

The Evaluation of Benefit of Newly Prepared Reversible Inhibitors of Acetylcholinesterase and Commonly Used Pyridostigmine as Pharmacological Pretreatment of Soman-Poisoned Mice

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ABSTRACT

Aim: The ability of four newly prepared reversible inhibitors of acetylcholinesterase (6-chlorotacrine, 7-phenoxytacrine, compounds 1 and 2) and currently used carbamate pyridostigmine to increase the resistance of mice against soman and the efficacy of antidotal treatment of soman-poisoned mice was evaluated. **Methods:** The evaluation of the effect of pharmacological pretreatment is based on the identification of changes of soman-induced toxicity that was evaluated by the assessment of its LD₅₀ value and its 95% confidence limit using probit-logarithmical analysis of death occurring within 24 h after administration of soman. **Results:** 6-chlorotacrine was only able to markedly protect mice against acute toxicity of soman. In addition, the pharmacological pretreatment with 6-chlorotacrine or compound 2 was able to increase the efficacy of antidotal treatment (the oxime HI-6 in combination with atropine) of soman-poisoned mice. The other newly prepared reversible inhibitors of acetylcholinesterase (7-phenoxytacrine, compound 1) as well as commonly used pyridostigmine did not influence the efficacy of antidotal treatment. **Conclusion:** These findings demonstrate that pharmacological pretreatment of soman-poisoned mice can be promising and useful in the case of administration of 6-chlorotacrine and partly compound 2.

KEYWORDS

soman; reversible inhibitors of acetylcholinesterase; antidotes; pharmacological pretreatment; mice

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INTRODUCTION

The highly toxic organophosphorus compounds, called nerve agents, are still considered to be the most dangerous chemical warfare agents. They pose potential threats to both military and civilian populations, as evidenced in terroristic attacks in Japan (1). Organophosphorus nerve agents exert their toxic effects mainly by inhibiting acetylcholinesterase (AChE, EC 3.1.1.7) and subsequent accumulation of acetylcholine (ACh) in the central and peripheral nervous systems and stimulation of both muscarinic and nicotinic cholinergic receptors. Death occurs due to an acute cholinergic crisis, with signs and symptoms such as excessive salivation, lacrimation, urination, defecation, sweating, bronchoconstriction, neuromuscular block, generalized seizures, respiratory distress and respiratory failure (2–3).

The current standard treatment for poisoning by nerve agents consists of the combined administration of anticholinergic drugs such as atropine sulfate and AChE reactivators such as pralidoxime, obidoxime or HI-6. Generally, anticholinergics (mainly atropine) are used for relieving muscarinic signs and symptoms whereas AChE reactivators (generally nucleophilic compounds with high affinity for phosphorus), also called oximes, are used to repair the biochemical lesion by dephosphorylation of AChE and restoring its activity. Although the antidotes against nerve agents and organophosphorus insecticides have been developed based on the knowledge of above-mentioned basic mechanism of acute toxicity, their efficacy is limited (4–5).

One of the most resistant nerve agents is soman (pinacolyl methylfluorophosphonate). Its deleterious effects are extraordinarily difficult to counteract due to the very rapid aging of soman-inhibited AChE (2, 6). In addition, the main action of soman is in the central nervous system where the reactivating efficacy of all oximes is low owing to their limited penetration through blood-brain barrier (7–8). The unsatisfactory antidotal treatment available for acute nerve agent poisonings, especially in the case of soman, cyclosarin and tabun exposure, has brought another approach how to protect the humans from nerve agent-induced acute lethal toxic effects – using “pharmacological pretreatment” in the case the threat of exposure to nerve agents occurs. This approach generally represents the medical countermeasures applied relatively shortly before the penetration of a toxic agent into the organism with the aim of protecting the organism against the toxic drug and increasing the effects of post-exposure antidotal treatment. It appears from toxicodynamic point of view that prophylactic countermeasures can bring two main actions: protection of AChE against irreversible inhibition and antagonisation of the action of accumulated ACh. Thus, the pharmacological pretreatment allows survival and increase the resistance of organisms exposed to nerve agents as previously described (9–11).

Up to date, the most common principle of pharmacological pretreatment is the protection of AChE against nerve agent-induced irreversible inhibition that is focused on the use of reversible cholinesterase inhibitors. Among reversible inhibitors of AChE, the carbamate pyridostig-

mine bromide is generally accepted and commonly used for the pharmacological pretreatment of nerve agent poisonings. However, pyridostigmine is only able to protect peripheral AChE from irreversible nerve agent-induced AChE phosphorylation, while nerve agents, especially fluorophosphonates, can cross the blood-brain barrier (BBB) and, thus, express their deleterious effects through their central toxic effects including centrally mediated seizure activity that can rapidly progress to *status epilepticus* and finally contribute to brain damage (2).

Thus, the replacement of pyridostigmine bromide with sufficiently effective reversible inhibitors of AChE with low toxicity and ability to cross the blood-brain barrier has been an important goal for the pharmacological pretreatment of nerve agent poisonings because the small decrease of the brain AChE activity (up to 20%) was found to be beneficial for an increase in the efficacy of pharmacological pretreatment and does not affect the behavioral and neurophysiological functions of experimental animals according to our neurobehavioral research (12). Recently, four novel reversible inhibitors of AChE – 6-chlorotacrine (6-chloro-1,2,3,4-tetrahydroacridine-9-amine hydrochloride), 7-phenoxytacrine (7-phenoxy-1,2,3,4-tetrahydroacridine-9-amine hydrochloride), compound 1 (6-hydrazil-N-{6-[(7-methoxy-1,2,3,4-tetrahydroacridine-9-yl)amino]hexyl}pyridine-3-carboxamide hydrochloride) and compound 2 (6-hydrazil-N-{6-[(1,2,3,4-tetrahydroacridine-9-yl)amino]hexyl}pyridine-3-carboxamide hydrochloride) (Figure 1) were synthesized at our Department of Toxicology and Military Pharmacy to improve the efficacy of pharmacological pretreatment against nerve agents and potentially for the treatment of Alzheimer's disease. All newly prepared reversible inhibitors of AChE are tacrine-related compounds. Tacrine itself failed as a prophylactic agent because of small difference between pharmacologically effective and toxic doses. Therefore, we were searching for tacrine-related compounds with bigger differences between therapeutic and toxic doses.

In the present study, the influence of pyridostigmine and four newly prepared reversible inhibitors of AChE (6-chlorotacrine, 7-phenoxytacrine, compounds 1 and 2) on the resistance of soman-exposed mice and on the therapeutic efficacy of currently used antidotal treatment (the oxime HI-6 in combination with atropine) of soman-induced acute poisoning was compared.

MATERIALS AND METHODS

ANIMALS

Male NMRI mice weighing 18–22 g were purchased from VELAZ (Prague, Czech Republic). They were kept in an air-conditioned room (22 ± 2 °C and $50 \pm 10\%$ relative humidity, with lights from 7.00 hrs a.m. to 7.00 hrs p.m.) and allowed access to standard food and tap water *ad libitum*. The rats were divided into groups of eight animals ($N = 8$). Handling of experimental animals was done under the supervision of the Ethics Committee of the Faculty of Military Health Sciences in Hradec Králové (Czech Republic).

CHEMICALS

Soman was obtained from the Military Technical Institute in Brno (Czech Republic) and was 95.0% pure. Its purity was assayed by acidimetric titration. The purity of all reversible inhibitors of AChE (Figure 1) was higher than 98%. They were synthesized earlier at the Department of Toxicology and Military Pharmacy of the Faculty of Military Health Sciences in Hradec Kralove (Czech Republic)

with the exception of pyridostigmine bromide that was purchased from LITOLAB (Chudobin, Czech Republic). The purity of newly synthesized reversible inhibitors of AChE was analysed using HPLC. All other drugs and chemicals of analytical grade were obtained commercially and used without further purification. All substances were administered intramuscularly (i.m.) at a volume of 10 mL/kg body weight (b.w.).

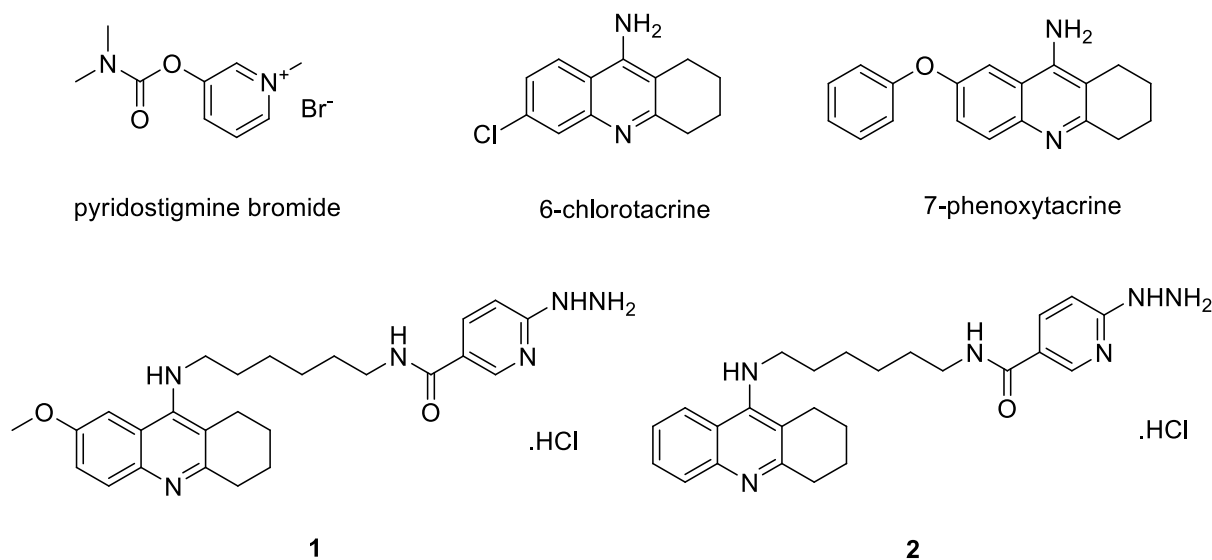


FIG. 1: Chemical structure of reversible inhibitors of AChE studied.

EVALUATION OF ACUTE TOXICITY OF REVERSIBLE INHIBITORS OF AChE

Before starting the evaluation of prophylactic efficacy of AChE reversible inhibitors, the acute toxicity of all tested inhibitors was evaluated in mice by the assessment of their LD_{50} values and their 95% confidence limits (CL) using probit-logarithmical analysis of death occurring within 24 hours after i.m. administration of each inhibitor at five different doses with eight animals per dose (13).

EVALUATION OF PROPHYLACTIC EFFICACY OF REVERSIBLE INHIBITORS OF AChE

To evaluate prophylactic efficacy of tested reversible inhibitors of AChE, the inhibitors (pyridostigmine, 6-chlorotacrine, 7-phenoxytacrine, compounds 1 and 2) were administered i.m. at doses corresponding to 5% of their LD_{50} values 30 minutes before i.m. soman challenge. The doses of tested reversible inhibitors of AChE were chosen to be sufficiently safe to avoid the potential adverse drug reactions in the peripheral as well as central compartment. Soman-induced toxicity was evaluated by the assessment of its LD_{50} value and its 95% confidence limit using probit-logarithmical analysis of death occurring within 24 h after administration of soman at five different doses with eight animals per dose (13). The efficacy of tested prophylactic drugs was expressed as protective ratio (LD_{50} val-

ue of soman in pretreated mice / LD_{50} value of soman in non-pretreated mice).

EVALUATION OF THE INFLUENCE OF REVERSIBLE INHIBITORS OF AChE ON THE THERAPEUTIC EFFICACY OF ANTIDOTAL TREATMENT

To evaluate the influence of reversible inhibitors of AChE on the therapeutic efficacy of antidotal treatment, all tested AChE reversible inhibitors were administered 30 minutes before soman poisoning while antidotal treatment (the oxime HI-6 at a dose corresponding to 5% of its LD_{50} in combination with atropine – 10 mg/kg) was carried out by i.m. injection 1 min after soman administration. Soman-induced toxicity was evaluated by the assessment of LD_{50} value and its 95% confidence limit using probit-logarithmical analysis of death occurring within 24 h after administration of soman at five different doses with eight animals per dose (13). The influence of tested prophylactic drugs on the antidotal treatment of soman poisoning was expressed as protective ratio A (LD_{50} value of soman in pretreated mice with antidotal treatment / LD_{50} value of soman in non-pretreated mice without antidotal treatment) and protective ratio B (LD_{50} value of soman in pretreated mice with antidotal treatment / LD_{50} value of soman in non-pretreated mice with antidotal treatment). The differences between LD_{50} values were considered to be significant when $p < 0.05$ (13).

RESULTS

The acute i.m. toxicity of all reversible inhibitors of AChE in mice is summarized in Table 1. The results show that the acute toxicity of newly prepared reversible inhibitors of AChE is quite different. While the acute toxicity of 7-phenoxytacrine, compound 2 and, especially, compound 1 is relatively low, the acute toxicity of 6-chlorotacrine is markedly higher although it is still lower than the acute toxicity of commonly used pyridostigmine. According to our results, the compound 1 seems to be the least toxic reversible inhibitor of AChE among all compounds studied.

A comparison of the prophylactic efficacy of pyridostigmine and all newly prepared reversible AChE inhibitors is presented in Table 2. Among all reversible inhibitors of AChE studied, 6-chlorotacrine was only able to markedly increase the resistance of experimental animals against acute toxicity of soman. Due to the prophylactic administration of 6-chlorotacrine, the LD₅₀ value of soman was increased from 49.5 µg/kg to 67.3 µg/kg. On the other hand, other reversible inhibitors of AChE including commonly used pyridostigmine were not able to influence the LD₅₀ value of soman.

A comparison of the benefit of pyridostigmine and all newly prepared reversible AChE inhibitors for the therapeutic efficacy of antidotal treatment of soman poisoning is presented in Table 3. Two newly prepared reversible inhibitors of AChE (6-chlorotacrine, compound 2) markedly increased the efficacy of the antidotal treatment of soman-poisoned mice consisting of the oxime HI-6 and at-

ropine. Due to the prophylactic administration of 6-chlorotacrine or compound 2, the protective ratio induced by antidotal treatment of soman poisoning was increased from 2.39 to 3.25, resp. 3.27. On the other hand, the prophylactic administration of other newly prepared reversible AChE inhibitors (7-phenoxytacrine, compound 1) as well as currently used pyridostigmine bromide did not influence the therapeutic efficacy of chosen antidotal treatment of soman-poisoned mice.

Reversible inhibitor of AChE	LD ₅₀ (mg/kg) ± 95% CL
Pyridostigmine bromide	3.24 (2.56–4.37)
6-chlorotacrine	10.08 (8.28–12.24)
7-phenoxytacrine	86.0 (61.0–109.1)
1	402.81 (314.7–513.0)
2	57.28 (46.5–78.0)

TAB. 1: Acute toxicity of reversible inhibitors of AChE in mice.

Pretreatment	LD ₅₀ (mg/kg) ± 95% CL	Protective ratio
–	49.5 (34.0–72.1)	–
Pyridostigmine bromide	47.0 (39.3–78.1)	0.95
6-chlorotacrine	67.3 (58.5–75.7)	1.36
7-phenoxytacrine	50.2 (38.5–65.7)	1.01
1	48.2 (35.1–63.1)	0.97
2	50.1 (34.6–54.0)	1.01

TAB. 2: Prophylactic effect of reversible inhibitors of AChE on the LD₅₀ value of soman in mice.

Pretreatment	Treatment	LD ₅₀ (mg/kg) ± 95% CL	Protective ratio A	Protective ratio B
–	–	92.3 (73.7–143.6)	–	–
–	HI-6 atropine	220.5 (189.8–297.3)*	2.39	–
Pyridostigmine bromide	HI-6 atropine	238.1 (202.4–309.4)*	2.58	1.08
6-chlorotacrine	HI-6 atropine	299.9 (240.8–408.2)*	3.25	1.36
7-phenoxytacrine	HI-6 atropine	196.7 (150.9–239.5)*	2.13	0.89
1	HI-6 atropine	201.4 (182.6–221.7)*	2.18	0.91
2	HI-6 atropine	302.0 (235.6–386.9)*	3.27	1.37

TAB. 3: The influence of pharmacological pretreatment on the effect of antidotal treatment on the LD₅₀ value of soman in mice. Statistical significance: * p < 0.05 (between non-pretreated and non-treated mice and pretreated and/or treated mice).

DISCUSSION

Sufficiently effective pretreatment is considered to be very important especially in the case of soman exposure because soman-induced deleterious effects are extraordinarily difficult to counteract due to very low reactivating efficacy of currently used oximes (14). The reason for the weak reactivating potency of the oximes is very rapid aging of phosphonylated AChE (15–16).

Better therapeutic effects of antidotal treatment can be achieved by pretreatment with reversible AChE inhib-

itors (9, 17). The protection of AChE against inhibition focused on the use of reversible AChE inhibitor is the most common principle of pharmacological pretreatment of nerve agent poisoning. Protection of AChE against inhibition – i.e. remaining intact AChE is a basic requirement for normal function of peripheral and central cholinergic nervous systems. Due to this pretreatment, the enzyme AChE became resistant to nerve agent-induced irreversible inhibition (18). It can be achieved by using reversible inhibitors (preferably carbamates) which are able to inhibit AChE reversibly with spontaneous recovery of the

activity (decarbamylation). Recovered activity of AChE serves as a source of the active enzyme (9). The reversible cholinesterase inhibitor pyridostigmine bromide, a well-known cholinesterase inhibitor, can be used for the treatment of Myasthenia gravis or for the prophylaxis against intoxication caused by organophosphorous nerve agents, especially against soman poisoning (19–21). Pyridostigmine is rapidly absorbed following oral administration determined as inhibition of the blood cholinesterases. The maximum inhibition is achieved 2–3 hours and lasts more than 8 hours. The half-life of inhibition is about 20 hours (18–19, 22). Based on these results, pyridostigmine was introduced into some armies as a prophylactic drug against nerve agents. Pyridostigmine bromide is stockpiled by various armed forces for pretreatment purpose against nerve agent poisoning and has been used by several thousand servicemen during UN operation against Iraq in 1991 (23). The important benefit of pyridostigmine bromide is the fact that it does not affect the combat readiness of the soldiers prior to nerve agent exposure and, therefore, it was chosen for the pretreatment of soldiers in the case of the threat of exposure to nerve agents. Nevertheless, our results demonstrate the shortage of effectiveness of pyridostigmine bromide alone to increase the resistance of nerve agent-exposed experimental animals (24). Its prophylactic effect is increased with its dose, however, higher doses cause more pronounced side effects. As pyridostigmine bromide is relatively toxic and the difference between its therapeutic and toxic dose is small, the safe dose for pharmacological pretreatment of nerve agent poisonings is limited and its prophylactic efficacy, when administered at safe doses, is unsatisfactory (25–26). In addition, it was demonstrated that exposure to physiologically relevant doses of pyridostigmine leads to neurobehavioral deficits and region-specific alterations in AChE and ACh receptors (27). It is known that commonly used pyridostigmine bromide as a prophylactic drug in the case of threat of exposure to nerve agents is able to protect just peripheral AChE. On the other hand, nerve agents, especially fluorophosphonates such as soman, sarin and cyclosarin, easily penetrate through BBB and markedly inhibit brain AChE. In addition, the currently used reactivators of nerve agent-inhibited AChE, monopyridinium or bispyridinium oximes poorly penetrate through BBB and, therefore, their ability to reactivate nerve agent-inhibited AChE in the brain is very limited. The usage of reversible inhibitors of AChE, that are able to cross the BBB, brings the protection of brain AChE from irreversible inhibition by nerve agents. This fact is important and useful for the increase of resistance of organism against nerve agents and the increase of the efficacy of post-exposure antidotal treatment. Of course, it is necessary to be careful with the dosage of centrally acting prophylactic drug. The doses of reversible inhibitors of AChE must be sufficiently safe to avoid peripheral as well as central adverse drug reactions and to maintain battle readiness of troops. Therefore, the searching for less toxic, more effective and centrally active reversible inhibitors of AChE seems to be rationale to increase the effectiveness of pharmacological pretreatment of nerve agent poisonings. During recent years, numerous alternative substances with known anti-cholinesterase

activity have been studied to evaluate their prophylactic efficacy in comparison with pyridostigmine bromide (28–31). Some of them are already in clinical use or have been developed as potential therapeutics for other indications such as Myasthenia gravis (32) or Alzheimer's disease (AD) (33–34).

One of the promising approaches how to find sufficiently effective reversible inhibitor of AChE for the pharmacological pretreatment of nerve agent poisonings is to develop substituted analogues of tacrine. Tacrine (9-amino-1,2,3,4-tetrahydroacridine) is a reversible inhibitor of AChE that was launched in 1993 as the first drug for the symptomatic treatment of AD (35). However, recent evaluations of the clinical effects of tacrine have confirmed the adverse events consisting in the elevated liver transaminase levels and in dose-related peripheral cholinergic effects (36). Therefore, its clinical use as a drug for the treatment of AD was discontinued. Substituents in position 6 of the tetrahydroacridine moiety exerted relative steric freedom and favorable electron-attracting effect that represents a possibility of a hydrophobic interaction between some amino acid residues and substituents in position 6 of tacrine in the active site of AChE. On the other hand, substituents in position 7 of the tetrahydroacridine moiety exerted detrimental steric effect that represents strongly negative contribution for their enzyme inhibitory activity (37). This conclusion corresponds to our results. While tacrine derivative substituted in the position 6 (6-chlorotacrine) due to its increased inhibitory potency towards AChE was able to increase the resistance of experimental animals against lethal toxicity of soman and to increase the therapeutic efficacy of recommended antidotal treatment of acute soman poisoning, the tacrine derivative with a substitution in the position 7 (7-phenoxytacrine) was not effective. Novel multi-target directed ligands (tacrine-pyridine-3-carboxamide hybrids) 1 and 2 were selected upon their IC_{50} values (1: $hAChE$ $IC_{50} = 3.81 \pm 2.4 \mu M$; 2: $hAChE$ $IC_{50} = 0.097 \pm 0.009 \mu M$) to confront the multifactorial nature of AD by combining $hAChE$ inhibitor with antioxidant action provided by hydrazinonicotinamide (38–39). Based on the inhibitory properties, compound 2 was able to increase the therapeutic efficacy of antidotal treatment of acute soman poisoning, while analogue of 7-methoxytacrine (compound 1) is not effective because the potency of 7-methoxytacrine to inhibit AChE is generally much lower compared to tacrine (7-methoxytacrine: $hAChE$ $IC_{50} = 10.00 \pm 1.0 \mu M$; tacrine: $hAChE$ $IC_{50} = 0.32 \pm 0.01 \mu M$) (40). Generally, the tacrine analogues exerting the sufficient prophylactic efficacy due to their potency to reversibly inhibit AChE in the peripheral and central nervous systems are more toxic. However, the acute toxicity of effective tacrine analogues studied is significantly lower compared to commonly used pyridostigmine and, therefore, their safe dose is higher and more effective.

CONCLUSION

In spite of some promising results in the case of prophylactic administration of 6-chlorotacrine and compound 2, the basic principle of pharmacological pretreatment of

nerve agent poisonings – the protection of AChE from nerve agent-induced irreversible inhibition by administration of reversible AChE inhibitors is somewhat limited, especially by relatively high toxicity of sufficiently effective reversible inhibitors of AChE. On the other hand, our results also show that it is possible to develop sufficiently effective, centrally acting reversible inhibitors of AChE that are more effective and less toxic drugs for pharmacological pretreatment of nerve agent exposure than commonly used pyridostigmine bromide.

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