59979.1	54.5	40.0
K075 + atropine	148217.1	116493.0-188582.0	171.5	100.0
K203 + atropine	108918.1	82205.3-144311.2	126	63.0
HI-6 + atropine	20594.4	17261.2-24571.4	23.8	15.9
TMB-4 + atropine	28523.3	21527.8-37792.0	40.0	25.2

* LD_{50} (paraoxon)=864.3 $\mu\text{g kg}^{-1}$

PI-protective index

MDP-maximal dose of poison

that oxime reactivation is a very important treatment modality for OP compound poisoning.

Oximes exhibit their potency by enabling the recovery of an active AChE in contrast to the symptomatic treatment of excessive cholinergic stimulation with atropine largely in the periphery. By testing the therapeutic efficacy of newer oximes in paraoxon-poisoned mice, we aimed to single out compound(s) that would be more effective than the oximes used currently. With the exception of K033, all of the oximes showed better antidotal activity than HI-6 and TMB-4. Unfortunately, the high acute toxicity of these oximes is a limiting factor for their usage. The protective index of the two less toxic oximes, K027 and K048, ranged from 60.0 to 100.0. Moreover, therapy with both 5 and 25 % LD₅₀ doses of K027 plus atropine resulted in the survival of all animals at a 50.4 LD₅₀ dose of paraoxon. A similar result was obtained for K048, with the exception that a higher therapeutic efficiency was achieved with a lower dose of this oxime (5 % of the respective LD₅₀). Results of our *in vivo* experiments on mice showed a relatively good correlation with *in vitro* results obtained by other authors. To be more precise, oximes K027 and K048 were found to be potent reactivators of the erythrocyte AChE in *in vitro* studies with methyl-, ethyl-paraoxon and DFP (23, 25-27, 29). Thus, it seems that these compounds have a pharmacological effect indeed related to the reactivation of paraoxon-inhibited AChE, and - what is even more important - these oximes can be used at lower doses, applicable for human use.

In summary, this study indicates a higher potency of the majority of the tested K-oximes to protect against high lethal doses of paraoxon when compared to the conventional oximes HI-6 and TMB-4. Among the tested oximes, K027 and K048 stood out with low acute toxicities and very good antidotal effects. We can conclude that K027 and K048 might be antidotes for paraoxon intoxication therapy.

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Conflicts of interest

The authors declare no conflict of interest.

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Usporedno određivanje učinkovitosti bispiridinijskih oksima pri trovanju paraoksonom

Činjenica da standardna terapija ne omogućuje dovoljnu zaštitu pri trovanju organofosforim spojevima (pesticidima i živčanim bojnim otrovima) potaknula nas je na istraživanje novih, učinkovitijih oksima. U uvjetima *in vivo* ispitali smo farmakotoksikološka svojstva šest eksperimentalnih K-oksima (K027, K033, K048, K074, K075 i K203). Terapijski učinak kombinacije K-oksima (primjenjenih u dozi 5 ili 25 % njihove LD₅₀) i atropina testiran je na miševima otrovanim paraoksonom i uspoređen s konvencionalnim oksimima HI-6 i TMB-4. Bisoksim K074 je bio najtoksičniji (LD₅₀=21.4 mg kg⁻¹) za miševe, dok je monooksim K027 bio najmanje toksičan (LD₅₀=672.8 mg kg⁻¹). Osim K033, svi K-oksimi pokazali su bolji terapijski učinak u miševa trovanih paraoksonom u odnosu na HI-6 i TMB-4. Iz skupine testiranih oksima istaknuli su se K027 i K048 koji su pokazali nisku akutnu toksičnost i osigurali protektivne indekse u rasponu od 60.0 do 100.0 LD₅₀ paraoksona. Uzmemo li u obzir da su ta dva oksima pokazala sličan terapijski učinak bez obzira na primjenjenu dozu, prikazani rezultati upućuju na K027 i K048 kao perspektivne antidote u terapiji trovanja paraoksonom.

KLJUČNE RIJEČI: akutna toksičnost; miševi; terapijska učinkovitost