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SHORT COMMUNICATION

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Antidotal efficacy of quinuclidinium oximes against soman poisoning

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Abstract The efficiency of newly synthesized oxime derivatives of quinuclidinium were tested in vitro on soman inhibited acetylcholinesterase (AChE) of human erythrocytes and in vivo using soman poisoned mice. For this purpose, the inhibitory power of oximes (IC_{50}), acute toxicity (LD_{50}) as well as reactivating and protective capacities with respect to soman-inhibited AChE were determined for each of the oximes. All oximes tested were ineffective in vitro but protected mice very efficiently (BM-1 protects against $4LD_{50}$ of soman). The results indicate that the in vivo effectiveness of quinuclidinium oximes against soman poisoning may not be related to reactivation or protection of AChE but rather to some other mechanism of the cholinergic system.

Key words Acetylcholinesterase reactivators · Oximes · Quinuclidinium compounds · Soman

Abbreviations *BMP-1* 3-Hydroxyimino-1-methylquinuclidinium iodide · *BMP-2* 1,1-(2 oxapropyl) bis (3-hydroxyiminoquinuclidinium diiodide) · *BMP-3* 3-hydroxyimino-1-[3-(2-hydroxyiminomethyl-1-pyridinio)-2-oxapropyl] quinuclidinium diiodide · *BMP-4* 3-hydroxyimino-1-[3-(4-hydroxyiminomethyl-1-pyridinio)-2-oxapropyl] quinuclidinium diiodide) · *BMP-5* 3-oxo-1-[3-(2-hydroxyiminomethyl-1-pyridinio)-2-oxapropyl] quinuclidinium diiodide · *BMP-6* 3-oxo-1-[3-(4-hydroxyiminomethyl-1-pyridinio)-2-oxapropyl] quinuclidinium diiodide · *BM-1* 1-[3-(2-hydroxyiminomethyl-3-methylimidazolio)-2-oxapropyl]-3-(N,N-dimethylcarbamoyloxy) quinuclidinium diiodide · *HI-6* (2-hydroxyimino methyl pyridinium-1-methyl-4-carbamoyl pyridinium)-1-methyl ether dichloride · *PAM-2* hydroxyimino methyl-1-methyl pyridinium iodide

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Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7.) is an extremely active enzyme. Irreversible inhibition by organophosporus compounds (OPs) results in the accumulation of endogenous acetylcholine in synaptic cleft and paralysis of nerve impulse transmission in the central and peripheral nervous system. Together with atropine, oximes are known to be successfully used as therapy against intoxication with many OPs. Studies dealing with the therapy of soman poisoning (one of the most toxic of OPs) have demonstrated that the usual therapy with atropine and oximes is not effective, primarily due to the rapid ageing of the phosphonylated enzyme (Aldridge and Reiner 1972; Gray 1984). The acute toxicity of soman causes an excessive accumulation of acetylcholine (ACh), the synthesis of which depends on the activity of high-affinity transport uptake mechanism (HAChU; Sterling et al. 1993). The inhibition of HAChU with consequent reduction of ACh synthesis is demonstrated for many compounds structurally similar to choline (Ch; Kuhar and Murin 1978). It is also reported that the therapeutic effect of quaternary quinuclidinium oximes is partially due to reduction of HAChU (Sterling et al. 1991). In this paper we have evaluated the efficiency of seven newly synthesized oximes, derivatives of quinuclidinium, which inhibit the HAChU mechanism and might protect AChE.

Materials and methods

Experiments in vitro

Enzyme assay and preparation

Activity of AChE was measured spectrophotometrically by the method of Ellman et al. (1961). All experiments were performed in

0.1 mol/l phosphate buffer, pH 7.4, and enzyme activities were measured in the presence of 1.0 mmol/l acetylthiocholine as substrate at 37 °C. Native human erythrocytes obtained at random among leftover sera from biochemistry laboratory of our Institute, were used as the enzyme source. The erythrocytes were washed twice and diluted with saline to the original blood volume.

Oximes

Derivatives of quinuclidinium were synthesized for the purposes of this work in the Department of Organic Chemistry, Faculty of Natural Sciences, University of Zagreb. The synthesis of oximes BMP-2 to BMP-6 was described by Amitai et al. (1987), but the oximes used in our work contain iodide anion. Oximes BMP-1 and BM-1 were not synthesized previously (Mesić M and Primožič I; in preparation). Oximes were characterized by elemental analysis, IR and proton NMR-spectroscopy. For all compounds 1×10^{-2} mol/l (M) stock solutions were prepared in water and further diluted immediately before use.

Soman

Stock solution of 1×10^{-6} mol/l (M) soman was prepared in propylene glycol. Further dilutions were made in water, shortly before use.

Inhibition of AChE by oxime

The concentration of the oxime giving 50% enzyme inhibition (IC_{50}) was determined by incubating erythrocytes with four or more different concentrations of each compound (ranging from 10^{-9} to 10^{-3} mol/l) at 4 °C and assaying for AChE activity after 15 min. The IC_{50} values were calculated by linear least-square regression of log enzyme activity vs concentration of the test compound. Only those values between 10 and 90% inhibition were used for calculation.

Protective potency

The protective potency of quinuclidinium oximes with respect to soman-inhibited AChE was tested by measurement of enzyme inhibition in the presence and absence of quinuclidinium compounds. Native human erythrocytes were diluted with phosphate buffer, pH 7.4, the quinuclidinium compound (concentrations of each compound ranged from 1×10^{-9} to 1×10^{-3} mol/l) and soman (4×10^{-7} mol/l) were added in succession. The reaction mixture was incubated for 30 min at 37 °C after the addition of soman and activity of AChE was measured. The in vitro protective efficiency of oximes against soman inhibition of human erythrocyte AChE was expressed as P_{50}.

Protective index

The in vitro efficiency of antidote expressed as the ratio IC_{50}/P_{50} and called the protective index (PI), theoretically predicts the efficiency of oxime in vivo (Binenfeld et al. 1982). Oxime with PI > 1.8 should be effective as antidote in vivo, while oxime with PI < 1.2 is inefficient in vitro and in vivo.

Reactivating potency

Native human erythrocytes were incubated for 15 min at 4 °C with soman to obtain approx. 90% inhibition of AChE activity. The reaction mixture was diluted (1:600) with phosphate buffer (pH 7.4) and the activity of AChE was measured. The activity of AChE in the erythrocytes diluted with phosphate buffer (pH 7.4) was determined in the presence of soman and oxime. Simultaneously the activity of AChE from native erythrocytes diluted identically, was measured in the presence of oxime. Prior to AChE assay, the reaction mixture was incubated for 30 min at 37 °C. The percentage of reactivation (%R) was calculated according to De Jong et al. (1989). The enzyme hydrolysis of acetylthiocholine in the presence of oximes was corrected in all measurements for non-enzymic hydrolysis. Enzyme activities were corrected for the substrate hydrolysis due to the reaction of acetylcholine with the oxime.

Experiments in vivo

Animals

Male Balb-C mice were selected by weight (18–25 g). The animals were fed on standard diet (Sljeme, Zagreb), which was withdrawn 24 h prior to the treatment, and with free access to water. The mice were kept in macrolone cages under controlled conditions (12:12 h light/dark, 21 °C). Each experimental group contained five to six mice.

Acute toxicity

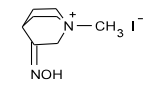
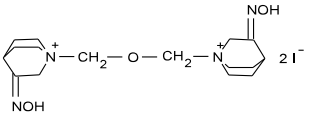
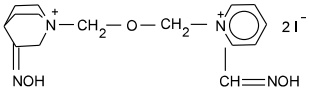
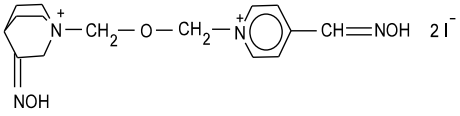
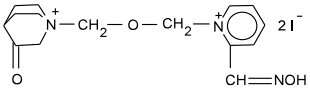
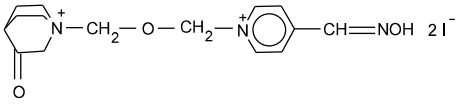
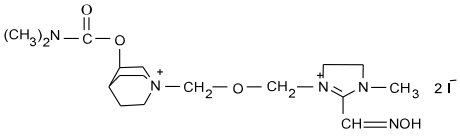
Acute toxicity (LD_{50}) based on 24 h mortality rates was calculated according to Thompson (1947) and Weil (1952). The therapeutic effect against soman toxicity in mice was tested by giving oxime intraperitoneally (i.p.) at 1/4 of the respective LD_{50} dose together with atropine (10 mg/kg body wt.) immediately after subcutaneous (s.c.) administration of soman. The therapeutic effect was expressed as therapeutic factor (TF) and therapeutic dose (TD). For therapeutic factor, relative efficiency was calculated as follows: TF = LD_{50} of poison with antidote/LD_{50} of poison without antidote. Therapeutic dose was expressed as the highest multiple of LD_{50} of soman, which could be counteracted by the antidote injected i.p. 1 min after s.c. injection of the poison. (All animals survive).

Results and discussion

Quinuclidinium derivatives (monoquarternary, symmetric bisquarternary or nonsymmetric containing pyridinium nucleus: BMP-1 to BMP-6) are poor inhibitors of human erythrocyte AChE in vitro. The IC_{50} values range from 9.8×10^{-5} mol/l for the BMP-4 oxime to 2.8×10^{-3} mol/l for BMP-1. The exact value for other oximes tested was not determined due to their insolubility at higher concentrations. The results indicate a low affinity of these compounds for AChE although some of them contain a quarternary pyridinium moiety in the molecule, which is known to have a certain affinity for human erythrocyte AChE (IC_{50} for PAM-2 is 3.5×10^{-3} mol/l). Effects of quinuclidinium oximes on soman-inhibited AChE in vitro and in vivo are given in Table 1.

The protective activity of quinuclidinium oximes, as tested on human erythrocyte AChE inhibited by soman and expressed as P_{50}, indicates low protective potency of these compounds. BMP-1 and BMP-3 oximes did not increase the activity of AChE in human erythrocytes to 50%: residual activity after soman inhibition was 10%. P_{50} values for other oximes ranged from 1.7×10^{-4} to 4.5×10^{-4} mol/l. Therefore, for those compounds the IC_{50}/P_{50} ratio (or protective index) was < 1.8, which leads to the assumption that those compounds displaying poor antidotal potency in vitro should be also ineffective in vivo (Binenfeld et al. 1982).

Table 1 Structure and biological efficiency[a] of quinuclidine oximes on acetylcholinesterase (AChE) inhibited by soman in vitro and in vivo

Oximes	In vitro IC_{50} mol/l	%R	P_{50} mol/l	PI	In vivo LD_{50} mg/kg	TF	TD
BMP-1 	2.8×10^{-3}	b	$>4 \times 10^{-3}$	<1.8	56.0	3.18	2
BMP-2 	$>1 \times 10^{-3}$	b	4×10^{-4}	<1.8	224	2.52	2
BMP-3 	$>1 \times 10^{-3}$	b	$>1 \times 10^{-3}$	<1.8	>100	2.52	1.59
BMP-4 	9.8×10^{-5}	33	1.7×10^{-4}	<1.8	107	2.34	1.59
BMP-5 	$>1 \times 10^{-3}$	b	3.8×10^{-4}	<1.8	>1000	2.73	2.52
BMP-6 	$>1 \times 10^{-3}$	b	4.5×0^{-4}	<1.8	>200	3.83	2.52
BM-1 	6×10^{-6}	b	3.9×10^{-3}	≪1.8	202.2	5.34	4

[a]IC_{50} is the concentration of the test compounds which inhibits 50% of AChE activity; %R is percentage of reactivation of human erythrocyte AChE inhibited by soman (1×10^{-6} mol/l) with oximes (2×10^{-5} mol/l or 1/4 IC_{50}); P_{50} is the antidote concentration allowing AChE to preserve 50% of normal activity in the presence of ~100% inhibitory concentration of soman; b, cannot be determined; LD_{50}-values (mice) were based on 24 h mortality; PI is the ratio IC_{50}/P_{50}, a numerical expression for antidotal efficiency of the compound; TF is LD_{50} of soman with antidote/LD_{50} of soman, the highest multiple of soman LD_{50} which could be counteracted by the antidotes (1/4 LD_{50} /kg or 50 μmol/kg) i.p. injected 1 min after the injection of soman; TD is multiple LD_{50} of soman with antidote/LD_{50} of soman (all animals survived)

Apart from BMP-4, the reactivating potency of all other oximes used (applied in final concentration of 1/4 of their IC_{50} values), was not significant when tested on AChE inhibited by soman (Table 1). For those oximes for which IC_{50} values could not be determined, the concentration of 2×10^{-5} mol/l was used as final concentration in checking the reactivating ability. At this concentration the administration of oximes led to enhanced inhibition of AChE activity. Although BMP-4 reactivated the enzyme by 33% when applied in a concentration of 1/4 of the IC_{50} value, the PI only amounted to 0.9. A low PI (< 1.8) shows that oxime BMP-4 does not have protective potency in vitro and should also be ineffective in vivo (Binenfeld et al. 1982). In experiments on mice, acute toxicity (LD_{50}) for each oxime was determined. The values obtained varied from 56 mg/kg body wt. (BMP-1) to > 1000 mg/kg body wt. (BMP-5) indicating low toxicity of some of the oximes used (Table 1).

In soman-intoxicated mice, a therapeutic effect of quinuclidinium oximes in combination with atropine was also determined. The values obtained for the therapeutic factor of quinuclidinium and quinuclidinium-pyridinium oximes (BMP-1 to BMP-6) ranged from 2.34 to 3.83 LD_{50} of soman. The therapeutic dose of these compounds was from 1.59 to 2.52 of LD_{50} of soman. All mice survived when given subcutaneously 1.59 LD_{50} of soman followed by intraperitoneal dosing of atropine (10 mg/kg body wt.) and 1/4 of LD_{50} of every oxime, but did not survive when therapy only comprised atropine (data not presented).

Quinuclidinium-imidazolium oxime (BM-1) was found to be the most effective against soman intoxication in mice. Applied in vivo at a dose of ¼ LD_{50}

(LD_{50} = 202.2 mg/kg body wt.) together with atropine-sulphate, BM-1 ensures survival of all experimental animals at as much as four LD_{50} of soman. However, the inhibitory potency (IC_{50}) of BM-1 determined in vitro on human erythrocyte AChE was 6×10^{-6} mol/l, and protective potency (P_{50}) of BM-1 tested on AChE inhibited by soman was 3.9×10^{-3} mol/l; the calculated PI was < 1.8. Reactivation of soman inhibited AChE was not observed. The results obtained in vitro showed weak affinity of BM-1 to AChE. A very good therapeutic effect obtained in vivo without in vitro activity indicates that quinuclidinium oximes, especially BM-1, protect mice against soman poisoning by an unknown mechanism that is not related to AChE interactions.

Our results are in accordance with those of other authors. Amitai et al.(1987) have shown that quinuclidinium oximes give good protection in soman intoxicated mice, together with atropine and benactyzine following pyridostigmine pretreatment. It was proved that the oximes are also efficient in soman intoxicated dogs and monkeys (Amitai et al. 1995). Two of the oximes tested (AB-8 and AB-13) correspond to the structure of our oximes (BMP-5 and BMP-6), but instead of chloride anions, our oximes contain iodide.

The fact that the basic structural differences between the compounds tested and good AChE reactivators in vitro (HI-6, PAM-2) lies only in the existence of quinuclidinium quaternary core, indicates that this part of the molecule is responsible for the weak in vitro affinity of these compounds to AChE. According to Sterling et al. (1993), the quinuclidinium quarternary core has no affinity for AChE except in the presence of a carbamoyl group, which would be linked to the ester position of the enzyme. This assumption may be true for the results obtained for BM-1 having both the quinuclidinium ring and the carbamoyl group, but not for BMP-1 to BMP-6 where the carbamoyl group is lacking.

However, quinuclidinium derivatives are proved to inhibit presynaptic synthesis of ACh involving high-affinity transport uptake mechanism (Sterling et al. 1991). Inhibition of this system efficiently blocks uptake of Ch with consequent reduction of ACh synthesis and its release in the synaptic cleft (Kuhar and Murin 1978). According to Sterling et al. (1988), quinuclidinium derivatives given intramuscularly (i.m.) at 1 μmol/kg 30 min prior to soman, together with atropine sulphate and PAM-2 administrated i.m. 1 min after soman, dramatically reduced soman toxicity from LD_{70} to LD_{20} at 1 h and from LD_{76} to LD_{20} at 24 h post soman.

In our experiments the best effects of quinuclidinium oximes were found to be obtained with the dose approximately equal to ¼ LD_{50} (given with atropine). With this treatment of soman poisoning animals demonstrated reduced central, nicotinic and muscarinic symptoms in the first 30–60 min. After this delay, all symptoms increased (salivation, fasciculation, ataxia and dyspnoea) but the animals survived. With adequate choice of the oxime dose ACh synthesis is not completely disabled, but is markedly reduced. Therefore, it could be supposed that ACh does not accumulate in the synaptic cleft and the transfer of impulses is possible although to lower intensity than under normal conditions. This might ensure minimal transfer of impulses, because a certain number of free active positions of AChE still remain for hydrolysing the reduced quantity of ACh in the synaptic cleft. The potency of quinuclidinium compounds to protect against severalfold LD_{50} of soman makes these compounds important from the point of view of therapy of soman intoxication.

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