The effect of HI-6 on cholinesterases and on the cholinergic system of the rat bladder

Ondrej Soukup [1], **Miroslav Pohanka** [1], **Gunnar Tobin** [3], **Daniel Jun** [1,2], **Josef Fusek** [1], **Kamil Musilek** [1], **Jan Marek** [1], **Jiri Kassa** [1], **Kamil Kuca** [1]

1. Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic
2. Center of Advanced Studies, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic
3. Institute of Neuroscience and Physiology, Department of Pharmacology, the Sahlgrenska Academy at Goteborg University, Sweden

Correspondence to: Kuca Kamil, PhD.
Centre of Advanced Studies and Department of Toxicology,
Faculty of Military Health Science, University of Defense
Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
TEL.: +420 973 251 523, FAX: +420 495 518 094
E-MAIL: kucakam@pmfhk.cz

ORIGINAL ARTICLE

Abstract

OBJECTIVES: The current standard treatment of organophosphate poisoning consists of an administration of anticholinergic drugs and cholinesterase reactivators (oximes). Oximes can react – except their reactivating effect on cholinesterases – directly with cholinoreceptors. HI-6 is an oxime that may have an inhibitory effect on the muscarinic receptors, too.

METHODS: In our work, we have investigated an influence of HI-6 on the acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and on the muscarinic receptors *in vitro*. The study was conducted using biosensor technique and on the rat bladder using *in vitro* test (tissue bath; methacholine as muscarinic agonist). IC50 for BChE from human serum was determined to be 1.01×10^{-6} M and for human erythrocytes AChE 3.31×10^{-6} M, respectively.

CONCLUSION: We assume that the demonstrated contractile response can be attributed to the inhibition of the AChE at the lower concentration and to a predominant inhibition of muscarinic receptor at higher concentration of compound tested.

INTRODUCTION

Organophosphates represent extremely toxic group of compounds. Members of this group can be both nerve agents (sarin, soman, cyclosarin, tabun and VX) and pesticides (paraoxon, chlorpyrifos, parathion). These compounds inhibit enzyme acetylcholinesterase (AChE, EC 3.1.1.7.) via its phosphorylation or phosphonylation at the serine hydroxyl group in its active site. Due to the inhibition, the enzyme is not able to fulfill its physiological role in the organism – splitting the neuromediator acetylcholine (ACh) at the synaptic clefts – and subsequently, ACh accumulates at the cholinergic synaptic junctions (Marrs 1993). The poisoning manifests as a cholinergic syndrom. Clinical effects of excessive stimulation of cholinoreceptors depend on their localization and the type of receptor. Muscarinic, nicotinic and central symptoms are typical for organophosphate poisoning (Bajgar 2004). Two main groups are used in the treatment of poisoning. (1) Anticholinergics like atropine are used as the functional antidotes. They are able to antagonize the effects of excessive ACh by a blocade of mainly muscarinic receptors.(2) Reactivators of AChE (causal antidotes) – oximes – are able to reactivate inhibited AChE (Kassa 2002). Anticholinergics and reactivators are usually administered together because of their synergistic effect (Kuca *et al*., 2007).There are a few compounds used it the treatment of organophosphate poisoning-pralidoxime, trimedoxime, methoxime, obidoxime and HI-6. However, the reactivators differ in their efficacy against individual nerve agent. No universal antidote has been developed yet. Besides these two main groups, anticonvulsant drugs (benzodiazepines) may be used (Marrs 1993).

HI-6 is a bisquaternary oxime that seems to be the most efficacious oxime for the antidotal treatment of acute poisonings because of the highest reactivating and therapeutic efficacy (Kassa 2002; Kassa *et al*., 2006). It is the very effective antidote against soman, sarin and VX (Kassa 2002) but it is less effective against tabun (Puu *et al*., 1986). The high therapeutic efficacy of HI-6, besides its reactivating potency, may be also due to other antidotal mechanisms based probably on direct anticholinergic actions (Hamilton and Lundy 1989). It has been discovered that HI-6 blocks the nicotinic receptor and therefore contribute to a recovery of diaphragm muscle from soman poisoning (Tattersall 1993). Loke *et al*., has also reported that pralidoxime related compounds without oxime group (weak cholinesterase inhibitors) have a protective effect after soman poisoning. This effect may be the outcome of interaction with muscarinic receptors (Loke *et al*., 2002).

There are five known distinct subtypes of muscarinic acetylcholine receptors (mAChRs) M_1-M_5. These receptors that mediate metabotropic actions belong to the G-protein coupled receptors. The distribution of individual subtypes differs in the whole body. Former studies showed that in the bladder, M2 and M3 subtypes are predominant (Abrams *et al*., 2006). Even though the M2 subtypes outnumber M3 subtype, it is mainly the M3 subtype that plays a key role in the contraction of the bladder. The M2 subtype seems to only enhance the contractile response to M3 receptor activation (Abrams *et al*., 2006).

The aim of this pilot study was to investigate the anticholinesterase effect of HI-6 on the AChE and BChE by the biosensor and the influence on the muscarinic receptors as well. The contraction study on a rat bladder has been chosen for this purpose.

MATERIALS AND METHODS

Methacholine, 2-acetyloxypropyl-trimethyl-azanium (Sigma Chemicals Co., St. Louis, MO, USA), HI-6 dichloride(1-[[[4(aminocarbonyl)-pyridinio]methoxy]methyl]-2(hydroxyimino)pyridinium dichloride) (Department of Toxicology, Faculty of Military Health Science, University of Defense, Hradec Kralove, Czech Republic).

The procedure to measure the actions of muscarinic agents in contracting rat bladder has been described previously by Giglio *et al*. (2007).

Sprague-Dawley rats (weighing 250–300g) were used. The rats were killed by carbon dioxide and the bladders were dissected and bladder strips (6×2mm) were prepared. The detrusor strip was then mounted by a thread between two steel rods where one was fixed and the other adjustable and connected to an isometric force transducer (Linton, Norfolk, UK) in 25 ml organ baths. The organ baths contained Krebs solution of the following composition (in mM): NaCl 118, KCl 4.6, $CaCl_2$ 1.25, KH_2PO_4 1.15, $MgSO_4$ 1.15, $NaHCO_3$ 25 and glucose 5.5 (all substances from Sigma Chemicals Co., St. Louis, MO, USA) , gassed with 5% CO_2 in O_2 and kept at 37 °C by a thermo-regulated water circuit and at pH 7.4. The detrusor strip was thereafter prestretched and let at rest for 30 min to get a stable tension of around 5 mN. Data were recorded using an MP100WSW data acquisition system and Acquire software (Biopac, Goleta, CA, USA). Agonist (methacholine) and antagonist (HI-6) administered to the organ baths were given at a volume of 200 µl and the concentrations mentioned in the text refer to the final ones in the organ bath chambers. Methacholine was given in a cumulative manner from the concentration of 10^{-7} M to 10^{-3} M and the next dose of methacholine was administered immediately when the maximal contractile response was observed. Antagonist was added 30 min prior the agonist.

Biosensor was prepared in the same manner as in the reference (Pohanka *et al*., 2007). Human erythrocytes AChE and human serum BChE were obtained from Sigma-Aldrich (Czech Republic). Enzymes were suspended into phosphate buffered saline (PBS). Final activity towards acetylthiocholine chloride (ATChCl) was adjusted up 600 ncat/ml (Ellman´s assay). After

that, glutaraldehyde and bovine serum albumin were admixed up 3% respectively 3 mg/ml. 2 μl of the mixture was split over the screen printed platinum working electrode (diameter 1 mm; purchased from BVT, Brno, Czech Rep.) and let to dry in a fridge.

Biosensor and Ag/AgCl reference electrode were immersed into stirred reaction cell (1 ml volume). Reaction medium consisted of solution with given concentration of HI-6 (10^{-2}–10^{-8} M) and 5 mM ATChCl. The applied voltage needing for produced thiocholine oxidation was set up at +0.45 V. Achieved current was recorded after 5 minutes stabilization. The achieved data were processed by software Origin 6.1. The Boltzmann function was found suitable for IC_{50} evaluation.

RESULTS

It is obvious that contractile response in the absence of the antagonist (basal response) showed a concentration-dependent curve. The highest contractile response was evoked by muscarinic agonist methacholine of the concentration 10^{-3} M (375,29%standard deviation-SD 0,919) whereas concentration of 10^{-7} should be considered as ineffective (103,94%, SD 0,01). Interestingly, not the clear concentration dependence could be observed in the presence of antagonist. HI-6 potentiates the contractile effect of methacholine with the maximal contractile response at the HI-6 concentration of 10^{-6} M. Observing the curves of methacholine of the most effective concentration – 10^{-4} M and 10^{-3} M – a certain "drop" in the contractile response at the concentration of HI-6 10^{-4} M and higher can be seen (Figure 1). Standard deviations of values are shown in Table 1.

IC_{50} for BChE from human serum was found 1.01×10^{-6} M and for human erythrocytes AChE 3.31×10^{-6} M.

DISCUSSION

Reactivator HI-6 seems to be a strong reversible inhibitor of cholinesterases. The inhibitory effect on BChE and AChE was very similar. However, AChE seems to be a little more resistant to inhibition caused by HI-6. On the other hand, the inhibition is completely reversible. Simple washing of biosensor and new performance in a fresh reaction medium with only buffered ATChCl provided current responding to a new biosensor. No hysteresis was observed.

Methacholine is a potent muscarinic agonist with a small effect on nicotinic receptors. It causes the contraction of the smooth muscles via IP_3 and Ca^{2+} (Collins and Crankshaw 1986). Comparing the contractile responses in the absence and in the presence of HI-6, the effect on muscarinic receptors can be observed.

Tentatively, since weak cholinesterases inhibitors have a protective effect against soman poisoning (Loke *et al.*, 2002) stronger inhibitors like HI-6 could have the same effect. Moreover we have observed modulatory effect on muscarinic receptors in the contraction study. This could lead us, that HI-6, besides the reactivation of AChE, can prevent from the excessive ACh in the junction via the direct inhibitory effect on the cholinoreceptors as it was described previously (Lundy and Tremblay 1979; Kuhnen-Clausen *et al.*, 1983; Tattersall 1993). Tentatively, obtained orientation results mean that HI-6 acts as both the inhibitor of AChE and the muscarinic receptors inhibitor. This could be demonstrated by the "drop" in the curves in Figure 1. This "drop" occurred in response to more effective doses of methacholine (i.e. not in the lower concentration) at the concentration of HI-6 of 10^{-6} M. We may suppose that in lower concentration of HI-6 the inhibitory effect on AChE predominates and at the higher doses than 10^{-6} M HI-6 seems to inhibit muscarinic receptors more significantly so a relative decrease in contraction could be observed.

Since HI-6 is soluble in the blood we suppose that is possible to expect the same effect on the bladder *in vivo* too. Moreover we can expect the similar effect on other peripheral tissues with smooth muscles, where the same receptors occur.

In conclusion, current study should be considered as a pivotal study that showed a possibility of testing modulators of the AChE (reactivators and inhibitors)
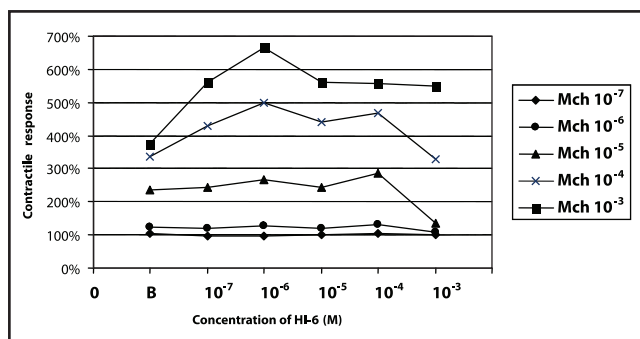

Figure 1. Contractile response to methacholine in the absence (B) and in the presence of HI-6. B stands for basal

Table 1. Standard deviations (SD) for all values

SD	Basal	HI-6 10^{-7}	HI-6 10^{-6}	HI-6 10^{-5}	HI-6 10^{-4}	HI-6 10^{-3}
Mch 10^{-7}	0.010	0.017	0.008	0.014	0.033	0.007
Mch 10^{-6}	0.007	0.085	0.237	0.158	0.193	0.081
Mch 10^{-5}	0.241	0.385	0.955	0.871	0.809	0.139
Mch 10^{-4}	0.652	1.183	2.317	1.937	1.739	0.954
Mch 10^{-3}	0.919	2.166	3.689	2.898	2.588	1.917

on the rat bladders with a possibility to distinguish the effect on the AChE and the muscarinic receptors. We assume that the response that has been demonstrated can be attributed to the inhibition of the AChE at the lower concentration and to a predominant inhibition of muscarinic receptor at higher concentration of HI-6. To confirm our hypothesis, more experiments using another tissues, agonists and antagonist will be performed. Also studies focused on the binding to the muscarinic receptors should be in future done.

ACKNOWLEDGEMENT

REFERENCES

1 Abrams P, Andersson KE, Buccafusco JJ, Chapple C, de Groat WC, Fryer AD, *et al.*, (2006). "Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder." Br J Pharmacol **148**(5): 565–78.
2 Bajgar J (2004). "Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment." Adv Clin Chem **38**: 151–216.
3 Collins SM and Crankshaw DJ (1986). "Dissociation of contraction and muscarinic receptor binding to isolated smooth muscle cells." Am J Physiol **251**(4 Pt 1): G546–52.
4 Giglio D, Aronsson P, Eriksson L and Tobin G (2007). "*In vitro* characterization of parasympathetic and sympathetic responses in cyclophosphamide-induced cystitis in the rat." Basic Clin Pharmacol Toxicol **100**(2): 96–108.
5 Grossmuller M, Antony J, Trankle C, Holzgrabe U and Mohr K (2006). "Allosteric site in M2 acetylcholine receptors: evidence for a major conformational change upon binding of an orthosteric agonist instead of an antagonist." Naunyn Schmiedebergs Arch Pharmacol **372**(4): 267–76.
6 Hamilton MG and Lundy PM (1989). "HI-6 therapy of soman and tabun poisoning in primates and rodents." Arch Toxicol **63**(2): 144–9.
7 Kassa J (2002). "Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents." J Toxicol Clin Toxicol **40**(6): 803–16.
8 Kassa J, Kuca K, Cabal J and Paar M (2006). "A comparison of the efficacy of new asymmetric bispyridinium oximes (K027, K048) with currently available oximes against tabun by *in vivo* methods." J Toxicol Environ Health A **69**(20): 1875–82.
9 Kuca K, Jun D, Musilek K and Bajgar J (2007). "Reactivators of Tabun-Inhibited Acetylcholinesterase:Structure-Biological Activity Relationship." Frontiers in Drug Design and Discovery(3): 381–394.
10 Kuhnen-Clausen D, Hagedorn I, Gross G, Bayer H and Hucho F (1983). "Interactions of bisquaternary pyridine salts (H-oximes) with cholinergic receptors." Arch Toxicol **54**(3): 171–9.
11 Loke WK, Sim MK and Go ML (2002). "O-Substituted derivatives of pralidoxime: muscarinic properties and protection against soman effects in rats." Eur J Pharmacol **442**(3): 279–87.
12 Lundy PM and Tremblay KP (1979). "Ganglion blocking properties of some bispyridinium soman antagonists." Eur J Pharmacol **60**(1): 47–53.
13 Marrs TC (1993). "Organophosphate poisoning." Pharmacol Ther **58**(1): 51–66.
14 Pohanka M, Kuca K and Jun D (2007). "Amperometric biosensor for pesticide methamidophos assay." Acta Medica (Hradec Kralove) **50**(4): 239–41.
15 Puu G, Artursson E and Bucht G (1986). "Reactivation of nerve agent inhibited human acetylcholinesterases by HI-6 and obidoxime." Biochem Pharmacol **35**(9): 1505–10.
16 Tattersall JE (1993). "Ion channel blockade by oximes and recovery of diaphragm muscle from soman poisoning *in vitro*." Br J Pharmacol **108**(4): 1006–15.
17 Trankle C, Dittmann A, Schulz U, Weyand O, Buller S, Johren K, *et al.*, (2005). "Atypical muscarinic allosteric modulation: cooperativity between modulators and their atypical binding topology in muscarinic M2 and M2/M5 chimeric receptors." Mol Pharmacol **68**(6): 1597–610.