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Note

In vitro and *in vivo* Reactivation of Cholinesterases Inhibited by Highly Toxic Organophosphorus Compounds

A Comparison Between 1,3-Acetone-bis-(4-hydroxyiminoformyl pyridinium) dibromide, 1,3-Trimethylene-bis-(4-hydroxyiminoformyl pyridinium) dibromide, and 1,3-Dimethylether-bis-(4-hydroxyiminoformyl pyridinium) dibromide

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The *in vitro* and *in vivo* reactivating power of 1,3-acetone-bis-(4-hydroxyiminoformyl pyridinium) dibromide (MBM-3) was compared with that of 1,3-trimethylene-bis-(4-hydroxyiminoformyl pyridinium) dibromide (TMB-4) and 1,3-dimethylether-bis-(4-hydroxyiminoformyl pyridinium) dibromide (Toxogonin). As a source of cholinesterase rat's whole blood, plasma, erythrocytes, and brain homogenate, were used. The cholinesterase inhibition was performed by ethyl *N*-dimethylaminophosphorocyanidate (Tabun); ethyl 4-nitrophenyl phosphonate (Armin), *O,O*-diethyl-*S*-(2-diethylaminoethyl) phosphorothioate (Amitone-3); pinacolyl-methyl-phosphorofluoridate (Soman); and *O*-ethyl-*S*-(2-diethylamino ethyl phosphorothioate (Edemo-3).

The cholinesterase activities were measured by a modified Michel electrometric method. The molar oxime concentrations which cause a 50% reactivation of the phosphorylated enzyme (MRD_{50}) were as follows a) *erythrocytes:* from 3.5×10^{-7} to 7.8×10^{-6} (TMB-4); from 1.5×10^{-7} to 7.5×10^{-5} (Toxogonin); from 8×10^{-7} to 7.5×10^{-5} (MBM-3). b) *plasma:* from 1×10^{-6} (TMB-4); from 6.5×10^{-7} to $9.3 \times \times 10^{-6}$ (Toxogonin); from 3.5×10^{-6} to $> 1 \times 10^{-4}$ (MBM-3), c) *brain:* from 1.5×10^{-7} to 1×10^{-5} (TMB-4); from 1.3×10^{-7} to $> 1 \times 10^{-4}$ (Toxogonin); from 5.0×10^{-7} to $> 1 \times 10^{-4}$ (MBM-3). Cholinesterase inhibited by Soman could not be reactivated.

In vivo experiments with Amitone-3 gave a variable percentage of reactivation of the whole blood cholinesterase, from 65 to 87%. A statistically significant difference was observed between Toxogonin and MBM-3 ($P < 0.01$).

The results indicate that MBM-3 belongs to the class of the most powerful reactivators of phosphorylated cholinesterase, *e. g.* TMB-4 or Toxogonin.

Continuing our earlier studies which have shown that 1,3-acetone-bis-(4-hydroxyiminoformyl pyridinium) dibromide (MBM-3) is an effective antidote in Paraoxon and Sarin poisoning[1,2], we have measured the *in vitro* and *in vivo* reactivating activity of MBM-3 towards cholinesterases inhibited by a number of the most powerful anticholinesterases[3]. As reference reactivators we used 1,3-trimethylene-bis-(4-hydroxyiminoformyl pyridinium) dibromide (TMB-4), and 1,3-dimethylether-bis-(4-hydroxyiminoformyl pyridinium) dibromide (Toxogonin).

MATERIALS AND METHODS

Organophosphorus Compounds

Ethyl-4-nitrophenyl phosphonate (Armin); pinacolyl methyl phosphonofluoridate (Soman); *O,O*-diethyl-*S*-(2-diethylaminoethyl) phosphorothioate (Amitone-3); ethyl-*N*-dimethylphosphoro cyanidate (Tabun); *O*-ethyl-*S*-(2-diethylaminoethyl) phosphorothioate (Edemo-3) were of 98—99% purity. All compounds were made up as 0.1% stock solutions in propylene glycol and diluted to desired concentrations with isotonic saline. The concentrations of the solutions were checked by spectrophotometric measurements[4] and by determining their LD_{50} subcutaneously in white mice.

Oximes

TMB-4 (m. p. 235°), Toxogonin (m. p. 225°) and MBM-3 (m. p. 219°) were dissolved in redistilled water immediately before use.

Cholinesterase Preparations

As sources of enzyme we used: 1) Rat erythrocytes, washed three times with isotonic saline and haemolysed; the haemolysate volume was adjusted with distilled water to the corresponding blood volume. 2) Rat blood plasma. 3) Rat brain homogenate. 4) Rat whole blood.

In vitro *Cholinesterases Reactivation* — Male wistar rats weighing between 200 and 250 g. were injected subcutaneously with $^2/_3$ of LD_{50} dose of a given organophosphorus compound, and the animals sacrificed half an hour later. The volume of the injected solution was 2 ml./kg. The activity of erythrocite, plasma and brain cholinesterases was about 10—20% of the activity of untreated animals. The enzyme preparations were incubated for 10 min. with an oxime solution, and acetylcholine chloride (3×10^{-3} *M* final) was then added. Cholinesterases activity was measured electrometrically by the Michel's method, as modified by Stevanović and Jović[5]. The final oxime concentrations before addition of substrate varied between 1×10^{-7} and 1×10^{-4} *M*.

In vivo *Cholinesterases Reactivation of Rat's Whole Blood.* — Blood was taken from the rat's tail. One hour after the subcutaneous application of $\sim ^1/_2$ LD_{50} dose of Amitone-3 the oximes ($\sim ^1/_8$ LD_{50}) were given subcutaneously. The volume of the oxime and organophosphorus solution together was 1 ml./kg. One hour after the administration of oxime the rats were sacrificed and the cholinesterases activity was measured as described. Cholinesterases measurements were made on blood samples obtained before administration of the organophosphorus compounds, immediately before administration of oximes and immediately after the rats had been sacrificed. The cholinesterase activity before oxime administration was about 35 to 45% of the normal value.

The *in vitro* and *in vivo* degree of cholinesterases reactivation was calculated according to the method of Childs *et al.*[6]; Statistical calculations were carried out by the Eramens method[7].

RESULTS AND DISCUSSION

The results of the *in vitro* cholinesterases reactivation are presented in Table I. From these experiments and results obtained in our previous experiments with Sarin[2] the following conclusions can be drawn. MBM-3 possesses a reactivating property similar to that observed with other oximes used so far and its efficacy varies from one inhibitor to the other. From these results it is not possible to draw a theoretical conclusion or to predict the antidotal activity of the three oximes studied.

MBM-3, like Toxogonin and TMB-4, is very effective in reactivating brain cholinesterases *in vitro*. Recent experiments of Milošević and Anđelković[8] and Hobbiger and Vojvodić[9] have shown that it is also possible to reactivate cholinesterases inhibited by organophosphorus compounds in different brain areas with oximes *in vivo*. It seems likely that MBM-3 would also reactivate

TABLE I

In Vitro *Reactivation of Rat Erythrocyte, Plasma, and Brain Cholinesterases*

Inhibitor	MRD_{50} (μM)								
	Erythrocyte			Plasma			Brain		
	Toxogonin	TMB-4	MBM-3	Toxogonin	TMB-4	MBM-3	Toxogonin	TMB-4	MBM-3
Tabun	75	7.8	75	8.0	4.3	> 100	> 100	10	> 100
Soman	none	none	none	none	none	none	none	none	none
Armin	1.0	0.75	0.95	8.0	5.0	20	0.80	0.45	0.83
Amitone-3	0.15	0.35	0.80	0.65	4.0	6.3	0.17	0.15	0.50
Edemo-3	8.0	4.8	6.8	9.3	1.0	3.5	4.3	0.83	3.8

MRD_{50} = μmolar concentration of oximes which causes a 50% reactivation of inhibited enzyme.

TABLE II

In Vivo *Reactivation of Rat's Whole Blood Cholinesterase*

Inhibitor	Reactivation of cholinesterase (%) $\bar{x} \pm 2\sigma$		
	TMB-4	Toxogonin	MBM-3
Amitone-3	78 ± 15 $P > 0.1$	87 ± 11 $P < 0.01$	65.0 ± 4.2 —

brain cholinesterases *in vivo*, since its chemical structure is similar to that of TMB-4 and Toxogonin[1].

The results of the *in vivo* reactivation of whole blood cholinesterases are presented in Table II. These results are only preliminary and each number presents the mean obtained on 4 animals:

The *in vivo* experiments with Amitone-3 showed good agreement with the *in vitro* results, although they were performed with whole blood preparations which contain both erythrocyte and plasma cholinesterase. The statistically significant difference between Toxogonin and MBM-3 in the *in vivo* experiments is probably due to the 10 and 5 fold respectively, higher reactivating power of Toxogonine as compared to MBM-3 in reactivating plasma and erythrocyte cholinesterase inhibited by Amitone-3. In the case of TMB-4, where this difference was only 1.5 and 2.3 fold, respectively, no statistically significant difference was observed.

The promissing results obtained with MBM-3 as an antidote in Sarin and Paraoxon poisoning[1,2] were confirmed in the present experiments where MBM-3 showed equally effective reactivating properties as TMB-4 and Toxogonine against cholinesterases inhibited by some of the most toxic organophosphorus compounds.

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IZVOD

In vitro i *in vivo* reaktivacija kolinesteraza inhibiranih najtoksičnijim organofosfornim spojevima

Aktivnost 1,3-aceton-bis-(4-hidroksiiminoformilpiridinijum) dibromida u usporedbi s aktivnošću 1,3-trimetilen-bis-(4-hidroksiiminoformilpiridinijum) dibromida i 1,3-dimetileter-bis-(4-hidroksiiminoformilpiridinijum) dibromida

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Ispitana je *in vitro* reaktivatorska moć 1,3-aceton-bis-(4-hidroksiiminoformilpiridinijum) dibromida (MBM-3), 1,3-trimetilen-bis-(4-hidroksiiminoformilpiridinijum) dibromida (TMB-4) i 1,3-dimetileter-bis-(4-hidroksiiminoformilpiridinijum) dibromida (Toxogonin) na kolinesteraze eritrocita, plazme i mozga štakora inhibirane etiletoksi-*p*-nitro-fenolatom (Armin), dimetilamidoetoksifosforilcijanidom (Tabun), pinakoloksimetilfluorofosfatom (Soman), *O,O*-dietoksi-*S*-(2-dietil-aminoetil)-tiofosfatom (Amiton-3), i *O*-etoksi-*S*-(2-dietilaminoetil)-metiltiofosfonatom (Edemo-3).

Za mjerenje upotrebljena je modificirana Michelova elektrometrijska metoda a kao izvor enzima služili su eritrociti, nativne plazme i homogenizati mozga štakora prethodno otrovanih navedenim spojevima. Molarna koncentracija oksima, koja dovodi do 50% reaktivacije inhibiranog enzima kreće se ovisno od alkilfosfatu za eri-

trocitne kolinesteraze: od $3,5 \times 10^{-7}$ do $7,8 \times 10^{-6}$ (TMB-4), od $1,5 \times 10^{-7}$ do $7,5 \times 10^{-5}$ (Toxogonin); od 8×10^{-7} do 7.5×10^{-5} (MBM-3); za kolinesterazu plazme: od 1×10^{-6} do 5×10^{-6} (TMB-4); od $6,5 \times 10^{-7}$ do $9,3 \times 10^{-6}$ (Toxogonin); od $3,5 \times 10^{-6}$ do $> 1 \times 10^{-4}$ (MBM-3); za kolinesterazu mozga: od $1,5 \times 10^{-7}$ do 1×10^{-5} (TMB-4); od $1,3 \times 10^{-7}$ do $> 1 \times 10^{-4}$ (Toxogonin); od 5×10^{-7} do $> 1 \times 10^{-4}$ (MBM-3); osim za Soman gdje ni u jednom slučaju nije došlo do reaktivacije.

U *in vivo* eksperimentima sa Amitonom-3 procenat reaktivacije inhibirane kolinesteraze pune krvi štakora kretao se od 65 do 87% s tim da se statistički značajna razlika pokazala između Toxogonina i MBM-3 ($P < 0,01$).

Na temelju ovih rezultata zaključeno je da MBM-3 spada u grupu najefikasnijih oksima reaktivatora kolinesteraza inhibiranih alkilfosfatima u koje spadaju i TMB-4 i Toxogonin.

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