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The Acute Toxicity of Acetylcholinesterase Reactivators in Mice in Relation to Their Structure

LUCIE BARTOSOVA*, KAMIL KUCA, GABRIELA KUNESOVA and DANIEL JUN

Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Trebešská 1575, Hradec Králové 500 01, Czech Republic.bartosova@pmfhk.cz

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Oximes in combination with atropine, are an integral part of the treatment of acute intoxications with organophosphorus insecticides or with the nerve agents such as tabun, sarin, soman, cyclosarin or VX. Organophosphorus compounds are extremely potent inhibitors of the enzyme acetylcholinesterase (AChE, 3.1.1.7). The pharmacological action of oximes is multiple: they are able to reactivate the inhibited AChE, but they affect acetylcholine release in peripheral and central cholinergic synapses, allosterically modulate the muscarinic receptors in peripheral and central synapses and influence the nicotinic receptor associated ion-channels. In our study, we have determined the acute toxicity of different structures of oximes after intramuscular application in mice. The acute toxicity of oximes is crucial for the assesment of a dose applied as a treatment for organophosphorus intoxications. We have tested 7 oximes of different structures (HS-6, K033, BI-6, MMB-4, K048, HI-6 and obidoxime) and during experiment we have observed the intoxication process including typical signs of intoxication, and times of death. K033 was the most toxic oxime with LD_{50} only 48 mg/kg, while the least toxic oxime - HI-6 has value of LD50 671 mg/kg. All the oximes tested were of bispyridinium type with different length or shape of the connecting chain and positions of oxime groups at the pyridinium rings. All these structure features play important role in biological activity of these compounds performed by their acute toxicity as well as by their reactivation potency.

Keywords: Oxime; Mice; Acute toxicity; Acetylcholinesterase

Abbreviations

HS-6 [1-(2-hydroxyiminomethylpyridinium)-3-(3-carbamoylpyridinium)-2-oxa-propane dichloride]
BI-6 [1-(2-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)-but-2-ene dibromide]
K033 [1,2-bis(2-hydroxyiminomethylpyridinium)butane dibromide]
MMB-4 [methoxime; 1,1-bis(4-hydroxyiminomethylpyridinium)-methane dibromide]
K048 [1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)-butane dibromide]
HI-6 [1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride]

INTRODUCTION

Nerve agents (sarin, soman, cyclosarin and tabun) are extremely toxic organophosphorus (OP) compounds that pose potential neurotoxic threat to both military and civilian populations, as evidenced in recent terrorist attacks. The acute toxicity of OP compounds in mammals is generally believed to be due to their irreversible inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) and subsequent accumulation of the neurotransmitter acetylcholine (ACh) in synapses of the central and peripheral nervous systems, resulting in over-stimulation of post-synaptic cholinergic receptors (Marrs, 1991). The current standard antidotal treatment usually includes a muscarinic receptor antagonist to block the over-stimulation of cholinergic receptors by ACh, and an oxime to reactivate OP-inhibited AChE. Oximes are nucleophilic substances that reactivate the OP-inhibited AChE by removing the organophosphoryl moiety and thus restoring AChE activity. The oximes differ in their required doses, their toxic-

*Corresponding author. Tel. +420 973 251 534; Fax: +420 495 512 430; E-mail: bartosova@pmfhk.cz
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ity, and their effectiveness (*e.g.*, trimedoxime is more effective against tabun poisoning than pralidoxime) (Kassa, 2006). The effective dose of the oxime depends on the nerve agent, the time between poisoning and oxime administration, and other factors (Sidell, 1997). Two oximes, obidoxime and pralidoxime, that are presently commercially available, are considered to be of insufficient efficacy against certain nerve agents, *e.g.*, soman and cyclosarin. Thus, some new oximes were developed in order to improve treatment of intoxication by oxime-resistant nerve agents (Simeon-Rudolf *et al.*, 1998; Dube *et al.*, 2000). There is not yet a single broad-spectrum oxime suitable for the treatment of poisoning with all highly toxic OP agents (Kuca *et al.*, 2004b).

This study was undertaken to investigate the acute toxicity of oximes that were designed for treatment of nerve agent intoxications. The other aim of this study was to address the question about the relationship of the structure of oxime and its toxicity, determined after intramuscular administration in mice.

MATERIAL AND METHODS

Animals

Adult albino female mice (Konarovice, Czech Republic) weighing 28-30 g were used throughout this study. They were housed six in a cage, in a temperature-controlled (20-24°C) environment with 12-h light/dark cycles (lights on from 0600 to 1800 h) and with free access to food and water except during the experimental period. Mice were divided into groups of six animals each. The animals used in this study were handled under the supervision of the Ethics Committee of the Medical Faculty of Charles University and the Military Medical Academy in Hradec Kralove, Czech Republic.

Chemicals

HS-6 oxime was a gift of Dr. Stojiljkovic, Serbia and Montenegro. All other oxime reactivators used in this study were synthesized earlier at our laboratory (Bielavsky *et al.*, 1972; 1998; Kuca *et al.*, 2003; 2004a). All other chemicals and drugs were obtained from commercial sources and were of reagent grade.

Animal Experiments

In our experiment seven oximes (FIG. 1), HS-6 (1-(2-hydroxyiminomethylpyridinium)-3-(3-carbamoylpyridinium)-2-oxa-propane dichloride), BI-6 (1-(2-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)-but-2-ene dibromide), K033 (1,2-bis(2-hydro xyiminomethylpyridinium)butane dibromide), MMB-4 (methoxime; 1,1-bis(4-hydroxyiminomethylpyridinium)-methane dibromide), K048 (1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)-butane dibromide), HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propanedichloride)) and obidoxime (1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride) were tested for acute toxicity after intramuscular (im) administration. Different concentrations of solutions of oximes in saline were prepared. Mice received the solution of oxime in saline intramuscularly in a volume of 0.1 ml/10 g of body weight.

Table I LD_{50} values (mg/kg) of oximes in mice after im administration

OXIME	LD_{50} (mg/kg) ± 95% confidence limits
HS-6	220.4 (185.7 – 261.6)
K033	47.9 (44.9 – 53.5)*
BI-6	224.2 (208.2 – 241.5)
MMB-4	441.4 (418.7 – 465.4)*
K048	233.5 (217.8 – 250.3)*
HI-6	671.3 (627.4 – 718.3)*
Obidoxime	188.4 (156.3 – 208.0)

*significantly different from obidoxime acute toxicity, $P < 0.05$

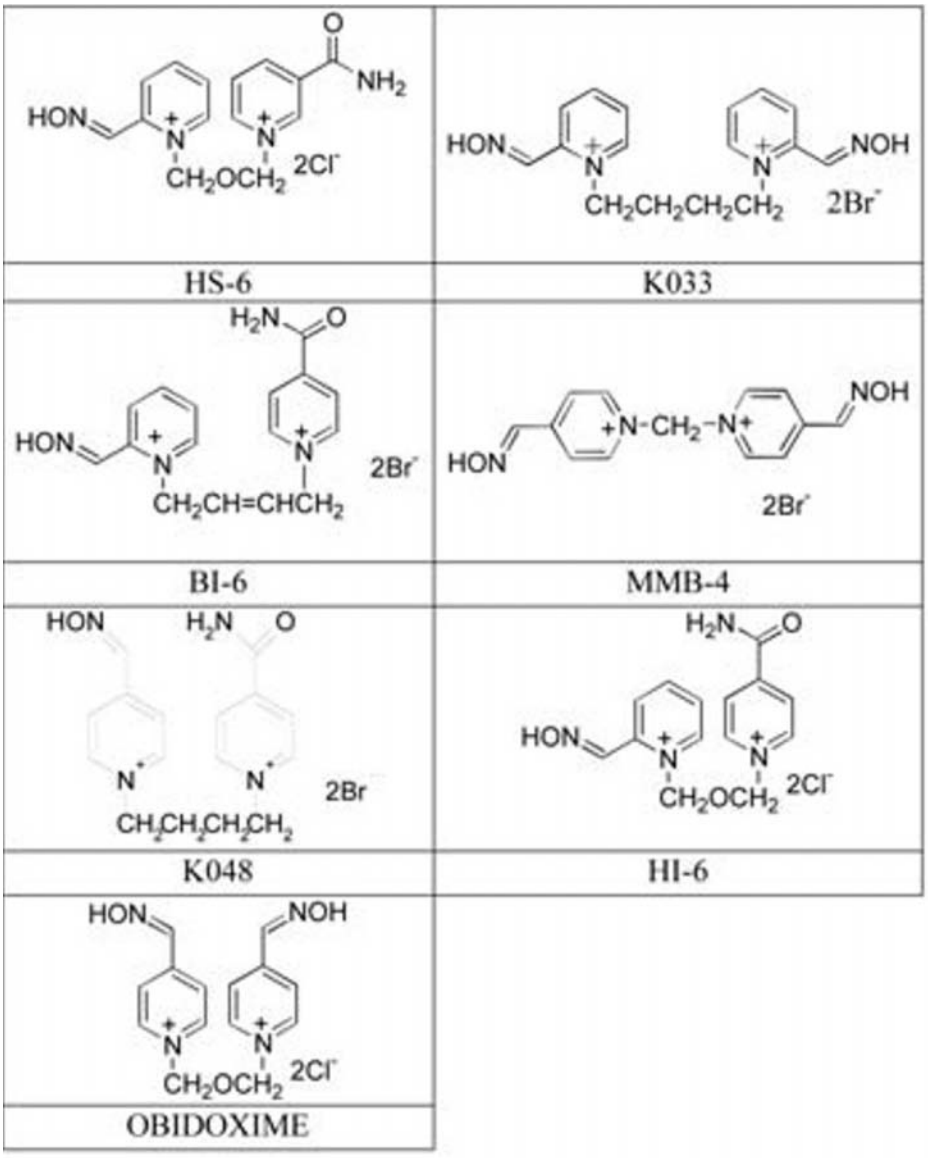

FIGURE 1 Structures of the oximes

Data Analysis

Oxime-induced toxicity was evaluated by the assessment of LD_{50} values and their 95% confidence limits that were calculated by probit analysis of deaths occuring within 24 h after administration of the oxime at five different doses, with six mice per dose (Tallarida and Murray, 1987). The differences between LD_{50} values were considered to be significant at $P < 0.05$ (Roth *et al.*, 1962).

RESULTS

A comparison of the acute toxicity of oximes after im administration is presented in Table I. Symptoms of intoxication with oximes were similar to those observed during intoxication with nerve agents - convulsions, fasciculations, muscular weakness, breathlessness, and these signs were displayed from 1-15 min after oxime challenge. The least toxic oxime was HI-6 with one oxime group (position 2) and one carbamido group (position 4), and with oxapropane as the connecting chain. MMB-4, the compound with oxime groups in position 4 at both pyridinium rings, was found to be a relatively low-toxic oxime, versus others tested. The acute toxicity of the compounds with the same position of the oxime group, but different position of the carbamido group in their structures - HS-6 and BI-6 - was similar. Structures of the oximes K048 and BI-6 are quite similar chemically (different position of oxime group and double bond in the connecting chain) as well as in their acute toxicities. Obidoxime was more toxic than HS-6, BI-6 and K048, but the difference was significant only in comparison to K048. K033- the only oxime with both functional (oxime) groups in position 2 at the pyridinium rings - was the oxime with the highest acute toxicity in mice after im administra-

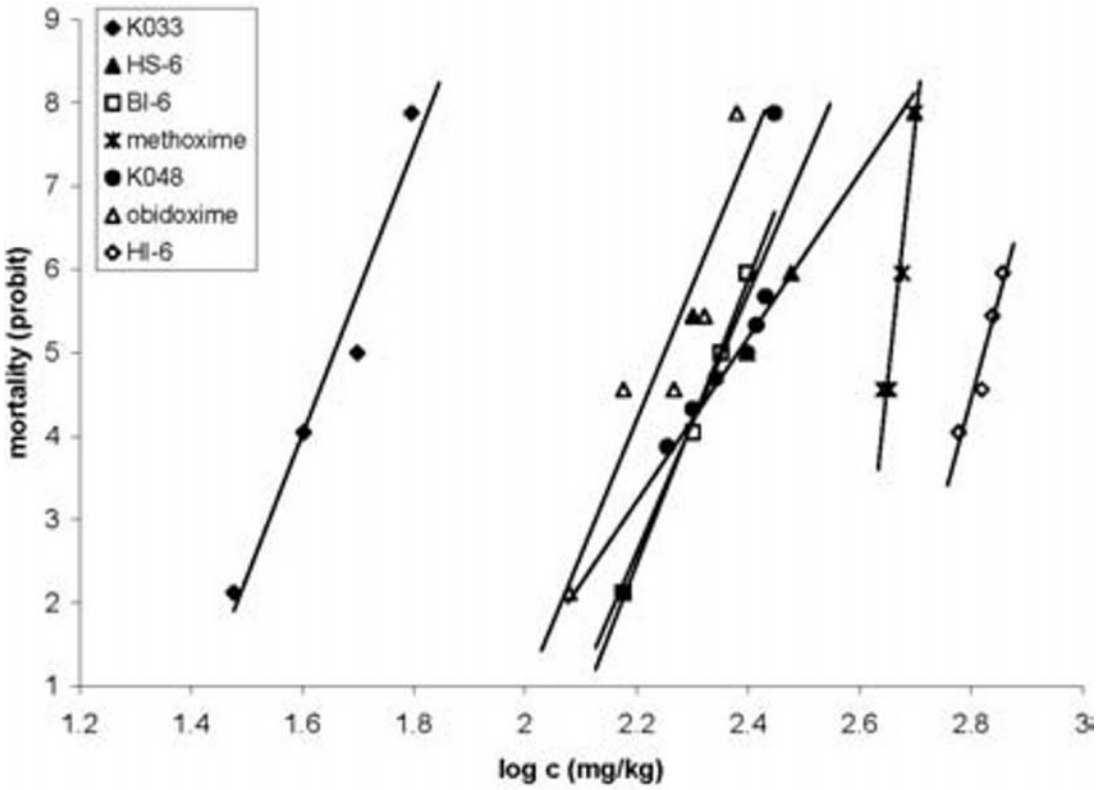

FIGURE 2 Effect of the oxime dose on the mortality of mice

tion. Although HI-6 and HS-6 oximes differ only in the position of a carbamido group on the pyridinium ring, the acute toxicity of HS-6 was 3-fold higher than that of HI-6. Different connecting chains in the strucutures of MMB-4 and obidoxime accounted for the 2-fold lower acute toxicity of MMB-4. The same structural difference (connecting chain) caused nearly 3-fold lesser acute toxicity of HI-6 in comparison with BI-6.

The relationship of the mortality of mice on the dose of oxime is presented in figure 2. The tangents of the dose-mortality curves were similar for K033 (17.18), obidoxime (16.16), K048 (15.15) and BI-6 (17.05). The steepest slope of the dose-mortality curve was found for MMB-4 (61.7), while the flattest curve occurred with HS-6 (9.83).

DISCUSSION

In our study, acute toxicity was determined only in those oximes that were proved to be effective reactivators of cyclosarin-inhibited AChE during *in vitro* reactivation tests (Kuca *et al.*, 2004a). In animal experiments, generally each oxime that is used for treatment of nerve agent intoxication is applied in a dose proportionate to its LD_{50}. In this manner acute toxicity is determined. All tested oximes were of the bispyridinium type, with different length or shape of the connecting chain and positions of oxime groups on the pyridinium rings. All of these structural features play an important role in the biological activity of these compounds, as evidenced by their acute toxicity as well as by their reactivation potency (Kuca and Patocka, 2004). Testing of acute toxicity in a homologous series of oximes (effective, as well as ineffective, reactivators during *in vitro* screening) is not possible for ethical reasons. Our results showed that the compound with both functional groups in positions 2 (K033) is the most toxic reactivator. The values of LD_{50} for HS-6, obidoxime, K048 and BI-6 were comparable, even if the positions of the carbamido group as well as the length and shape of connecting chains were different. The relatively low acute toxicity was found for MMB-4 - the reactivator with both oxime groups in positions 4 at the pyridinium rings and the shortest connecting chain. However, HS-6 is quite similiar to HI-6 in its structure (different positions of carbamido groups). The value of the acute toxicity of HI-6 is nearly 3-fold higher (671 mg/kg) than that of HS-6.

The slope of the dose-response relationship provides useful and important information: a steep slope indicates little variation in the population response, while a comparatively shallower slope indicates that the response is much more variable over a greater dose range. A shallow slope indicates a greater margin of

safety (comparatively larger changes in dose result in small changes in response) (Lüllmann *et al.*, 2002). A steep slope of the dose-mortality curve, as was determined for MMB-4, indicates that individuals within a species will behave very similarly to each other in their response to the chemical (whereas a shallow slope of the curve indicates considerable variation in susceptibility to that particular chemical within a species). It should be mentioned that a steep dose response curve for significant toxicities in the most appropriate species or in multiple species may indicate a greater risk to the humans.

There are few data regarding side effects and acute toxicity of available pyridinium oximes. When bispyridinium oximes were tested on HeLa cells, they were shown to possess an inhibitory effect on cell growth and finally to provoke cell death. Bispyridinium oxime-affected cells displayed reduced mitochondrial oxygen consumption, reduced ATP stores, and were blocked in the G1 phase of the cell cycle. Bispyridinium oximes could act by penetrating energized mitochondria, inducing a dysfunction of their energetic metabolism and eliciting a programmed cell death process in exposed cells (Nocentini *et al.*, 1997). Respiratory paralysis is considered to be a major factor in the toxicity of pyridine aldoximes, chemicals related to the common oximes. Therefore, death due to respiratory paralysis is probable in oxime toxicity (Faff and Bak, 1978). Cholinomimetic symptoms, including retching, hypersalivation and enhanced intestinal motility were observed after administration of HI-6 to dogs (Eyer *et al.*, 1987). There has been some concern about the hepatotoxicity of obidoxime (Marrs, 1991; Balali-Mood and Shariat, 1998), thus pralidoxime has become the most widely-used oxime. Side effects of pralidoxime when administered intravenously to humans, in the absence of nerve agent poisoning, were only of a transient type (i.e., dizziness and blurred vision). Transient diplopia was observed when high doses of pralidoxime (10 mg/kg) were applied. Occasionally, nausea and vomiting may occur. The most serious side effect of pralidoxime is hypertension (Sidell, 1992). On the one hand high doses of pralidoxime revealed no serious side effects and significantly improved the outcome of patients poisoned with OP compounds (Balali-Mood and Shariat, 1998). On the other hand, there are some case reports of severe cardiac side effects - *e.g.*, repeated asystole after administration of pralidoxime in OP self-poisoning (Scott, 1986).

In conclusion, there is a lack of toxicity data of other bispyridinium oximes to make a qualified decision about the acute toxicity-structure activity relationship. Thus LD_{50} values have to be recorded and evaluated.

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