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Efficacy of antidotes and their combinations in the treatment of acute carbamate poisoning in rats

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ABSTRACT

Background: Physostigmine and its analogues neostigmine, pyridostigmine and rivastigmine are carbamates nowadays used in many indications, including antidotal effects against antimuscarinic poisonings, reversal of competitive neuromuscular block, myasthenia gravis, Alzheimer's disease and prophylaxis against nerve agent intoxications. Use of these medicinal carbamates, but also of carbamate insecticides, created need for research into the potential and mechanisms of action of several antidotes against carbamate poisonings, including anticholinergics and oximes.

Aim: The goal of this experimental study was to ascertain the life-preserving potential of anticholinergics atropine, hexamethonium and D-tubocurarine, oxime HI-6 and their combinations in rats poisoned with physostigmine or pyridostigmine.

Materials and methods: Experiments were performed in Wistar rats. Carbamates were injected subcutaneously (*sc*) and antidotes intramuscularly (*im*). Median lethal dose (LD_{50}) in animals treated with antidotes were compared to the ones in saline-treated rats and protective ratios (PRs) were calculated. Atropine (5, 10 and 20 mg/kg), hexamethonium (5, 10 and 20 mg/kg), D-tubocurarine (0.005, 0.010 and 0.020 mg/kg) and oxime HI-6 (25, 50 and 100 mg/kg) were used as monotherapies and in dual combinations, where atropine was the obligatory antidote. Biochemical experiments consisted in measuring of the cholinesterase activities in brain, whole blood and diaphragm in rats 5, 15, 30, 60, 120 and 240 min after poisoning with 0.8 LD_{50} of physostigmine or pyridostigmine.

Results: All the tested antidotes assured some degree of protection against the two carbamates. Atropine and hexamethonium produced better protection in physostigmine-poisoned rats, while D-tubocurarine and HI-6 were more efficacious in pyridostigmine-intoxicated animals. Oxime HI-6 50 mg/kg reactivated acetylcholinesterase (AChE) in brain inhibited by physostigmine and in diaphragm inhibited by pyridostigmine.

Conclusions: Mechanism of physostigmine-induced lethal effect is predominantly central and it involves inhibition of brain AChE, while pyridostigmine produces the same effect exclusively outside the central nervous system, by inhibiting AChE in the respiratory muscles. As a consequence, increasing doses of atropine and their combination with hexamethonium assure excellent protection against physostigmine toxicity, while the best protection against pyridostigmine is provided by a strictly peripherally acting antinicotinic D-tubocurarine and bispyridinium oxime HI-6. The oxime acts as antidote against physostigmine and pyridostigmine poisoning by reactivating AChE in the brain and diaphragm, respectively.

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1. Introduction

1.1. Mechanism of action of carbamates

Physostigmine and other carbamates produce pseudo-irreversible inhibition of acetylcholinesterase (AChE) and cholinesterase (Perola et al., 1997) and thus produce both pharmacological and toxic effects based on the excess of the accumulated acetylcholine in cholinergic synapses (Jokanović, 2009). They carbamylate the active centre of these enzymes, which is different from *e.g.* ambenonium or edrophonium that leave the active centre of the enzyme unchanged (producing thus a true reversible inhibition) becoming a substrate for the enzyme, with 10,000-fold higher affinity for the active centre of the enzyme than acetylcholine (Wilson et al., 1960; Aldridge and Reiner, 1972). The resulting building-up of acetylcholine in the cholinergic synapses causes overstimulation of central and peripheral muscarinic and nicotinic receptors. As a consequence, muscarinic (miosis, hypersalivation, lacrymation, bronchorrhoea, bronchoconstriction, nausea, vomiting, increased motility of the bowels, bradycardia and hypotension), nicotinic (mydriasis, tachycardia, hypertension, fasciculations and necrosis of skeletal muscles) and central (motor incoordination, tremor, convulsions, respiratory depression and coma) signs of toxicity occur (Vale and Lotti, 2015). These signs belong to the acute cholinergic syndrome and are shared by both carbamate and organophosphate cholinesterase inhibitors (Suzuki et al., 2017). The severity and outcome of the poisoning, however, usually differ because of the reversibility of the enzyme inhibition and the self-limited nature of the carbamate-induced intoxication (Hoffman et al., 2009). At the same time, in case of massive and untreated or untimely treated intoxications with medicinal or agricultural carbamates, death may also occur (Ameno et al., 2001; Pinakini and Kumar, 2006).

1.2. Treatment of carbamate intoxications

Like in cases of organophosphate poisonings, atropine remains the basic symptomatic antidote against toxicity of carbamates. It is lipophilic and therefore passes through the blood-brain barrier into brain and antagonises both central and peripheral muscarinic sings in carbamate poisoning (Stojiljković et al., 1989). As a consequence, atropine proved to be effective in treating experimental intoxications in rodents poisoned with centrally and peripherally acting carbamate physostigmine (Natoff and Reiff, 1973; Harris et al., 1989) and only peripherally acting carbamates neostigmine (Natoff and Reiff, 1973) and pyridostigmine (Harris et al., 1989; Caldwell et al., 1989). Some authors suggested that atropine should be replaced by even more lipophilic and thus centrally more active anticholinergic scopolamine (Janowsky et al., 1984, 1985b, 1987) or by its combination with either rapidly acting anticholinergic benactyzine (Klemm, 1983) or aprophen, an anticholinergic with higher lipophilicity (D'Mello, 1983; Leadbeater et al., 1985).

Use of oximes as causal antidotes – reactivators of the inhibited AChE - in carbamate poisonings is controversial (Jokanović, 2009). In some experiments pyridinium-2-aldoxime (pralidoxime chloride, 2-PAM) and pralidoxime mesylate (P2S) did not influence the toxicity of physostigmine and neostigmine in mice and guinea pigs (Kewitz et al., 1956; Bethe et al., 1957; Hobbiger and Sadler, 1959). In some poisonings, induced by *N*-monomethylcarbamates and especially in case of intoxication with insecticide carbaryl, these two monopyridinium oximes, but also some bispyridinium oximes, even potentiated their toxicity in humans, dogs and rodents (Carpenter et al., 1961; Farago, 1969; Natoff and Reiff, 1973; Bošković et al., 1976; Harris et al., 1989). Other authors reported on the increased atropine protection in mice poisoned with physostigmine and treated also with trimedoxime (TMB-4), but not with 2-PAM (Klemm, 1983). Good protection with 2-PAM was found in mice poisoned with pyridostigmine (Klemm, 1983) and in rats poisoned with physostigmine, but both with 2-PAM and HI-6 (Harris et al., 1989).

Table 1
Medicinal carbamates and their indications.

Indication	Carbamate(s)	Reference(s)
Antidote against antimuscarinic poisoning	Physostigmine	Frascogna (2007); Rosenbaum and Bird (2010)
Glaucoma	Physostigmine	Realini (2011)
Myasthenia gravis	Pyridostigmine, neostigmine	Aquilonius et al., (1983)
Reversal of neuromuscular blockade	Neostigmine	Parida et al. (2017)
Adynamic ileus	Neostigmine	Dodds et al. (2016)
Alzheimer's disease	Rivastigmine	Onor et al. (2007)
Hypotension in sepsis	Physostigmine	Pinder et al. (2015) Zimmermann et al. (2017)
Prophylactic antidotes against nerve agents	Physostigmine, pyridostigmine	Berry and Davies (1970); Inns and Leadbeater (1983)

Benzodiazepines, mainly diazepam (Todorović et al., 2012) and midazolam (Bokonjić and Rosić, 1991; Reddy and Reddy, 2015), are effective anticonvulsants and adjunct to atropine and oxime therapy against organophosphate poisonings. At the same time, its effects are beneficial when combined with atropine, in those cases of carbamate intoxications that are complicated by seizures (Klemm, 1983; Bokonjić and Rosić, 1991; Burgess et al., 1994).

1.3. Use of carbamates in medicine

Nowadays, carbamates are widely used in medicine and these indications are listed in Table 1.

A special indication is use of carbamates, mainly physostigmine and pyridostigmine, as pretreatment against organophosphate cholinesterase inhibitors, such as the nerve agents tabun, sarin, soman and VX. This line of research is based on the finding by Koster (1946) that physostigmine, administered at a large dose along with atropine 3.5 h before an organophosphate, di-isopropyl fluorophosphates (DFP) protected a cat against 30 LD_{50}s of DFP. The whole concept consists of the pseudo-irreversible inhibition with a carbamate of 20–40% of AChE, after which this part of the enzyme activity, crucial for survival, remains protected from the subsequent irreversible inhibition by an organophosphate and undergoes spontaneous decarbamylation (Eckert et al., 2007; Herkert et al., 2011a, 2011b). Pyridostigmine bromide prophylactic tablets, although included in the standard antidotal kits of several armies, do not protect the brain AChE, since pyridostigmine cannot pass the blood-brain barrier (Layish et al., 2005). Physostigmine, on the other hand, protects both central and peripheral AChE and is therefore more efficient than pyridostigmine (Miller et al., 1993). Best results with physostigmine prophylaxis were obtained in non-human primates after subchronic administration (Philippens et al., 2000). For this reason, transdermal delivery systems that enable long-term delivery of small, non-toxic doses of physostigmine, are being developed (Meshulam et al., 1995). One of such prophylactic combinations of physostigmine and procyclidine, followed by atropine/HI-6 treatment, assured protection against 5 LD_{50} of soman in Rhesus monkeys (Cho et al., 2012). The efficacy of these carbamate prophylactic regimens followed by atropine, oxime and diazepam treatment, is very high and assures protective ratios (PRs) of up to 76, 380, 20 and 410 in guinea-pigs poisoned with tabun, sarin, soman and VX, respectively (Berry and Davies, 1970; Inns and Leadbeater, 1983).

1.4. Toxicological significance of carbamates

The mentioned widespread use of carbamates – physostigmine, neostigmine, pyridostigmine and rivastigmine – in medicine creates a

potential for overdoses and toxicity that must be adequately treated (Cumming et al., 1968; Lai and Moen, 2005; Sener and Ozsarac, 2006; Hoffman et al., 2009; Suzuki et al., 2017). This is even more important when we have in mind the threat from poisonings with carbamate insecticides. Although the clinical picture of carbamate poisonings resembles the one in organophosphate-induced intoxication and dictates use of similar antidotes, there is still need for research in the quantitative aspects of use of anticholinergics and oximes.

Use of oximes in carbamate poisonings is still controversial, since in some cases of carbaryl intoxication even fatal potentiation of its toxicity was obtained with pralidoxime chloride (2-PAM) (Farago, 1969). In other cases, 2-PAM did not reduce the toxic effects of benfucarb (Ichikawa et al., 1995), aldicarb and methomyl (Brittain et al., 2016), nor could 2-PAM or obidoxime (LüH-6) reactivate cholinesterase inhibited by aldicarb or methomyl (Lifshitz et al., 1994). It was also reported that higher, but not lower doses of oximes 2-PAM or HI-6 decreased the LD_{50} of carbaryl in rodents (Stojiljković, 1994; Mercurio-Zappala et al., 2007). On the other hand, 2-PAM, administered without atropine, effectively antagonised peripheral nicotinic effects in a case of rivastigmine overdose (Hoffman et al., 2009).

1.5. Aim

The goal of this experimental study was to ascertain the life-preserving potential of anticholinergics atropine, hexamethonium and D-tubocurarine, oxime HI-6 and their combinations in rats poisoned with physostigmine or pyridostigmine.

2. Material and methods

2.1. Experimental animals

Experiments were carried out in male Wistar rats, weighing 180–220 g and bred under the standard controlled conditions and with access to food and water *ad libitum*.

2.2. Chemicals

Bispyridinium oxime HI-6 dichloride monohydrate was synthesised at the SBS Institute, Sarajevo, Bosnia & Herzegovina, while physostigmine salicylate, pyridostigmine bromide, atropine sulphate monohydrate, hexamethonium bromide, D-tubocurarine dichloride were obtained from commercial sources.

Almost all the substances were dissolved before injection in saline (0.9% NaCl). The exception was physostigmine, which was dissolved in dimethylsulphoxide, due to the low water-solubility of its salicylate salt.

Application volume of the chemical was 1 ml/kg. Carbamates were injected subcutaneously (*sc*) into the abdominal region, while the treatment solutions were administered intramuscularly (*im*) into the left or right thigh.

2.3. Experimental procedures

2.3.1. Protection experiments

In these experiments potential of an antidote or of an antidotal combination to protect from lethal outcome due to physostigmine or pyridostigmine intoxication was ascertained based on the 24-h survival. The outcome was protective ratio (PR), i.e. ratio of the median lethal dose (LD_{50}) in protected and in unprotected rats. Rats, in groups of 6, were poisoned with increasing doses of carbamates (by factor 2) and by applying the “up and down method”, number of dead animals was registered in each group. For each LD_{50} calculation 3–6 dosage levels of carbamates were needed. The LD_{50} values were computed by means of the statistical software, according to the Litchfield and Wilcoxon (1949).

2.3.2. Routes of administration, timing and doses

Carbamates were injected subcutaneously (*sc*) because the usual route for the administration of antidotes is intramuscular (*im*). For practical reasons, it would not be convenient to pursue *im* administration for the carbamates, too, especially when a combination of multiple antidotes was to be applied also *im*.

The syringes with solutions of antidotes were always prepared in advance, so that they could be injected within 15 s after the administration of carbamates.

In the present experiment, the LD_{50} values for physostigmine salicylate and pyridostigmine bromide administered *sc* in rats were 1.32 and 4.19 mg/kg, respectively. For biochemical experiments a high, but non-lethal dose of either carbamate was chosen – 0.8 LD_{50} in order to induce significant AChE inhibition and signs of severe poisoning, yet allowing the animals to survive long enough to endure the experimental procedures.

Although various doses of atropine can be found in literature, the usual one is 10 mg/kg (Parkes and Sacra, 1954) and therefore we used this one, but also half of it and the double one – 5 and 20 mg/kg, in order to study the dose-dependency of atropine protection. The *im* LD_{50} was reported to be as high as 920 mg/kg (Lewis, 1996a), which means that the current dose range (5–20 mg/kg) corresponds to 0.54–2.17% of the LD_{50} of atropine.

Because of the different mechanism of action, the doses of antinicotinic drugs did not have to be paired with certain micromolar doses of atropine or between each other and therefore they were chosen based on the effective doses found in the literature – hexamethonium 5, 10 and 20 mg/kg and D-tubocurarine 0.05, 0.1 and 0.2 mg/kg (Parkes and Sacra, 1954; Bošković and Stern, 1970; Dekleva et al., 1989).

The *sc* LD_{50} of hexamethonium is 200 mg/kg (Santa Cruz Biotechnology, Texas, USA, 2018). Since its bioavailability after *sc* and *im* administration appears to be very similar - 96% and 93%, respectively (Mason, 1980), it is reasonable to assume that the *im* LD_{50} would be similar. It means that the dose range of hexamethonium used in the present study, i.e. 5–20 mg/kg *im*, corresponds to 2.5–10% of its LD_{50}.

The *im* LD_{50} of D-tubocurarine in rats is 0.5 mg/kg (Lewis, 1996b). It means that the dose range of D-tubocurarine used in the present study (0.005–0.020 mg/kg *im*) equals 10–40% of the *im* LD_{50}.

The basic dose of the oxime HI-6 was 50 mg/kg as this one was frequently used against intoxications with organophosphates (Bošković et al., 1984) and carbamates (Harris et al., 1989). Yet again, its half and a double dose – 25 and 100 mg/kg were chosen to investigate the antidotal dose-dependency. The *im* LD_{50} of HI-6 in rats is 857 mg/kg (Bošković et al., 1984), which means that its dose range used in the present study (25–100 mg/kg) equals 2.9–11.67% of the corresponding LD_{50} value.

2.3.3. Biochemical analyses

Biochemical analytical methods were used in order to ascertain the cholinesterase activity in rat brain, diaphragm and whole blood, in animals intoxicated with physostigmine, while in animals poisoned with pyridostigmine brain cholinesterase was not studied, due to negligible passage of pyridostigmine through the blood-brain barrier.

In this series of experiments, rats were treated with 0.8 LD_{50} of physostigmine (1 mg/kg *sc*) or pyridostigmine (3.4 mg/kg *sc*) and, immediately thereafter, with HI-6 (50 mg/kg *im*). Control animals, instead of carbamates *sc* or HI-6 *im* or instead both of them, received saline 1 ml/kg. Rats, divided in groups of 4–8, were anaesthetised with ether and sacrificed by decapitation at following times after injections: 0, 5, 15, 30, 60, 120 and 240 min. Samples of 3 ml of blood were taken immediately after cutting the main neck arteries into the heparinized tubes, after which samples (100–150 mg) of muscular parts of left hemidiaphragm were excised. Following craniotomy, brain parts rostral from rombencephalon (excluding thus cerebellum, pons, medulla oblongata and medulla spinalis) were cut for further processing.

Brain tissue samples were weighed and transferred into a glass

homogeniser. After the addition of saline (4 ml/g of tissue), samples were homogenised for 30 s. Samples of diaphragm muscles were homogenised manually by grinding in a porcelain mortar with pestle after addition of saline (20 ml/g of tissue).

Test tubes with whole blood samples and heparin were carefully shaken in order to avoid haemolysis and blood clotting. This suspension was kept for determination of whole blood cholinesterase activity.

Brain and diaphragm total cholinesterase activity was determined spectrophotometrically (Ellman et al., 1961), by means of a method modified by Wilhelm (1968). Whole blood cholinesterase activity was determined titrimetrically (Augustinsson, 1971).

Statistical difference between LD_{50} values was tested according to the Litchfield and Wilcoxon (1949) computerized method. Data on AChE activity at certain points in time were compared by means of the analysis of variance (ANOVA) and Student t-test. Statistical significance was set at $p < 0.05$.

2.4. Ethics

All the experiments were carried out according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

3. Results

3.1. Anticholinergic monotherapies

In the first series of experiments protective ratios (PR) of the increasing doses of antimuscarinic drug atropine and antinicotinic drugs hexamethonium and D-tubocurarine were ascertained in rats poisoned with physostigmine or pyridostigmine (Figs. 1–3).

Atropine, in doses of 5, 10 and 20 mg/kg *im*, assured much higher PRs in physostigmine- than in pyridostigmine-intoxicated animals, producing a dose-dependent increase of PRs (10.87, 16.94 and 31.88, respectively). At the same time, in pyridostigmine-treated rats, atropine assured a very limited protection that even decreased with the increase of its dose (PRs 3.14, 2.6 and 1.82) (Fig. 1).

Increasing doses of hexamethonium (5, 10 and 20 mg/kg *im*) significantly protected rats from both carbamates, however, without sign of a dose-dependency. In physostigmine- and pyridostigmine-poisoned animals the range of PRs was 7.97–8.7 and 2.17–2.97, respectively (Fig. 2).

Treatment of rats with increasing doses of D-tubocurarine (5, 10 and 20 µg/kg *im*) protected them equally from physostigmine and pyridostigmine. There was no linear dose-dependency; rather, it seems that doses equal to 10 µg/kg *im* or higher reached a PR plateau (Fig. 3).

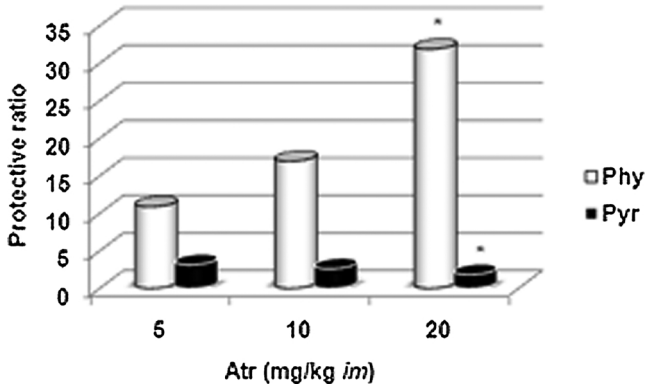

Fig. 1. Protective ratios (PRs) of three *im* doses of atropine (Atr) in rats poisoned with physostigmine (Phy) or pyridostigmine (Pyr) *sc*. Atr was injected immediately after Phy or Pyr. *p < 0.5 vs PR of Atr 5 mg/kg.

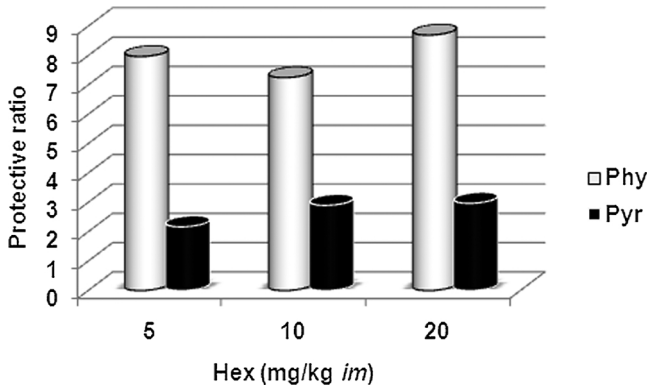

Fig. 2. Protective ratios of three *im* doses of hexamethonium (Hex) in rats poisoned with physostigmine (Phy) or pyridostigmine (Pyr) *sc*. Hex was injected immediately after Phy or Pyr.

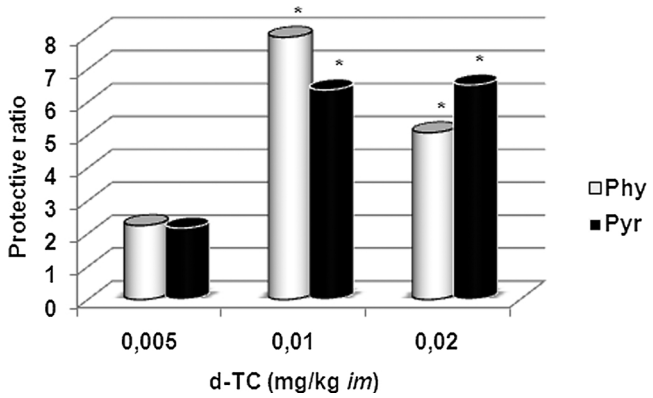

Fig. 3. Protective ratios (PR) of three *im* doses of d-tubocurarine (d-TC) in rats poisoned with physostigmine (Phy) or pyridostigmine (Pyr) *sc*. d-TC was injected immediately after Phy or Pyr. *p < 0.5 vs PR of d-TC 0.005 mg/kg.

3.2. Anticholinergic combinations

In further experiments in animals poisoned with either of the two carbamates PRs of combinations of the increasing doses of atropine (5, 10 and 20 mg/kg *im*) with increasing doses of hexamethonium (5, 10 and 20 mg/kg *im*) or D-tubocurarine (5, 10 and 20 µg/kg *im*) were obtained (Figs. 4–7).

All the atropine/hexamethonium combinations provided high PRs in physostigmine-intoxicated animals, with highest PR being 51.23, assured by the combination of atropine 10 mg/kg and hexamethonium

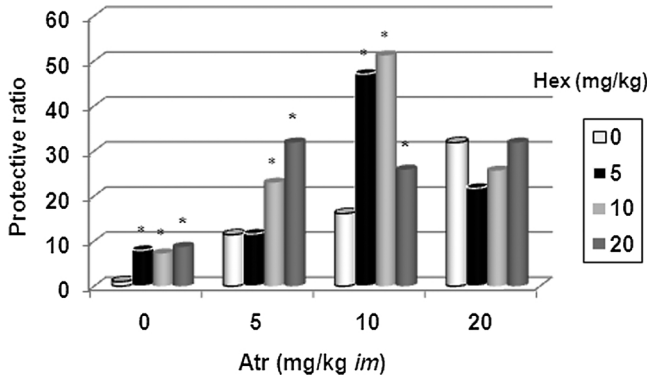

Fig. 4. Protective ratios (PRs) of combinations of three *im* doses of atropine (Atr) and three *im* doses of hexamethonium (Hex) in rats poisoned with physostigmine (Phy) *sc*. Atr and Hex were injected immediately after Phy. *p < 0.5 vs PR of the corresponding dose of Atr.

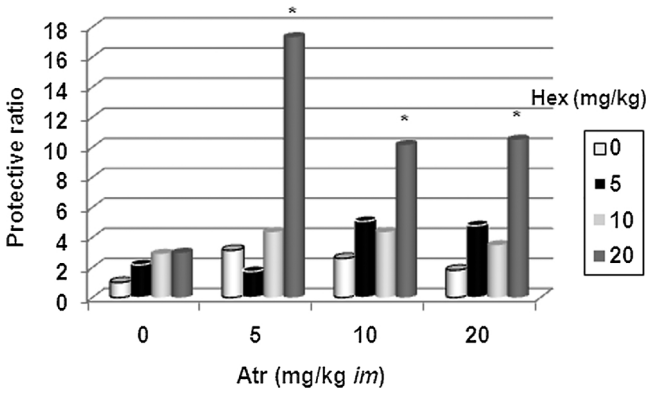

Fig. 5. Protective ratios (PRs) of combinations of three *im* doses of atropine (Atr) and three *im* doses of hexamethonium (Hex) in rats poisoned with pyridostigmine (Pyr) *sc*. Atr and Hex were injected immediately after Pyr. *p < 0.5 vs PR of the corresponding dose of Atr.

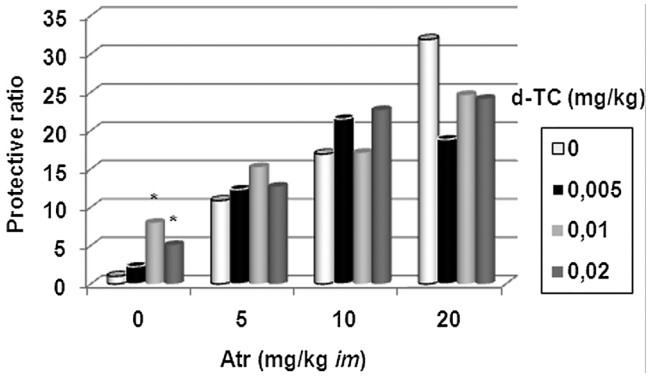

Fig. 6. Protective ratios (PRs) of combinations of three *im* doses of atropine (Atr) and three *im* doses of d-tubocurarine (d-TC) in rats poisoned with physostigmine (Phy) *sc*. Atr and d-TC were injected immediately after Phy. *p < 0.5 vs PR of the corresponding dose of Atr.

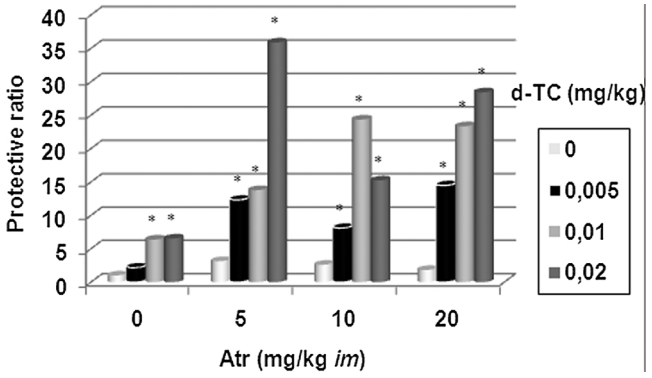

Fig. 7. Protective ratios (PRs) of combinations of three *im* doses of atropine (Atr) and three *im* doses of d-tubocurarine (d-TC) in rats poisoned with pyridostigmine (Pyr) *sc*. Atr and d-TC were injected immediately after Pyr. *p < 0.5 vs PR of the corresponding dose of Atr.

10 mg/kg. Addition of hexamethonium to the highest dose of atropine of 20 mg/kg did not increase its PR (Fig. 4).

In pyridostigmine-poisoned rats the same combinations of atropine and hexamethonium produced very different PRs. It is notable that only the highest dose of hexamethonium (20 g/kg) significantly potentiated PR of every dose of atropine, reaching in the case of atropine dose of 5 mg/kg the highest PR of 17.32. When added to atropine 10 and 20 mg/kg, it assured significant, but lower PRs of 10.15 and 10.50, respectively (Fig. 5).

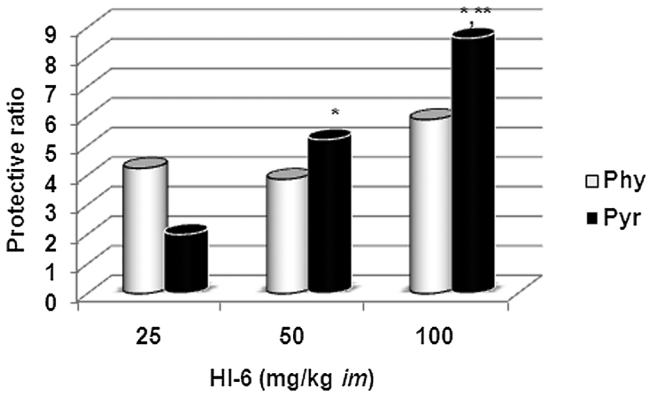

Fig. 8. Protective ratios (PRs) of three *im* doses of oxime HI-6 in rats poisoned with physostigmine (Phy) or pyridostigmine (Pyr) *sc*. HI-6 was injected immediately after Phy or Pyr. *p < 0.5 vs PR of HI-6 25 mg/kg, **p < 0.5 vs PR of HI-6 50 mg/kg.

Addition of D-tubocurarine (5, 10 and 20 μg/kg) had no significant effect on PRs of the increasing doses of atropine in rats intoxicated with physostigmine (Fig. 6).

At the same time, in animals poisoned with pyridostigmine, every dose of D-tubocurarine dose-dependently potentiated the effects of the increasing doses of atropine, with highest PR of 35.87 obtained by combination of atropine 5 mg/kg and D-tubocurarine 20 μg/kg (Fig. 7).

3.3. Oxime HI-6

In the following experiments HI-6 was administered as monotherapy (Figs. 8 and 9).

Increasing doses of HI-6 (25, 50 and 100 mg/kg *im*) resulted in different PRs in physostigmine- and pyridostigmine-poisoned rats. In case of physostigmine, only the lowest dose of HI-6 was more effective than in pyridostigmine-intoxicated animals; the other two doses resulted in PRs higher in pyridostigmine than in physostigmine-treated rats. In case of pyridostigmine, HI-6 showed a clear dose-dependent effect on survival, yielding the highest PR of 8.64 with the highest dose of 100 mg/kg (Fig. 8).

3.4. Tissue AChE activity

In next experiments the effect of oxime HI-6 on AChE activity of brain, whole blood and diaphragm in rats intoxicated with 0.8 LD_{50} of physostigmine (Figs. 9–11) and of diaphragm and whole blood in animals poisoned with equitoxic dose of pyridostigmine (Figs. 12 and 13) was investigated.

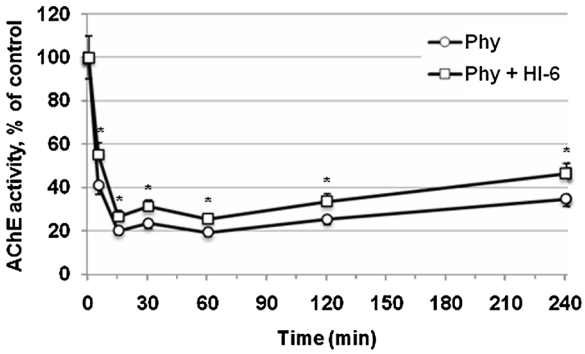

Fig. 9. Effect of physostigmine (0.8 LD_{50} *sc*) and HI-6 (50 mg/kg *im*) on brain acetylcholinesterase (AChE) activity in rats. Each point represents mean value of tissue AChE of six animals. Bars represent standard errors of the mean. *p < 0.05 vs Phy.

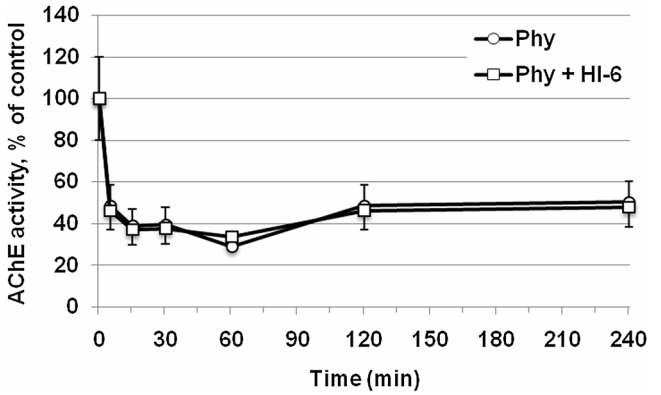

Fig. 10. Effect of physostigmine (0.8 LD_{50} *sc*) and HI-6 (50 mg/kg *im*) on whole blood acetylcholinesterase (AChE) activity in rats. Each point represents mean value of tissue AChE of six animals. Bars represent standard errors of the mean.

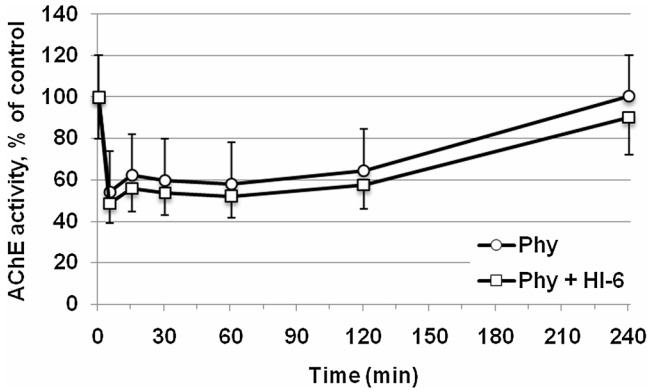

Fig. 11. Effect of physostigmine (0.8 LD_{50} *sc*) and HI-6 (50 mg/kg *im*) on diaphragm acetylcholinesterase (AChE) activity in rats. Each point represents mean value of tissue AChE of six animals. Bars represent standard errors of the mean.

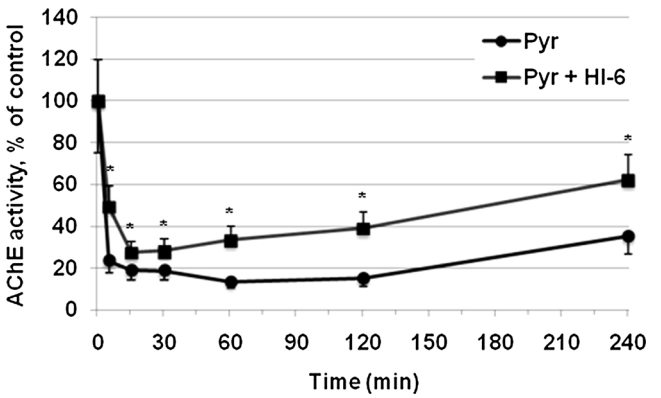

Fig. 12. Effect of pyridostigmine (0.8 LD_{50} *sc*) and HI-6 (50 mg/kg *im*) on diaphragm acetylcholinesterase (AChE) activity in rats. Each point represents mean value of tissue AChE of six animals. Bars represent standard errors of the mean. *p < 0.05 vs Pyr.

It is obvious that physostigmine induces strongest AChE inhibition in the brain, followed by the whole blood and diaphragm (Figs. 9–11).

It can be seen that high sublethal dose of physostigmine rapidly causes inhibition of brain AChE, bringing it to a minimum of just below 20% after 15 min. The same level of inhibition remains until 60 min after poisoning, after which slow spontaneous reactivation takes place, with AChE activity of slightly over 25% of the control after 240 min.

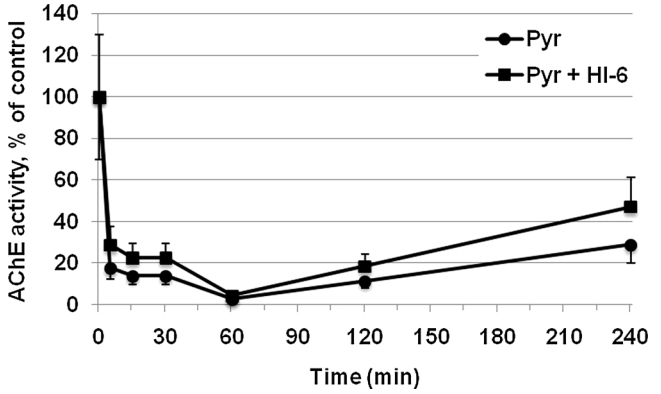

Fig. 13. Effect of pyridostigmine (0.8 LD_{50} *sc*) and HI-6 (50 mg/kg *im*) on whole blood acetylcholinesterase (AChE) activity in rats. Each point represents mean value of tissue AChE of six animals. Bars represent standard errors of the mean.

Animals treated with HI-6 demonstrated similar course of AChE inhibition, but with AChE values slightly but significantly higher than in rats that were treated with saline, instead of HI-6 (Fig. 9).

The dose of physostigmine used decreased AChE activity of whole blood less than in the brain, reaching the lowest level of 29% after 60 min and slowly recovering to 50% after 240 min. Oxime HI-6 did not offer any protection from physostigmine-induced AChE inbition (Fig. 10).

Potential of physostigmine to inhibit diaphragm AChE was the weakest among the three tissues studied; the remaining AChE activity was in the range of 54% and 58% of the control from 5th to 60th min after poisoning. The following spontaneous reactivation was complete, reaching the control values after 240 min. Again, HI-6 did not have any effect of this process (Fig. 11).

In case of pyridostigmine, only AChE activities in diaphragm and whole blood were monitored, since this carbamate does not pass the blood-brain barrier. Following a sharp decrease to about 24% of the control values after only 5 min, the lowest diaphragm AChE activities were found after 60 min – 13.6%. Thereafter, a gradual AChE activity recovery can be seen, with a maximum value of 35.42% after 240 min. In rats that received also HI-6, during the whole period a significantly less accentuated decrease in diaphragm AChE activity were seen, with values of 33.42% and 62.08% registered after 60 and 240 min, respectively (Fig. 12).

The effects of pyridostigmine and HI-6 on AChE activity in rat whole blood are shown in Fig. 13.

Injection of 0.8 LD_{50} of pyridostigmine resulted in a very sharp decrease in whole blood AChE activity that reached 17.64% as early as 5 min after the intoxication, with a gradual tendency to further decrease. The lowest AChE activity was registered after 60 min – only 2.78% of the control and was followed by a very slow spontaneous recovery, with the value of 28.89% after 240 min. At all times were the AChE values in animals treated with HI-6 numerically higher than in saline-treated rats, but the difference was not significant.

3.5. Atropine/HI-6 combinations

In the final series of experiments, PRs were calculated for combinations of atropine and HI-6 (Figs. 14–17).

Addition of the small dose of atropine (5 mg/kg) very significantly potentiated PRs obtained by HI-6 monotherapy (25, 50 and 100 mg/kg) in rats intoxicated with physostigmine. The range of HI-6 PRs without and with atropine was 3.94–6.03 and 16.19–20.95, respectively (Fig. 14).

In pyridostigmine-treated animals, this phenomenon was even more accentuated and clearly dose-dependent, with ranges of HI-6 PRs

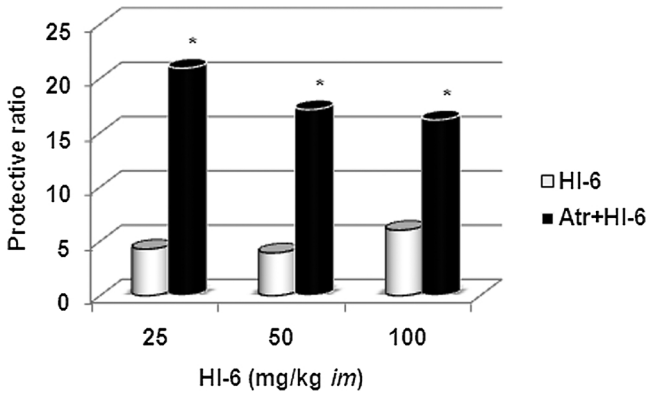

Fig. 14. Effect of atropine (Atr) 5 mg/kg *im* on protective ratios (PRs) of the increasing *im* doses oxime HI-6 in rats poisoned with physostigmine (Phy) *sc*. Atr and HI-6 were injected immediately after Phy. *p < 0.05 vs the PR of the corresponding dose of HI-6.

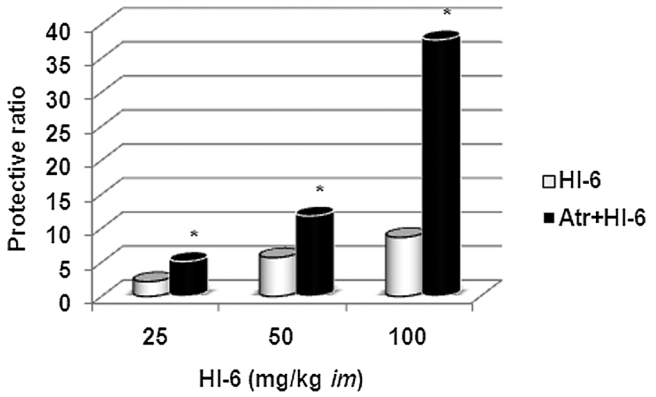

Fig. 15. Effect of atropine (Atr) 5 mg/kg *im* on protective ratios (PRs) of the increasing *im* doses oxime HI-6 in rats poisoned with pyridostigmine (Pyr) *sc*. Atr and HI-6 were injected immediately after Pyr. *p < 0.05 vs the PR of the corresponding dose of HI-6.

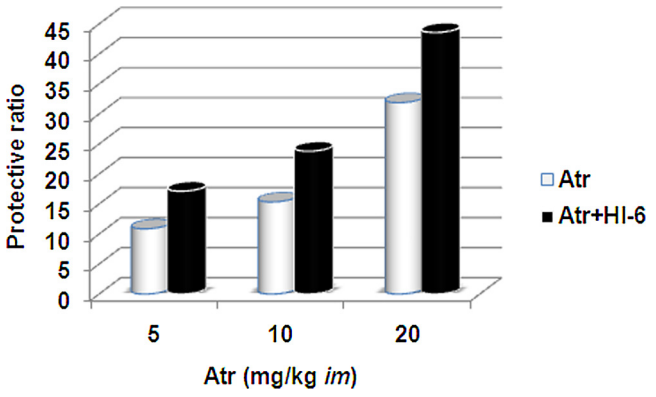

Fig. 16. Effect of oxime HI-6 50 mg/kg *im* on protective ratios (PRs) of the increasing *im* doses atropine (Atr) in rats poisoned with physostigmine (Phy) *sc*. Atr and HI-6 were injected immediately after Phy.

without and with atropine of 2.17–8.68 and 5.14–37.71, respectively (Fig. 15).

When adding HI-6 50 mg/kg to the increasing doses of atropine in physostigmine-poisoned rats, higher PRs were obtained in cases of combination of atropine/HI-6, but this difference was not significant (Fig. 16).

At the same time, HI-6 strongly potentiated the antidotal effects of atropine in pyridostigmine-intoxicated rats. It was demonstrated by the

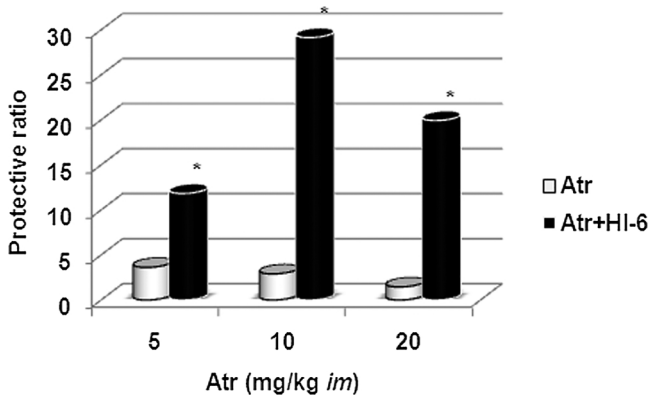

Fig. 17. Effect of oxime HI-6 50 mg/kg *im* on protective ratios (PRs) of the increasing *im* doses atropine (Atr) in rats poisoned with pyridostigmine (Pyr) *sc*. Atr and HI-6 were injected immediately after Pyr. *p < 0.05 vs the PR of the corresponding dose of Atr.

Table 2
Maximum protective ratios (MPRs) and maximum degrees of potentiation (MDPs) of the corresponding dose of atropine in rats poisoned with carbamates.

Treatment	Physostigmine		Pyridostigmine	
	MPR	MDP	MPR	MDP
Atropine	31.88	–	3.14	–
Atropine + hexamethonium	51.23	3.18	17.32	5.52
Atropine + D-tubocurarine	24.63	0.77	35.87	11.42
Atropine + HI-6	43.57	1.37	29.14	10.05

difference in the ranges of PRs without and with HI-6, which were 1.45–3.62 and 11.83–29.14, respectively (Fig. 17).

Table 2 contains data on the obtained maximum PRs for each antidotal combination and the maximum degree of potentiation (MDP) of the protective effect of atropine, which is considered as the basic part of therapy in carbamate intoxications.

It is obvious that atropine produced some 10 times higher PRs in physostigmine- than in pyridostigmine-poisoned animals. Although higher PR is obtained with the atropine/hexamethonium combinations in physostigmine than in pyridostigmine intoxication, the MDP is higher for the latter. Addition of D-tubocurarine in some cases even numerically decreases the PR of atropine in rats poisoned with physostigmine. On the contrary, in pyridostigmine-intoxicated rats, the addition of D-tubocurarine multiplied the atropine PR about 11 times. Very similar results were obtained with the oxime HI-6, where very high PR was obtained in case of physostigmine poisoning, but with a low potentiation of the effect of atropine monotherapy, while addition of HI-6 in pyridostigmine intoxication resulted in a 10-fold increase in PR of atropine (Table 2).

4. Discussion

To summarise, all the antidotes investigated – atropine, hexamethonium, D-tubocurarine and HI-6 – when given as monotherapy, provided some degree of protection against lethal effect of carbamates in rats. Bispyridinium oxime HI-6 induced a significant reactivation of AChE in the tissues crucial for each of the tested carbamates – in brain and diaphragm in case of physostigmine and pyridostigmine intoxication, respectively. Atropine *per se* and its combination with hexamethonium assured highest PRs in physostigmine-poisoned animals, while D-tubocurarine and HI-6 significantly potentiated the atropine PR in pyridostigmine-intoxicated rats.

4.1. Protective efficacy of atropine

Antidotal potential of an antimuscarinic depends on the type of a cholinesterase inhibitor and the rate and extent of its entering into the brain. In support of the first notion, it was shown in the present research that atropine protected animals much better in case of physostigmine than in case of pyridostigmine poisoning and that this difference only increased with the increase in the dose of atropine. Literature data suggest that atropine monotherapy gives much better results in carbamate than in organophosphate poisonings. Atropine's PR against physostigmine poisoning was reported to be 3.5 in rabbits (Fraser, 1870), 7.8 in guinea-pigs (Bethe et al., 1957), 9.3 and 7.2 in rats (Natoff and Reiff, 1973; Harris et al., 1989). The PRs for atropine in physostigmine intoxications in the present study were higher, due to higher doses of atropine employed - up to 20 mg/kg - vs. 17.4 mg/kg (Natoff and Reiff, 1973) and 8 mg/kg (Harris et al., 1989) and the different routes and timing of physostigmine and atropine injection. Atropine assured similar PRs in animals poisoned with yet another *N*-monomethyl carbamate, insecticide carbaryl – 6.5 and 6.6 (Natoff and Reiff, 1973; Harris et al., 1989). At the same time, in experimental animals intoxicated with organophosphates, atropine, irrespective of the injected dose, provided 3–4 times lower PRs, up to 2–2.5 (Holmstedt, 1959; Kords et al., 1968). The assumed reason for this higher potential of atropine in carbamate poisonings is the reversible character of cholinesterase inhibition induced by carbamates that makes the atropine's task less demanding.

Physostigmine is a lipophilic *N*-monomethyl carbamate that readily passes the blood- brain barrier (Deyi et al., 1981; Domino, 1987; Becker and Giacobini, 1988; Somani and Dube, 1989). As further proof of it, it was reported that anticholinergics with a quaternised N-atom cannot pass through the blood-brain barrier into the brain (Janowsky et al., 1985a) and that the more lipophilic anticholinergics protect better against lethal effects of physostigmine (Janowsky et al., 1984, 1985b, Stojiljković et al., 1989). Although atropine is by far the most frequently used centrally- and peripherally-acting antimuscarinic drug (Weger and Szinicz, 1981), its liphophilicity is only moderate, which impedes its access to the brain cholinoceptors (Bertram et al., 1977; Briggs and Simons, 1986). Finkelstein et al (1988) hypothesised that lower doses of atropine in humans (*e.g.* several milligrams) induced only peripheral muscarinic blockade and can antagonise only nausea and vomiting, among all the central cholinergic signs of intoxication, and even that only due to the lack of the blood-brain barrier around the haemoreceptor trigger zone in the medulla oblongata. Further in support to this notion, the increase in the dose of atropine did induce the linear increase in the PRs in the present study, implying that higher doses compensate for the relative low lipophilicity of atropine. It is quite possible that atropine prevents the occurrence of the cholinergically induced convulsions and central respiratory depression via the same mechanisms that this antimuscarinic drug does it in case of the nerve agent soman (Škrbić et al., 2017). As an additional proof to that, Janowsky et al. (1984) clearly showed that both atropine and a much more lipophilic antimuscarinic drug scopolamine afforded equal protection of mice intoxicated with one LD_{50} of physostigmine; however, in mice poisoned with higher dose of physostigmine, scopolamine was clearly more effective than the same number of milligrams of atropine.

Pyridostigmine, on the other hand, contains a quaternised N-atom and is lipophobic and therefore it cannot enter the brain, exerting its effects only outside the central nervous system (Birtley et al., 1966; Maxwell et al., 1988). In the present experiment, in pyridostigmine-poisoned rats, atropine assured only a marginal protection, similar to the ones in mice, reported by Parkes and Sacra (1954). They found that atropine 10 mg/kg *iv* assured PR of 2.16 in mice intoxicated with neostigmine *iv*. Moreover, even the doses of atropine up to 100 mg/kg *iv* could not produce better protection (Parkes and Sacra, 1954). In the present experiment, the obtained PR even decreased with the increase in the dose of atropine, which confirms the mentioned notion of Finkelstein et al. (1988) that even the lower doses of atropine saturate the peripheral muscarinic receptors. Therefore, further increase of the dose of atropine can only bring about the issue of its own toxicity and, like in our case, even significantly diminish the PR. Since the general *in vivo* lethality potential of atropine in rats is not high - the *im* LD_{50} was reported to be as high as 920 mg/kg (Lewis, 1996a) – higher doses of atropine in the present study tended to offer weaker protection against pyridostigmine poisoning because of some specific mechanism. One option might be the blockade of the neuromuscular presynaptic muscarinic receptors that enable acetylcholine to inhibit its own release from the nerve endings (Bowman et al., 1990). Indeed, Qiu et al (2001) showed that large concentrations of atropine could significantly diminish and even stop the contractions of the isolated rat diaphragm *in vitro*. *In vitro* studies have shown that both M1 and M2 muscarinic receptors exist in the presynaptic part of the neuromuscular synapse increasing and decreasing the release of acetylcholine, respectively, with the net effect depending on the functionality of AChE (Minić et al., 2002).

The aforementioned peripheral protective effect of atropine consists of blocking the muscarinic receptors primarily in the bronchial smooth muscles and exocrine glands, preventing thus the occurrence of the "pulmonary muscarinic syndrome", i.e. of bronchoconstriction and bronchorrhoea (Finkelstein et al., 1988; Caldwell et al., 1989). Atropine also blocks muscarinic receptors in the heart and endothelium, preventing in this way the occurrence of bradycardia, asystole and hypotension (Perera et al., 2008).

4.2. Protective effect of antinicotinic drugs

Like antimuscarinics, antinicotinics, when given as monotherapy, also fail to exert any protection from poisoning with organophosphate cholinesterase inhibitors, probably because of the too short duration of blockade of nicotinic receptors under the conditions of the permanent cholinesterase inhibition (Bošković and Stern, 1970). Based on the present experiments, it does not apply to carbamate intoxications, since even antinicotinic monotherapies yielded good PRs that are usually potentiated by the addition of atropine. However, the concrete efficacy depends both on the carbamate in question – predominantly centrally acting physostigmine or exclusively peripherally acting pyridostigmine - and on the choice of the antinicotinic – ganglionic nicotinic receptor blocker hexamethonium or antagonist of the neuromuscular nicotinic receptors D-tubocurarine.

Literature data support synergism between atropine and ganglionic blockers in animals intoxicated with physostigmine (Nose and Kojima, 1970; Niemegeers et al., 1982), neostigmine and organophosphates (Parkes and Sacra, 1954; Kords et al., 1968; Berry and Davies, 1970; Bošković and Stern, 1970; Chiou et al., 1986). It is obvious that the brain AChE is the main point of attack of physostigmine, since even the sublethal dose of this carbamate produced the 80% inhibition of the brain AChE and only less than 50% inhibition of the enzyme in the diaphragm. Similar results were reported by Deyi et al. (1981) and Maxwell et al. (1988). Significant potentiation of the atropine PRs obtained in the physostigmine-poisoned rats with hexamethonium – maximum PR of 51.23, with maximum degree of potentiation of 3.18 - could be explained rather by the protection of the neuromuscular than of the ganglionic nicotinic receptors. Large doses of hexamethonium (up to 20 mg/kg) might lose their selectivity for the ganglionic nicotinic receptors and are hypothesised to block the neuromuscular ones, as well (Parkes and Sacra, 1954). At the same time, use of large doses of atropine allows for tolerability of much higher doses of physostigmine that in turn inhibits even the peripheral cholinesterase and thus necessitates additional protection of neuromuscular nicotinic receptors. It should be however kept in mind that the experiments *in vitro* show that the concentration of hexamethonium necessary for the blockade of neuromuscular nicotinic receptors *in vitro* is 200-fold higher than the one needed for the ganglionic blockade (Wien et al., 1952). This

explanation, although tempting, is seriously challenged by the fact that in the present study, D-tubocurarine, a specific antagonist of the neuromuscular nicotinic receptors, contrary to hexamethonium, failed to afford any additional protection in rats poisoned with physostigmine and treated with atropine. In case of physostigmine, it might be that in the presence of large doses of hexamethonium some quantities manage to pass the blood-brain barrier and block the cerebral nicotinic receptors (Laurence and Stacey, 1953; Levine, 1959; Asghar and Roth, 1971; Tripathi et al., 1982), offering thus an explanation why the addition of hexamethonium, but not of D-tubocurarine, potentiates the atropine-induced protection in rats poisoned with physostigmine. This assumption is based on the findings that hexamethonium 5 mg/kg *iv* partially antagonises antinociceptive effect of nicotine that is of central origin (Tripathi et al., 1982) and that [^{14}C]-hexamethonium, after administration of 10 mg/kg *iv*, enters the central nervous system and penetrates especially the cortex and basal ganglia (Asghar and Roth, 1971). Central nicotinic receptors are responsible for the nicotine-induced seizures and this phenomenon can be abolished by the pretreatment with either hexamethonium (Laurence and Stacey, 1952) or a centrally acting antinicotinic agent, mecamylamine (Iha et al., 2017). At the same time, it seems that the blockade of central nicotinic receptors plays an important role in the protection of animals exposed to lethal doses of organophosphates or carbamate cholinesterase inhibitors (Fleisher et al., 1970; Klemm, 1983; Chiou et al., 1986).

It was to be expected that D-tubocurarine showed in the present study complete absence of the potentiation of the antidotal effects of atropine against physostigmine toxicity, since major point of attack for this carbamate is brain. Logically, in pyridostigmine poisoning, D-tubocurarine assured supra-additive synergism with atropine, with maximum PR of 35.87 and maximum degree of potentiation of 11.42. Similar synergism of atropine, hexamethonium and D-tubocurarine was described by Parkes and Sacra (1954). They noticed that the antidotal effects of hexamethonium and D-tubocurarine against neostigmine toxicity partly overlap, since triple combination atropine/hexamethonium/D-tubocurarine assured PRs similar to the ones after dual combinations. Reports by other authors indeed suggest that competitive neuromuscular relaxants can be successfully used to treat the so-called "peripheral nicotinic syndrome" in humans intoxicated with physostigmine or with some other cholinesterase inhibitor (Cumming et al., 1968; Poirier et al., 1987; Besser et al., 1990).

Since the doses of hexamethonium and D-tubocurarine used in present experiments are not sufficiently high to produce the neuromuscular blockade *per se*, a possible explanation for its effect might be the blockade of the presynaptic nicotinic receptors in the neuromuscular junction, the result of which is a decreased liberation of acetylcholine, a decrease in the postsynaptic membrane depolarisation and of the neuromuscular block (Haering et al., 1988; Hartman et al., 1988). Indeed, there are both nicotinic and muscarinic presynaptic receptors at the nerve endings of the myoneural synapse; the former being stimulatory for the release of acetylcholine and the latter being inhibitory (Vizi and Somogyi, 1989). Cholinesterase inhibitors, such as neostigmine, tend to abolish the tetanic contraction of skeletal muscles occurring normally as the consequence of repetitive nerve stimulations at a frequency of 50 Hz or higher (Chang et al., 1986) and this effect is antagonised by D-tubocurarine 15 μg/kg (Rump and Kaliszan, 1968), a dose within the range used in current study (5–20 μg/kg). In anaesthetised cats neostigmine induced augmentation of single twitches, muscle fasciculations and repetitive firing in the soleus muscle and antidromic firing in its nerve branches and all of these effects were antagonised not only by D-tubocurarine, but also by ganglionic-blocking doses of hexamethonium, suggesting a presynaptic effect on acetylcholine release (Webb and Bowman, 1974). Similar neostigmine-induced on train-of-four fade was obtained in the rat phrenic nerve-diaphragm preparation *in vitro*; the effect being antagonised by hexamethonium and potentiated with atropine and methoctramine, a selective M2 receptor antagonist (de Paula Ramos et al., 2014).

Composition of nicotinic acetylcholine receptors is fairly complex. Basically, the presynaptic nicotinic receptor responsible for higher acetylcholine release into the synaptic cleft in case of higher frequency stimulation was identified to be of neuronal type and containing two α3 and three β2 subunits. Postsynaptic skeletal muscle nicotinic receptors are divided in foetal $\alpha1\beta1\gamma\delta$ and adult $\alpha1\beta1\varepsilon\delta$ forms and they occur stoichiometrically with the ratio 2:1:1:1. Sometimes even neuronal α7 neuronal nicotinic receptor type can be found at the postsynaptic membrane (Fagerlund and Eriksson, 2009). *In vitro* studies suggested that the tetanic and train-of-four fade induced by nondepolarising neuromuscular relaxants, including D-tubocurarine, were results of their concentration-dependent antagonism of the presynaptic neuronal α3β2 receptors the result of which is the decreased release of acetylcholine into the synaptic cleft (Jonsson et al., 2006; Martyn et al., 2009).

4.3. Protective effect of oxime HI-6

There are three theoretical mechanisms of reactivation of carbamate-inhibited cholinesterase by HI-6 – direct interaction between the carbamate and the oxime, nucleophilic attack by the oxime on the O-bridge between the carbamyl-group of the carbamate and the active centre of the enzyme, and allosteric modulation. The direct *in vitro* interaction between an oxime and a carbamate is not a likely mechanism, since it could not be shown even in the case of the combination of carbaryl and 2-PAM, where the oxime definitely potentiated the toxicity of this *N*-monomethyl carbamate (Lieske et al., 1992). Other authors exclude the possibility of the nucleophilic attack on the carbamylated enzyme (Whiting and Byron, 1976; Dawson and Poretski, 1985), since it is much more characteristic for the mechanism of reactivation of cholinesterase irreversibly inhibited by organophosphates (Jokanović and Stojiljković, 2006; Jokanović, 2012). Therefore, it seems that the allosteric modulation remains the only available explanation for the oxime-induced decarbamylation, especially since increased rates of decarbamylation of physostigmine- or pyridostigmine-inhibited mammalian AChE can be induced by bispyridinium compounds lacking oxime groups (Dawson and Poretski, 1985).

Oxime monotherapy also achieves much better protective effects in case of carbamate than in case of organophosphate intoxications, which could be explained by the reversible, or pseudo-irreversible carbamate inhibition of AChE. The PRs produced by three doses of HI-6 in the present study were up to 6.03 and up to 8.68 in physostigmine- and pyridostigmine-poisoned rats, respectively. Sterri et al. (1979) reported somewhat lower PRs obtained with different oximes – obidoxime and P2S – 2 and 1.23 in physostigmine- and 3.13 and 2.64 in pyridostigmine-poisoned mice. All these results are in accordance with the notion that oximes reactivate cholinesterase and protect from lethality better in case of *N*, *N*-dimethyl carbamates, such as pyridostigmine than in case of *N*-monomethyl carbamates, such as physostigmine (Pelfrene, 1986). This is further confirmed by much higher maximum degree of potentiation of atropine obtained by HI-6 in the present study in pyridostigmine than in physostigmine intoxication (10.05 vs 1.37, respectively). The maximum PR of the atropine/HI-6 combination against physostigmine poisoning in rats in the present study was 20.95, which is close to PR of 23.3 reported by Harris et al. (1989). In addition, HI-6 and other bispyridinium oximes remain mainly outside the central nervous system, i.e. at the same side of the blood-brain barrier as pyridostigmine, which further explains its better efficacy in case of pyridostigmine- than in case of physostigmine-intoxicated rats. Although the mechanisms of antidotal action of HI-6 and D-tubocurarine differ, the fact that both the antidotes lack significant penetration into brain gives the plausible explanation why they are more effective against peripherally active carbamate pyridostigmine and suggests that the neuromuscular transmission is the function that they protect in case of AChE inhibition.

In vitro studies have shown that physostigmine and pyridostigmine have high carbamylation and decarbamylation constants in various

types of cholinesterases – AChE and butyrylcholinesterase from various animal species, including humans (Wetherell and French, 1991; Stojan and Zorko, 1997). Novel carbamates aimed at treatment of Alzheimer's disease, including the already approved rivastigmine, have higher constants of carbamylation and lower decarbamylation constants than the older carbamates physostigmine and pyridostigmine (Stojan and Zorko, 1997; Groner et al., 2007). Prolongation of the homologous row of the *N*-alkyl radicals in the molecule of *N,N*-dimethyl carbamates (Barak et al., 2009) or introduction of another alkyl substituent into the molecule of physostigmine that turns it into *N*-methyl, *N*-alkyl carbamate, results in a much more stable carbamate-AChE complex (Marta et al., 1992; Groner et al., 2007). It can be split *in vivo* in presence of an oxime, resulting in reactivation of AChE (Hoffman et al., 2009).

Oximes, and especially HI-6, significantly increase the rate of decarbamylation of physostigmine- or pyridostigmine-inhibited AChE (Harris et al., 1989; Eckert et al., 2008). Results on decarbamylation half-lives in case of these two carbamates and some other vary significantly depending on the species and tissue the AChE was taken from, as well as on the absence or presence of pyridinium oximes and on the *in vitro* experimental conditions. Eckert et al. (2008) studied human erythrocyte AChE in the conditions of the so-called dynamic and static models. They found spontaneous and HI-6-facilitated decarbamylation half-lives for physostigmine of 16.4 and 11.4 min respectively and for pyridostigmine and 26.9 and 22.0 min, respectively in the dynamic model. The corresponding values in the static model were 17.6 and 8.3 min for physostigmine and 29.0 and 18.2 min for pyridostigmine (Eckert et al., 2008). Since in the present study HI-6 was administered practically simultaneously with the carbamates, it cannot be ruled out that a portion of tissue AChE was protected from inhibition, since it was shown that the carbamylation constant of physostigmine was 2–3 times lower in the presence of oxime HI-6 (Dawson, 1994, 1995). It means that the oxime HI-6 probably binds to an allosteric site at the molecule of AChE, which results in slower carbamylation and faster decarbamylation of the active centre of the enzyme (Dawson and Poretski, 1985).

Based on the present biochemical experiments, it could be concluded that HI-6 exerts its antidotal effect by reactivating cholinesterase in organs crucial for survival – in rats intoxicated with physostigmine in brain and in animals poisoned with pyridostigmine in diaphragm. It is known that larger doses of oximes induce hypotension in various animal species, including non-human primates (Lipp and Dola, 1980). This effect is ascribed to their ganglion-blocking activity and this was confirmed when HS-6, an analogue of the oxime HI-6, blocked the hypotension induced by the electrical stimulation of the vagal nerve in cats (Lundy, 1978). It is therefore possible that HI-6 acts as antidote against carbamates by a combination of AChE-reactivating and hexamethonium-like effects. There are publications that accentuate that the antinicotinic potential of the oximes is important for survival, but not in ganglia, rather at the neuromuscular junction (Su et al., 1983). Electrophysiological studies showed that HI-6 blocks the open state of the sodium ion channel of these receptors, similarly to D-tubocurarine and hexamethonium, but with lower affinity (Alkondon et al., 1987; Alkondon and Albuquerque, 1989).

5. Conclusions

Increasing doses of atropine and their combination with hexamethonium assure excellent protection against physostigmine toxicity, while the best protection against pyridostigmine is provided by a strictly peripherally acting antinicotinic D-tubocurarine and bispyridinium oxime HI-6. The oxime acts as antidote against physostigmine and pyridostigmine poisoning by reactivating cholinesterase in the brain and diaphragm, respectively. Its protective effects and especially the potentiation of the basal atropine protection are much more accentuated in case of rats poisoned by pyridostigmine, since the actions of this carbamate are limited to the peripheral organs and tissues.

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Conflict of interest

None.

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