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

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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 phosphorylation 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 syndrome. 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 blockade 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) M1–M5. 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-acetoxypropyl-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, CaCl2 1.25, KH2PO4 1.15, MgSO4 1.15, NaHCO3 25 and glucose 5.5 (all substances from Sigma Chemicals Co., St. Louis, MO, USA), gassed with 5% CO2 in O2 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 200μl 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 acetyltiocholine 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 IC₅₀ 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.

IC₅₀ 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 IP₃ and Ca²⁺ (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)

Table 1. Standard deviations (SD) for all values

<table>
<thead>
<tr>
<th>Concentration of HI-6 (M)</th>
<th>Mch 10⁻⁷</th>
<th>Mch 10⁻⁴</th>
<th>Mch 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.010</td>
<td>0.007</td>
<td>0.241</td>
</tr>
<tr>
<td>HI-6 10⁻⁷</td>
<td>0.017</td>
<td>0.085</td>
<td>0.385</td>
</tr>
<tr>
<td>HI-6 10⁻⁴</td>
<td>0.008</td>
<td>0.237</td>
<td>0.955</td>
</tr>
<tr>
<td>HI-6 10⁻³</td>
<td>0.014</td>
<td>0.158</td>
<td>0.871</td>
</tr>
<tr>
<td>HI-6 10⁻⁵</td>
<td>0.033</td>
<td>0.193</td>
<td>0.809</td>
</tr>
<tr>
<td>HI-6 10⁻⁶</td>
<td>0.007</td>
<td>0.081</td>
<td>0.109</td>
</tr>
<tr>
<td>HI-6 10⁻⁴</td>
<td>0.652</td>
<td>2.317</td>
<td>2.298</td>
</tr>
<tr>
<td>HI-6 10⁻³</td>
<td>2.166</td>
<td>3.689</td>
<td>2.588</td>
</tr>
</tbody>
</table>

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

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REFERENCES