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ORIGINAL ARTICLE

The effect of trimedoxime on acetylcholinesterase and on the cholinergic system of the rat bladder

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Summary
Trimedoxime is a bisquaternary oxime that is widely used in the treatment of organophosphorous poisoning caused by tabun and paraoxon. We tested its affinity to acetylcholinesterase (AChE), its mechanism of interaction and effect on the cholinergic system of the rat bladder. The half maximal inhibitory concentration (IC_{50}) of trimedoxime to recombinant AChE was found to be 82.0 mM ± 30.1 mM. This represents a weak inhibition. Its interaction with AChE seems to be very similar to obidoxime – one aromatic nucleus interacts with the peripheral anionic site and the other with the residues TYR337 and TYR341 inside the cavity. Also the oxime moiety is moving towards the catalytic triade ready for the reactivation of the inhibited AChE. In the organ bath experiment no significant effect of trimedoxime was observed on the contraction of the detrusor caused by the muscarinic agonist metacholine.

Key words: acetylcholinesterase; trimedoxime; antidote; muscarinic receptors; reactivation

INTRODUCTION

Organophosphorous compounds are an extremely toxic group of substances. Members of this group are nerve agents (sarin, soman, cyclosarin) and pesticides (paraoxon, chlorpyrifos, parathion). Some of them are used in agriculture (chlorpyrifos, paraoxon etc.), in human and veterinary medicine as drugs, and as military weapons (tabun, sarin, soman, cyclosarin), most notably as nerve agents. The mechanism of OP poisoning involves phosphorylation of the serine hydroxyl group in the active site of the acetylcholinesterase (AChE) (Marrs 1993). The poisoning manifests as a cholinergic syndrome, and the clinical effects of excessive stimulation of the cholinoreceptors depend on their localization and the type of receptor. Muscarinic, nicotinic and central symptoms are typical of organophosphate poisoning (Bajgar 2004). There are two main groups of treatment for the poisoning: (1) anticholinergics such as atropine are used as functional antidotes since they are able to antagonize the effects of excessive ACh by a blockade of mainly muscarinic receptors, and (2)

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reactivators of AChE – oximes – are able to reactivate inhibited AChE (Kassa 2002). Anticholinergics and reactivators are usually administered together because of their synergistic effect. There are a number of compounds used in the treatment of organophosphate poisoning – pralidoxime, trimedoxime, methoxime, obidoxime and HI-6 (Kassa et al. 2009, Kuča et al. 2009). However, the reactivators differ in their efficacy against individual nerve agents. No universal antidote has yet been developed (Kuča et al. 2007, Žďárová Karasová et al. 2009) and other mechanisms not related to the reactivation are being investigated.

Trimedoxime (Fig. 1) is a bisquaternary oxime that is widely used in the treatment of organophosphorous poisoning caused by tabun (Kassa 2002, Kassa et al. 2006). *In vitro* and *in vivo* testing have shown its effect as significantly greater than the others in the case of tabun poisonings (Cabal et al. 2004, Kassa et al. 2005). Moreover it has very good reactivation potency *in vitro* on paraoxon inhibited AChE (Jun et al. 2008). Trimedoxime (and obidoxime), although relatively toxic, are the most potent in inducing reactivation of AChE inhibited by the majority of organophosphorous pesticides. Since there are currently no reports of controlled clinical trials on the use of trimedoxime in human organophosphate pesticide poisoning, obidoxime is prefered in the treatment of pesticide poisoning (Antonijević and Stojiljković 2006).

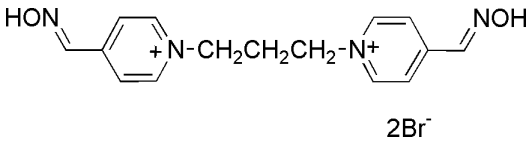


Fig. 1. **Structure of trimedoxime bromide.**

The high therapeutic efficacy of oximes, may in addition to its reactivating potency, be also due to other antidotal mechanisms based probably on direct anticholinergic actions (Hamilton and Lundy 1989). It has been discovered that oximes (HI-6) block the nicotinic receptor and thus contribute to a recovery of the diaphragm muscle from soman poisoning (Tattersall 1993). Moreover examination of the cholinolytic effect of trimedoxime has shown that a blockade of open end-plate ionic channels underlies the reactivating effect (Giniatullin et al. 1988).

The aim of this study was to investigate the binding of trimedoxime to the AChE using docking simulation, its inhibitory effect on AChE using Ellman's method and also its influence on the whole tissue containing muscarinic receptors. A contraction study on rat bladder has been chosen for this purpose.

MATERIALS AND METHODS

Chemicals

Trimedoxime: TMB-4, 1,3-bis(4-hydroxyimino-methylpyridinium)propane dibromide previously synthesized at the Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Králové, Czech Republic. Commercial recombinant AChE (Sigma-Aldridge, Czech Republic), acetylthiocholine, 5,5'-dithiobis(2-nitrobenzoic) acid (all Sigma-Aldridge, Czech Republic), methacholine: 2-acetyloxypropyl-trimethyl-azanium (Sigma-Aldridge, Czech Republic).

The assessment of AChE IC_{50}

The activity of AChE was assessed by the standard spectrophotometric Ellman's method with acetylthiocholine iodide as substrates and use of 5,5'-dithiobis(2-nitrobenzoic) acid as chromogen (Ellman et al. 1961); modified in wavelength (412 nm). The spectrophotometer Helios Alpha (Thermo Scientific, Czech Republic) was used for determination of absorbance. The inhibitor (K112) was added 10 min prior to measurement. The results were calculated using the Origin 6.1 software (OriginLab Corporation, Northampton, USA).

Docking studies

Docking studies were performed using the AUTODOCK 3.0.5 (Morris et al. 1998) to deduce conformation and orientation of trimedoxime in AChE. The initial model of AChE (*Mus musculus*, mAChE) for docking studies was built based on the X-ray crystal structure of the obidoxime – AChE complex which was obtained from the Protein Data Bank (PDB entry 2GYW) (Ekström et al. 2006). The original ligand was removed while water molecules present in the PDB file were maintained in their position. Residues with missing side chain atoms were rebuilt using the Swiss-PdbViewer 4.0. Hydrogen atoms were added to all amino acid residues. The search and scoring grid for Autodock was centered in the cavity. The grid size was set to 90 × 90 × 90 points with a spacing of 0.3 Å. The Lamarckian genetic search algorithm was performed 200 times by using a population size of 500, an upper limit for the number of energy evaluations of 5 000 000 and the maximum number of generations were set to 300 000. The parameters for mutation and crossover were kept at their default settings of 0.02 and 0.80, respectively. The local-energy-minimization algorithm was limited to 100 steps for 6% of the population. To explore the conformational space of

ligands, the overall translation steps was set to 0.2 Å, and the overall rotation and torsion rotation step were set to 5 in the docking studies. RMS tolerance during the clustering was set to 1.5 Å to reduce the number of clusters. All other values were used at default settings. VMD software (NIH Center for Research Resources, University of Illinois, USA) was used for structure drawing.

Contraction study

The procedure has been described previously (Giglio et al. 2007). In the current study, Sprague-Dawley rats (250–300 g) were used. The rats were killed by carbon dioxide and the bladders were dissected out and bladder strips (6 × 2 mm) were prepared. The detrusor strips were 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) in 25 ml organ baths. The organ baths contained Krebs solution with 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 strips were thereafter pre-stretched and let at rest for 30 min to produce a stable tension of around 5 mN. Data were recorded using an MP100WSW data acquisition system and Acquire software (Biopac, Goleta, USA). Methacholine and trimedoxime were administered to the organ baths at a volume of 200 µl and the concentrations mentioned in the text refer to the final ones in the organ bath chambers. The methacholine concentration was increased in a sequential manner from 10^{-6} M to 10^{-4} M. A subsequent dose of methacholine was added immediately when the maximal contractile response to the previous administration could be observed. Trimedoxime was added to the bath 30 min prior to the administration of the agonist. Student's t-test (two populations) has been applied.

RESULTS

IC_{50} for recombinant AChE was found 82.0 mM ± 30.1 mM (Fig. 2).

We selected the crystal structure of the complex Obidoxime-AChE for the docking study since obidoxime and trimedoxime (TMB-4) are analogical compounds. A similar interaction was expected. A redocking procedure was used in order to verify that the docking parameters specified in the input file for

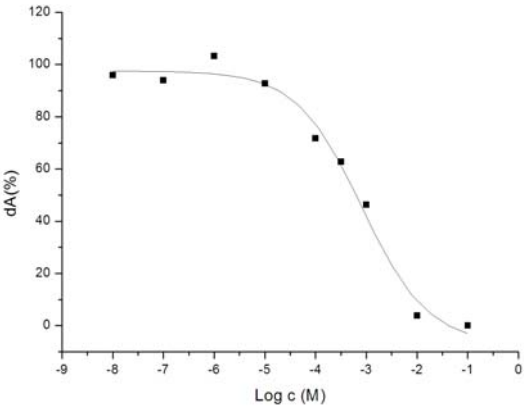
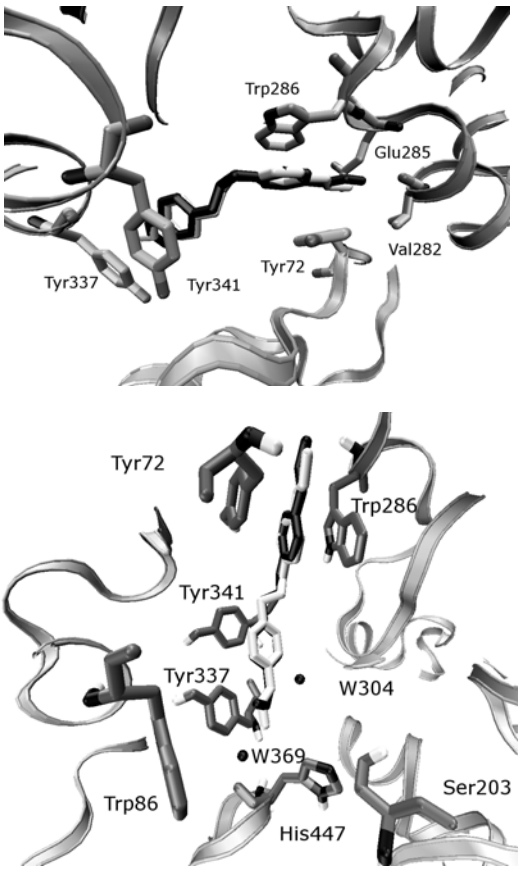
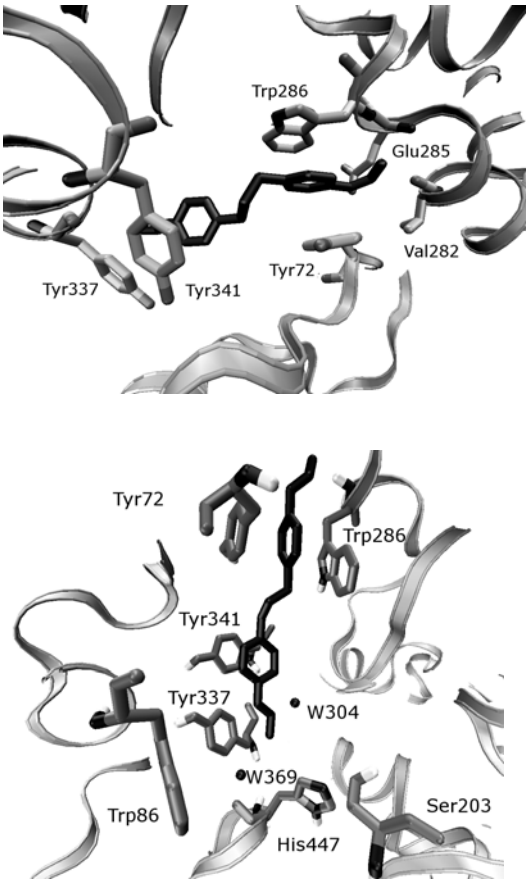


Fig. 2. **Inhibitory potency of trimedoxime on AChE.**



Figs 3, 4. **Comparison of the obidoxime gained from the crystal structure (white) with the results of docking (black).** The enzyme (PDB entry 2GYW) is represented by the grey ribbon, selected residues by tubes, water molecules are shown as black spheres.



Figs 5, 6. **Selected geometry of trimedoxime in the AChE obtained by AutoDock simulation.** The interaction between the Peripheral anionic site (PAS; TRP286, ASP74, TYR124, TYR72) of mAChE and trimedoxime (A), and the corresponding interaction with the catalytic site (SER203 and TRP86) shown in (B). The enzyme is represented by grey ribbon, selected residues by tubes, water molecules are shown as black spheres.

docking method are correct and able to recover a known complex's structure and interactions (Figs 3, 4). Docking studies show that the peripheral pyridinium ring is sandwiched via cation-π interactions with the side chains of Tyr124 and Trp286. The oxime moiety is able to form H-bond to the carbonyl oxygen of Val282, to the main-chain nitrogen of Trp286 or of Glu285 according to actual conformation. The central chain of trimedoxime is accommodated in the active-site gorge. The pyridinium ring that carries the attacking oxime forms cation-π interactions with the aromatic surface that is created by the side chains of Tyr337 and Tyr341.The oxime oxygen of trimedoxime can form the H-bond to the water molecule W369 or to the W304 (Figs 5, 6).

Contractions of the detrusor showed a concentration-dependent increase with the largest response to methacholine 10^{-3} M. However, trimedoxime does not cause any significant changes – according to Student's t-test – during the methacholine-evoked contractions (Fig. 7).

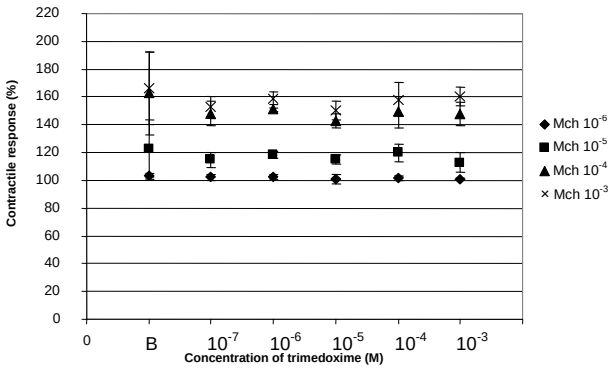


Fig. 7. **Effect of trimedoxime on methacholine-evoked contraction.** B stands for basal response – in the absence of trimedoxime.

DISCUSSION

Trimedoxime is a potent reactivator of AChE which also seems to be a weak reversible inhibitor of AChE. Compared to classic AChE inhibitors like donepezil or tacrine (Ogura et al. 2000) it is a much weaker inhibitor and compared to the reactivator HI-6, trimedoxime is also weaker (Soukup et al. 2008). This might be an advantage when comes to organophosphorous poisoning, where a blockade of AChE by organophosphates occurs.

Docking studies confirm the hypothesis that the interaction with AChE is similar to obidoxime. One aromatic nucleus interacts with the PAS and the other with the residues TYR337 and TYR341 inside the cavity. The oxime moiety is headed for the catalytic triade ready for reactivation of the inhibited AChE. However, obidoxime has been reported to be of higher affinity to AChE than trimedoxime.

Methacholine is a potent muscarinic agonist with also a slight effect on nicotinic receptors. It causes the contraction of the smooth muscles via IP_3 and Ca^{2+} (Collins and Crankshaw 1986). In the rat bladder cholinergic system M2 and M3 subtypes are predominant. Even though the M2 subtypes outnumber the M3 subtype, it is mainly the M3 subtype that plays a key role in the contraction of the

bladder. The M2 subtype seems only to enhance the contractile response to the M3 receptor activation (Abrams et al. 2006).

In the tissue bath experiment, the effect of trimedoxime on the whole cholinergic system (AChE and muscarinic receptors) should have been evident. However, comparison of the contractile responses in the absence and in the presence of trimedoxime, found no significant effect. Interestingly, after use of a similar reactivator – HI-6 – in the same *in vitro* experiment it was found that, at low concentrations an inhibitory effect on AChE was observed, and at higher concentrations an inhibitory effect on muscarinic receptors predominated (Soukup et al. 2008). This might be due to a low inhibitory potency on AChE or due to a balanced effect on AChE and on muscarinic receptors.

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REFERENCES

Abrams P, Andersson KE, Buccafusco JJ, Chapple C, de Groat WC, Fryer AD, Kay G, Laties A, Nathanson NM, Pasricha PJ, Wein AJ: Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. Br J Pharmacol 148:565–578, 2006.

Antonijević B, Stojiljković MP: Unequal efficacy of pyridinium oximes in acute organophosphate poisoning. Clin Med Res 5:71–82, 2006.

Bajgar J: Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. Adv Clin Chem 38:151–216, 2004.

Cabal J, Kuča K, Kassa J: Specification of the structure of oximes able to reactivate tabun-inhibited acetylcholinesterase. Basic Clin Pharmacol Toxicol 95:81–86, 2004.

Collins SM, Crankshaw DJ: Dissociation of contraction and muscarinic receptor binding to isolated smooth muscle cells. Am J Physiol 251:G546–552, 1986.

Ekström F, Pang YP, Boman M, Artursson E, Akfur C, Börjegren S: Crystal structures of acetylcholinesterase in complex with HI-6, Ortho-7 and obidoxime: Structural basis for differences in the ability to reactivate tabun conjugates. Biochem Pharmacol 72:597–607, 2006.

Ellman GL, Courtney KD, Andres V, Featherstone RM: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95, 1961.

Giglio D, Aronsson P, Eriksson L, Tobin G: *In vitro* characterization of parasympathetic and sympathetic responses in cyclophosphamide-induced cystitis in the rat. Basic Clin Pharmacol Toxicol 100:96–108, 2007.

Giniatullin RA, Shabunova IA, Nikolskii EN, Bukharaeva EA: Reactivating and cholinolytic action of trimedoxime bromide at the neuromuscular-junction of warm-blooded animals. Neurophysiology 20:256–260, 1988.

Hamilton MG, Lundy PM: HI-6 therapy of soman and tabun poisoning in primates and rodents. Arch Toxicol 63:144–149, 1989.

Jun D, Musilová L, Kuča K, Kassa J, Bajgar J: Potency of several oximes to reactivate human acetylcholinesterase and butyrylcholinesterase inhibited by paraoxon *in vitro*. Chem Biol Interact 175:421–424, 2008.

Kassa J: Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. J Toxicol Clin Toxicol 40:803–816, 2002.

Kassa J, Kuča K, Cabal J: Comparison of the efficacy of currently available oximes against tabun in rats. Biologia (Bratisl) 60:77–79, 2005.

Kassa J, Kuča K, Cabal J, Paar M: 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 69:1875–1882, 2006.

Kassa J, Žďárová Karasová J, Tesařová S: Evaluation of the neuroprotective efficacy of individual oxime (HI-6) and oxime mixtures (HI-6 + trimedoxime, HI-6 + K203) in tabun-poisoned rats. J Appl Biomed 7:189–199, 2009.

Kuča K, Jun D, Bajgar J: Currently used cholinesterase reactivators against nerve agent intoxication: Comparison of their effectivity *in vitro*. Drug Chem Toxicol 30:31–40, 2007.

Kuča K, Musílek K, Jun D, Pohanka M, Žďárová Karasová J, Novotný L, Musilová L: Could oxime HI-6 really be considered as "broad-spectrum" antidote? J Appl Biomed 7:143–149, 2009.

Marrs TC: Organophosphate poisoning. Pharmacol Ther 58:51–66, 1993.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ: Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J Comput Chem 19:1639–1662, 1998.

Ogura H, Kosasa T, Kuriya Y, Yamanishi Y: Comparison of inhibitory activities of donepezil and other cholinesterase inhibitors on acetylcholinesterase and butyrylcholinesterase *in vitro*. Methods Find Exp Clin Pharmacol 22:609–613, 2000.

Soukup O, Pohanka M, Tobin G, Jun D, Fusek J, Musílek K, Marek J, Kassa J, Kuča K: The effect of HI-6 on cholinesterases and on the cholinergic system of the rat bladder. Neuro Endocrinol Lett 29:759–762, 2008.

Tattersall JE: Ion channel blockade by oximes and recovery of diaphragm muscle from soman poisoning *in vitro*. Br J Pharmacol 108:1006–1015, 1993.

Žďárová Karasová J, Bajgar J, Novotný L, Kuča K: Is a high dose of Huperzine A really suitable for pretreatment against high doses of soman? J Appl Biomed 7:93–99, 2009.