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Cholinergic properties of new 7-methoxytacrine-donepezil derivatives

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Abstract. Organophosphorus nerve agents inhibit acetylcholinesterase (AChE) which causes the breakdown of the transmitter acetylcholine (ACh) in the synaptic cleft. Overstimulation of cholinergic receptors (muscarinic and nicotinic) by excessive amounts of ACh causes several health problems and may even cause death. Reversible AChE inhibitors play an important role in prophylaxis against nerve agents. The presented study investigated whether 7-methoxytacrine (7-MEOTA) and 7-MEOTA-donepezil derivatives can act as central and peripheral reversible AChE inhibitors and simultaneously antagonize muscarinic and nicotinic receptors. The possible mechanism of action was studied on cell cultures (patch clamp technique, calcium mobilization assay) and on isolated smooth muscle tissue (contraction study). Furthermore, the kinetics of the compounds was also examined. CNS availability was predicted by determining the passive blood-brain barrier penetration estimated *via* a modified PAMPA assay. In conclusion, this study provides promising evidence that the new synthesized 7-MEOTA-donepezil derivatives have the desired anticholinergic effect; they can inhibit AChE, and nicotinic and muscarinic receptors in the micromolar range. Furthermore, they seem to penetrate readily into the CNS. However, their real potency and benefit must be verified by *in vivo* experiments.

Key words: Acetylcholinesterase inhibitors — Nicotinic receptors — Muscarinic receptors — Bladder — Prophylaxis

Introduction

As recently evidenced in Syria, organophosphorus nerve agents such as sarin, soman or VX can still be a life-threat for the general public. They cause inhibition of acetylcholinesterase (AChE, 3.1.1.7), which is the enzyme responsible for the decomposition of the transmitter acetylcholine (ACh) in the synaptic cleft. AChE inhibition causes accumulation of ACh in the synaptic cleft and thereby overstimulation of nicotinic (nAChR) and muscarinic (mAChR) receptors. Muscarinic symptoms (blurred vision, hypersecretion in the airways, sweating, bronchoconstriction, gut hypermo-

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tility, etc.) and nicotinic symptoms (weakness and muscle fasciculation, tachycardia and hypertension) arise within a few minutes after intoxication. Effects in the CNS are usually non-specific and include headache, anxiety, confusion etc. (Bajgar 2005).

Besides treatment involving the combined administration of atropine and oxime reactivators (Kassa et al. 2002), prophylaxis against nerve agents is another approach. Reversible AChE inhibitors play the most important role in this approach. In general, the carbamate pyridostigmine (bromide) is the most common agent used by army personnel worldwide. The armed forces of the Czech Republic use PANPAL, containing pyridostigmine in combination with two anticholinergics – benactyzine and trihexyphenidyl. The latter two compounds prevent the adverse effects caused by pyridostigmine and protect the central nervous system from the cholinergic crisis. The latter protective effect is not exerted by pyridostigmine because of its poor penetration of the blood-brain barrier (BBB). The focus is on a new approach inspired by the PANPAL strategy, where two drugs with an "opposite" mechanism of action on the cholinergic system are combined. With these ideas in mind, we designed a prophylactic agent which should be able to act as a central and peripheral reversible AChE inhibitor and simultaneously antagonize muscarinic and nicotinic receptors.

Specifically, 7-methoxytacrine (7-MEOTA) was designed and approved for military use against incapacitating agents based on cholinergic overstimulation (BZ compound). 7-MEOTA is a pharmacologically equivalent but less toxic analogue of the parent tacrine. It also possesses a centrally-acting reversible AChE inhibition profile (Dejmek 1990) with complex cholinergic properties (Soukup et al. 2013). The novel series of compounds are based on 7-MEOTA and another potent AChE inhibitor (AChEI), donepezil, which also exerts low antimuscarinic and antinicotinic properties (Ago et al. 2011).

Recently, binary compounds with antimuscarinic and anticholinesterase effects have been synthesized (Leader et al. 2002). Some of them provided better *in vivo* protection than pyridostigmine alone (Meshulam et al. 2001). From this point of view, 7-MEOTA-donepezil-like hybrids represent an interesting group of modulators whose advanced cholinergic properties are of a high interest not only due to their prophylactic potential but also due to a unique combination of two such pharmacophores in a single molecule (Korabecny et al. 2014). The structure of these derivatives is detailed in Figure 1.

The aim of the presented study was to clarify the mechanism of action of 7-MEOTA on the cholinergic system. Three selected donepezil-like hybrids were selected and their effects were studied on AChE and muscarinic and nicotinic receptors. Furthermore, in order to predict their bioavailability in the CNS, the passive blood-brain penetration of the novel compounds was evaluated.

Materials and Methods

Chemicals

All assayed inhibitors were synthesized at the Department of Toxicology, Faculty of Military Health Science, University of Defence, Hradec Kralove, Czech Republic (Korabecny et al. 2014). Purity (>99%) was assessed using TLC and HPLC methods as described before (Jun 2007, 2008). Phosphate buffer, human recombinant AChE (*hr*AChE), DTNB (5,5´-dithiobis (2-nitrobenzoic) acid), acetylthiocholine (ATCh), D-MEM (Dulbecco's modified Eagle's medium), nicotine, ACh (acetylcholine), oxotremorine-M (Oxo-M), BQCA (benzylquinolone carboxylic acid), atropine, geneticin, Hank´s balanced salt solution (HBSS buffer), dimethylsulfoxide (DMSO), dodecane and donepezil were purchased from Sigma – Aldrich (Prague, Czech Republic). NaCl, KCl, $CaCl_2$, KH_2PO_4, $MgSO_4$, $NaHCO_3$ and glucose used for Krebs solution, and ATP (adenosine-5'-triphosphate) and metacholine (MCh) were from Sigma Chemicals (Co, St. Luis, MO, USA). Probenecid and Fluo-4 NW reagents were from Invitrogen (Carlsbad, CA). Porcine polar brain lipid (PBL) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

AChE kinetic study

The activities of *hr*AChE were evaluated by the adopted spectrophotometric Ellman's method. ATCh (various concentrations) was used as a substrate and DTNB (2.5×10^{-3} mol/l) was used as the chromogen. Based on previous experiments a wavelength of 412 nm was used. The absorbance was determined using a Helios Alpha (Thermo Scientific, Great Britain) spectrophotometer. The *in vitro* measurements were completed according to the following protocol: 650 µl of phosphate buffer (0.1 mol/l, pH 7.4) was pipetted into the cuvette. Subsequently, 25 µl of *hr*AChE, 200 µl DTNB and 25 µl of the selected AChEI in concentrations from 10^{-8} to 10^{-3} mol/l were added. This mixture was then incubated for 5 min at laboratory temperature (22 ± 2°C). The enzymatic reaction was started by adding ATCh (100 µl, various concentrations). The mixture was gently shaken and measured in duplicates. The values of the controls, in which phosphate buffer replaced the 25 µl of inhibitor, were subtracted from the experimental values.

The results were analyzed by GraphPad Prism 5.0 (San Diego, CA). The constants were calculated from enzyme kinetics using Lineweaver-Burk plot and a double reciprocal plot. The AChE dissociation constant for enzyme-

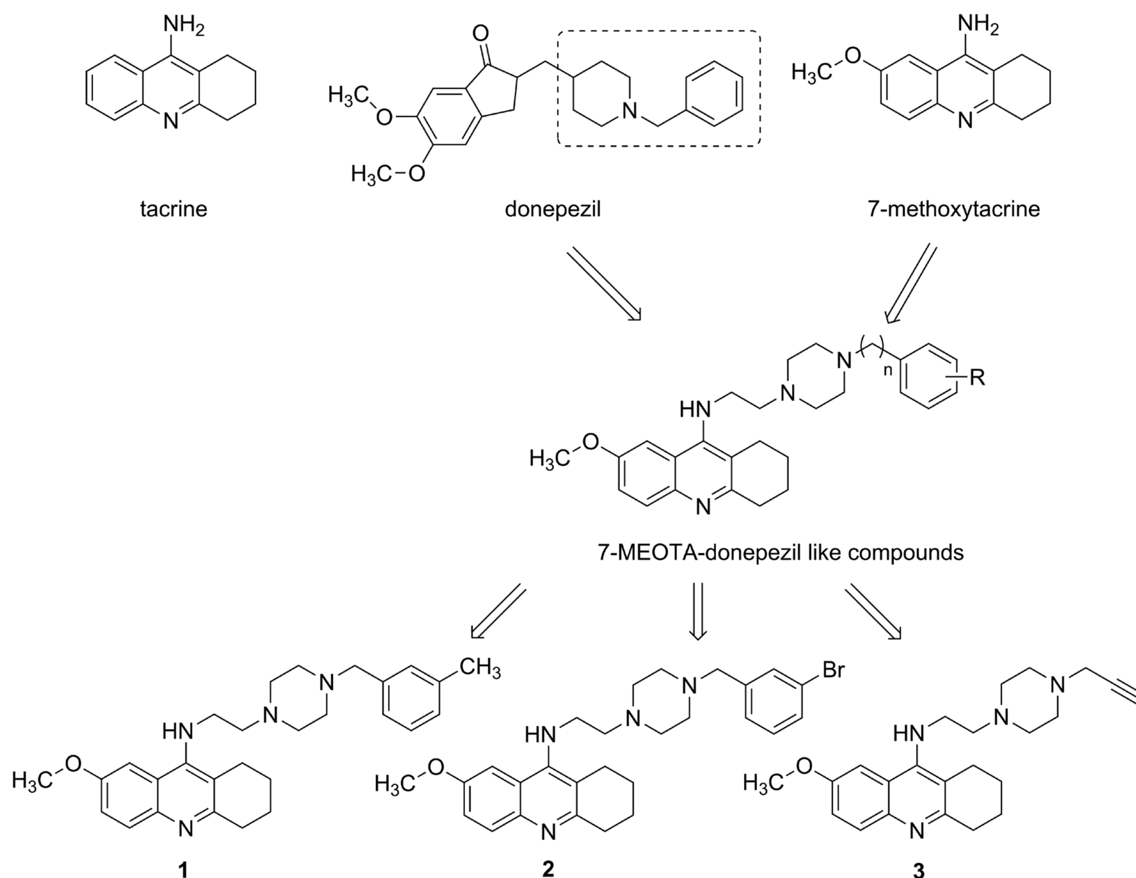

Figure 1. General structure of 7-MEOTA-donepezil like hybrids and derivatives **1**, **2** and **3** used in this study.

inhibitor complex (K_i) was calculated using the following equation:

$$K_{i1} = \frac{[E][I]}{[E\ I]} \qquad (1)$$

where K_{i1} is dissociation constant for enzyme-inhibitor complex; $[E]$, concentration of AChE; $[I]$, concentration of inhibitor; $[EI]$, enzyme-inhibitor complex concentration.

Patch clamp study

Cell culturing

Experiments were performed on the TE671 cell line (kindly provided by Dr. Jan Říčný), which is a medulloblastoma/rhabdomyosarcoma cell line endogenously expressing human embryonic muscle type receptor $\alpha_1\beta_1\gamma\delta$ (Schoepfer 1988). Cells were cultivated at 37°C under 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (D-MEM), which was supplemented with 10% fetal calf serum. Nicotine (100 µmol/l) was added to the cultivation medium 2–5 days before measurement to increase expression of nAChRs (Ke et al. 1998).

Experimental assay

The direct effects of AChEIs were measured according to the previous protocol (Soukup et al. 2011). Cells were held at –40 mV during the recordings. Fire-polished glass micropipettes with an outer diameter of approximately three µm were filled with a solution of the following composition (in mmol/l): CsF 110, CsCl 30, $MgCl_2$ 7, Na_2ATP 5, EGTA 2, HEPES-CsOH 10, pH 7.4. The resulting resistances of the microelectrodes were between 3 and 5 MΩ. The cell bath solution contained (in mmol/l): NaCl 160, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 2, HEPES-NaOH 10, glucose 10, pH 7.3. Solutions of the tested compounds were applied using a rapid perfusion system (Mayer et al. 1989) consisting of an array of ten parallel quartz-glass tubes, each approximately 400 µm in diameter. Tubes were positioned in the vicinity of the recorded cells and the flow of various solutions was

switched on/off under microcomputer control (Dittert et al. 1998). A complete change of the solution around the cell could be achieved in 30 to 60 ms. For signal recording and data evaluation, an Axon Instruments Digidata 1320A digitizer and pClamp9 software package were used (Axon Instruments, Foster City, CA). Data were low-pass filtered at 1 kHz and digitized at 2 kHz.

ACh (100 μmol/l) was used in the assay as control. Any tested compound was pre-applied (5 s) and then co-applied with ACh (100 μmol/l). The relative inhibition rate was determined as the ratio of the amplitude of the response to the agonist under the inhibitor to the arithmetic average of two adjacent experimental responses to the controls. At least a 30 s wash out period was applied between each measurement, so that the cell was washed by the extracellular solution and desensitized receptors were restored to the active state.

Concentration-response curves were fitted to the Hill equation:

$$I(C_a) = \frac{Y_{max} \times C_a^H}{C_a^H + EC_{50}^H} \quad (2)$$

where C_a is the agonist concentration, Y_{max} is the extrapolated maximal value of the response to a saturating concentration of the agonist, $I(C_a)$ is the relative value of the membrane current, EC_{50} is the agonist concentration inducing 50% of the maximal response, and H is the Hill coefficient.

Inhibition curves were fitted to the simple inhibition curve:

$$\frac{Y_L}{Y_{cont}} = \frac{IC_{50}^H}{IC_{50}^H + L^H} \quad (3)$$

where Y_L is the current response amplitude after agonist and lobeline application, Y_{cont} is the control response to the agonist, IC_{50} is the inhibitor concentration that inhibits 50% of the control response, H is the Hill coefficient for inhibition and L is the concentration of inhibitor.

Calcium mobilization assay

Cell culturing

Chinese hamster ovary cells (CHO-M1WT2, CRL-1984) stably expressing human recombinant M_1 mAChR (70 fmol/mg) were obtained from ATCC. Cells were cultured in Ham´s F-12 medium supplemented with fetal bovine serum (10%) and geneticin (50 ng/ml). Cells were maintained at 37°C in an atmosphere of 5% CO_2.

Fluo-4 NW Assay

Cells were plated out at a density of ≈ 70,000 cells *per* well in 100 μl of medium, in a black-walled, clear-bottomed 96-well plate (Biotech, Czech Republic), and were grown overnight at 37°C in an atmosphere of 5% CO_2. The next day, the medium was removed leaving the cells adhering to the bottom. 100 μl of Fluo-4NW solution was added. Fluo-4NW solution was prepared according to manufacturer's protocol – 10 ml of Hank´s balanced salt solution (HBSS buffer) and 100 μl of probenecid solution were added to the dye mixture. Cells were incubated with the dye for 30 min at 37°C/5% CO_2 in the dark and 30 min at room temperature.

The tested compounds were dissolved in DMSO and diluted 150 times with distilled water on the day of experiment, and stock concentrations were prepared in the HBSS buffer. Stock solution was pipetted to a well to achieve the final concentration. The final concentration of DMSO in the well did not exceed 0.3% (v/v).

The effect of the novel compounds was compared to BQCA, a positive allosteric modulator standard. Cells were pre-incubated with the tested compound (10 μl/well) for 10 minutes and then EC_{20} of oxo-M (30 nmol/l) was applied. Since the compounds inhibited the EC_{20} Oxo-M response, their inhibitory potency was measured at EC_{80} oxo-M (1 μmol/l). Ca^{2+} influx was measured by plate reader Synergy HT (Biotek, USA) at an excitation and emission wavelength of 485/20 nm and 528/20 nm, respectively. All measurements were made at room temperature ≈ 21°C.

Responses were quantified as the maximum response expressed as a percentage of the average baseline values and normalized to the control. The first and last wells received oxo-M (1 μmol/l or 30 nmol/l) and served as controls. The mean was considered to be a 100% response in order to minimize time-dependent errors (fading of responses), which were observed in the longer-lasting experiments. Data were fitted using a standard four-parameter equation GraphPad Prism 5.0 (San Diego, CA) to generate graphs, IC_{50} and SEM.

Contraction study

Tissue preparation

Male Wistar rats (bodyweight 250–320 g) were anaesthetized with pentobarbitone (45 mg/kg; *i.p.*). Thereafter, the urinary bladders were removed and two urinary strips (6 × 2 mm) were dissected. Strips were mounted into organ baths (25 ml) between two electrodes where one of the holders was fixed and the other moveable. The organ bath was filled with Krebs solution (NaCl 118, KCl 4.6, $CaCl_2$ 1.25, KH_2PO_4 1.15, $MgSO_4$ 2.26, $NaHCO_3$ 25 and glucose 5.5 in mmol/l) aerated with a mixture of 5% CO_2 and 95% O_2 and maintained at constant temperature (37°C) by a thermo-regulated water circuit. Strips were left 45 minutes in a solution with a stable tension of about 5 mN and repeatedly pre-stretched as needed.

Experimental protocol

The strips were left to equilibrate for 45–60 min before the experiment. The contractile responses (induced by electric field stimulation (EFS), by metacholine (MCh) or by adenosine triphosphate (ATP)) were used for the examination of the AChEI effect on rat bladder. Firstly, control responses were obtained. EFS was employed at increased frequencies (1, 2, 5, 10, 20 and 40 Hz) delivered as square wave pulses. The strip was then allowed to equilibrate for at least 20 min. MCh was administered in a cumulative manner in concentrations from 10^{-8} mol/l to 10^{-3} mol/l and then the strip was washed three times. Thereafter, ATP was added (10^{-6} mol/l to 5×10^{-3} mol/l). The tested compound, 7-MEOTA or compound **2**, was then added to achieve the final concentrations 10^{-6} mol/l, 10^{-5} mol/l and 10^{-4} mol/l in the organ bath chambers. The contraction responses to EFS, MCh and ATP were recorded after administration of the test compound in each concentration. A 20 min resting period was applied between each measurement, and after addition of MCh or ATP into the organ bath, the whole bath was washed three times. All drugs were administered cumulatively in a volume of 125 µl at a 200 times larger concentration in order to achieve the correct final concentration in the organ bath chambers. Responses were recorded by MP100WSW data acquisition system (Biopac, Goleta, USA). Results were evaluated by statistical program GraphPad Prism 5.0 (San Diego, CA) to generate the graphs, IC_{50} and SD.

Modified PAMPA assay

Penetration across the BBB is an essential property for compounds targeting the CNS. In order to predict the passive BBB penetration of the novel compounds, the parallel artificial membrane permeation assay (PAMPA) was used according to a slightly modified protocol (Di et al. 2003). The tested compounds were dissolved according to their solubility properties directly in PBS pH 7.4 buffer (7-MEOTA 300 µmol/l). Donepezil (100 µmol/l) and the 7-MEOTA-donepezil derivatives were first dissolved in DMSO in order to prepare a stock solution (1.2 mmol/l), which was subsequently further diluted in the PBS pH 7.4 buffer to achieve the final concentration in the donor well (300 µmol/l). The concentration of DMSO in the well was always below 0.5% (v/v). 250 µl of the solution was added to the donor wells. The filter membrane was coated with PBL in dodecane (4 µl of 20 mg/ml PBL in dodecane) and the acceptor well was filled with 500 µl of PBS pH 7.4 buffer. The donor filter plate was carefully put on the acceptor plate so that the coated membrane was "in touch" with both the donor solution and the acceptor buffer. The test compound diffused from the donor well through the lipid membrane and into the acceptor well. The set was left undisturbed for 24 h while permeation progressed. The concentration of drug in the acceptor well and in the reference wells (standard curve) was determined using the UV plate reader Biotek Synergy HT at the maximum absorption wavelength (360 nm for 7-MEOTA-donepezil derivatives, 270 nm for donepezil and 250 nm for 7-MEOTA). The concentration of the compounds was calculated from the standard curve and the membrane permeation was expressed in percentage; i.e. the ratio of the concentration assessed in the acceptor well and the concentration applied into the donor well.

Results

In order to characterize the therapeutic potential of novel 7-MEOTA – donepezil derivatives, a series of *in vitro* experiments were carried out involving evaluation of interaction of novel derivatives and their parent compound with AChE, with various subtypes of muscarinic muscle-type of nicotinic receptors. Furthermore, BBB penetration was estimated as a prime prerequisite for a centrally-acting drug. The investigation describing the mechanism of action was mainly of a comparative nature, i.e. it compares the properties of the new drugs with an already approved drug (7-MEOTA).

Interaction with AChE

The kinetic Lineweaver-Burk analysis was applied in order to investigate the affinity of the compounds and the mechanism of inhibition. IC_{50} (the concentration of tested compound that inhibits AChE activity to 50%), the K_i constants (the dissociation constant of the enzyme-inhibitor complex into free enzyme and inhibitor) and

Table 1. AChE inhibition constants for tested compounds

	IC_{50} (µmol/l)	Ki (µmol/l^{-1}·min^{-1})	Type of inhibition
7-MEOTA	10.50 ± 2.00[a]	1.50 ± 0.15	Noncompetitive
Compound **1**	1.38 ± 0.15[a]	0.02 ± 0.01	Mixed
Compound **2**	1.59 ± 0.32[a]	0.29 ± 0.05	Mixed
Compound **3**	12.91 ± 0.70[a]	4.62 ± 0.74	Mixed

Data are mean ± SD. [a] Korabecny et al. 2014.

type of inhibition are summarized in Tab. 1. The graphical analysis of steady state inhibition data is presented in Fig. 2. 7-MEOTA demonstrated classical noncompetitive inhibition. It binds reversibly, randomly and independently at the different sites of the enzyme. On the other hand, the data for compounds **1, 2** and **3** indicated a mixed type of inhibition. This type of inhibition generally resulted in a combination of partially competitive and pure noncompetitive inhibitions and points towards inhibitors having a dual-binding site feature. Such characteristics assume binding into the active site as well as into the peripheral anionic site (PAS) of AChE at the same time.

Interaction with nicotinic receptors

Interaction of new AChEIs (compounds **1, 2, 3**) on ACh-induced (100 μmol/l) with muscle-type nicotinic receptors has been investigated using the patch clamp technique. The inhibitory effect on ACh-induced (100 μmol/l) currents in the presence of increasing concentrations (3×10^{-6}–10^{-4} mol/l) of new drugs was investigated. AChEIs were administered 5 s before Ach.

We used human embryonic muscle type receptor $\alpha_1\beta_1\gamma\delta$ stably expressed by the TE671 cell line. All tested compounds inhibited the ACh-evoked response in a dose-dependent manner (Fig. 3). Compound **3** showed the most pronounced inhibitory potency of the ACh-evoked (100 μmol/l) response (IC_{50} = 1.7 ± 0.9 μmol/l). The other tested compounds inhibited the nicotinic muscle receptor response at the same order of magnitude, but with slightly lower potency (compound **1**: IC_{50} = 3.6 ± 0.3 μmol/l, compound **2**: IC_{50} = 6.8 ± 2.6 μmol/l). 7-MEOTA has previously been tested and shows a similar concentration-dependent curve (IC_{50} = 3.7 μmol/l). However, it should be pointed out that in the previous investigations the concentration of ACh was 30 μmol/l (data not shown).

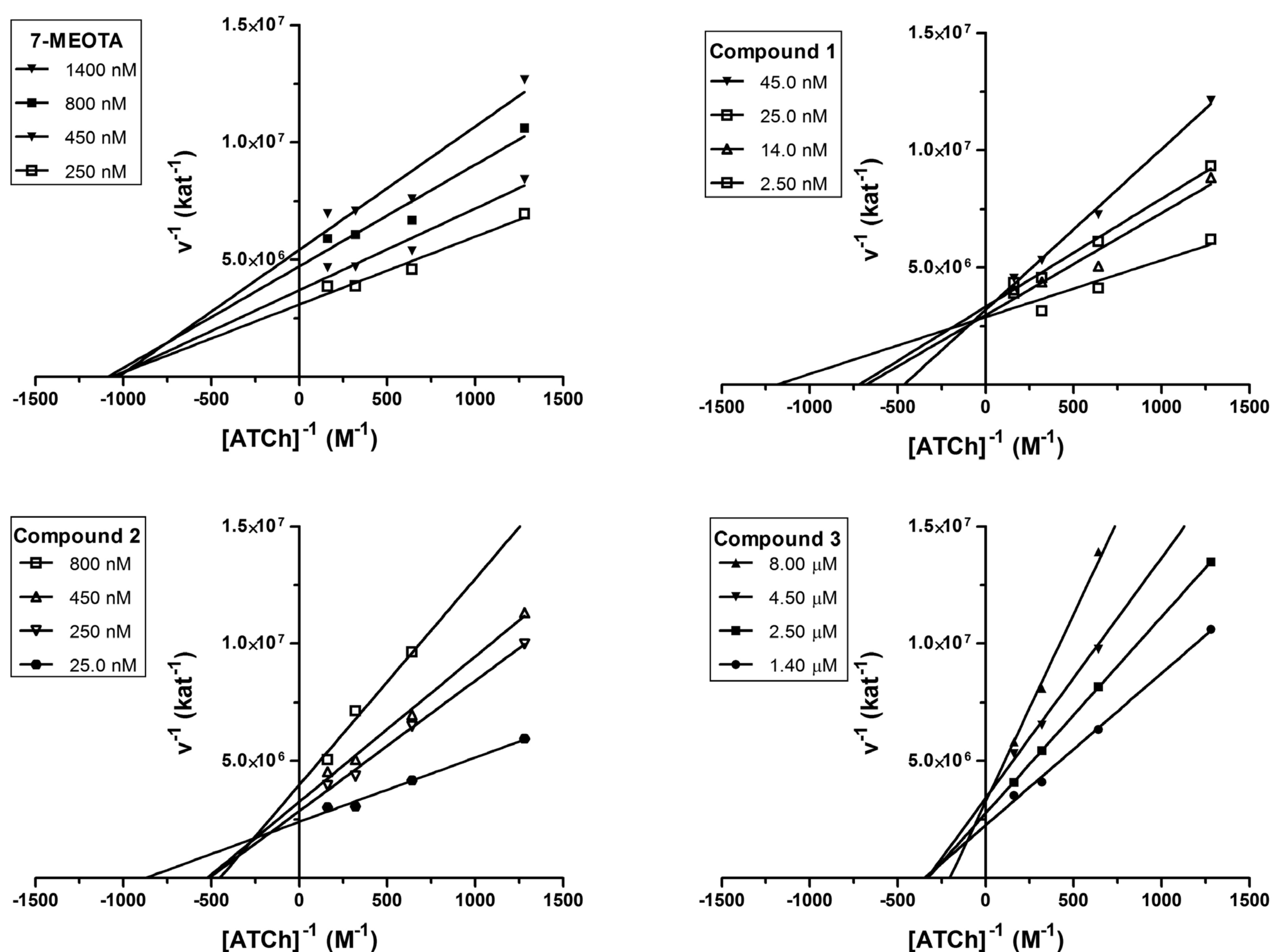

Figure 2. Lineweaver-Burk analysis of *hr*AChE inhibition by 7-MEOTA, compounds **1, 2** and **3**. The reciprocal plots show noncompetitive type inhibition for 7-MEOTA and compound **1**, and mixed type of inhibition for compounds **2**, **3**.

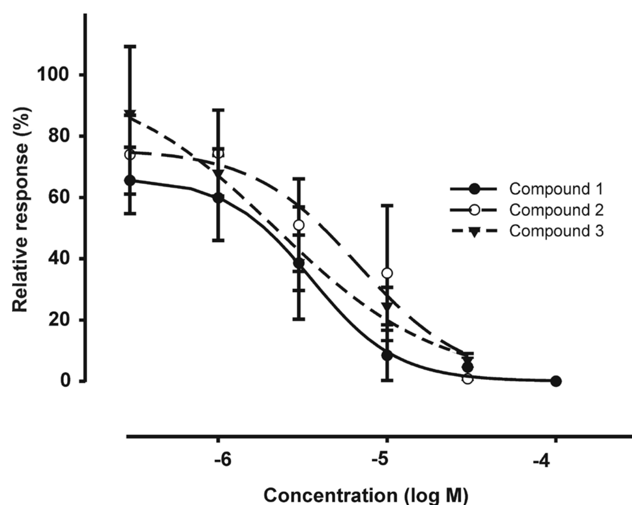

Figure 3. Antagonistic effect of tested AChEIs. Inhibitory effect of tested AChEIs (Compounds **1, 2, 3**) on ACh-induced currents. Decrease of ACh (100 μmol/l) response amplitudes with increasing concentrations (3×10^{-6}–10^{-4} mol/l) of AChEIs. AChEIs were pre-applied 5 s before Ach.

Interaction with M_1 muscarinic receptors

The effect of 7-MEOTA and its derivatives on the muscarinic receptor-mediated response was assessed in CHO-K1 cells stably expressing the M_1 subtype of mAChR, using calcium mobilization assay (Fig. 4). For comparison, atropine, the classic antagonist of mAChR, and BQCA, a standard allosteric agonist, were used in order to investigate the mechanism of interaction. The novel compounds **1**, **2**, **3** and 7-MEOTA, exhibited an antagonistic effect on M_1 muscarinic receptors, similar in type to that of atropine. However, such effect was observed at completely different concentration ranges. Atropine (IC_{50} = (59 ± 13) × 10^{-9} mol/l), was a 300-fold more effective antagonist than the novel inhibitors series. The parent compound 7-MEOTA

Table 2. Inhibition ability of tested compounds on nicotinic muscle-type and muscarinic M_1 receptor

	nACHR IC_{50} (μmol/l)	mAChR M_1 IC_{50} (μmol/l)
7-MEOTA[a]	3.7 ± 0.3	3.05 ± 0.47
Compound **1**	3.6 ± 0.3	23.17 ± 2.18
Compound **2**	6.8 ± 2.6	20.02 ± 7.49
Compound **3**	1.7 ± 0.9	38.18 ± 3.99
Atropine	nt	0.059 ± 0.013

Data are mean ± SD of three to independent experiments, each from different cells (nAChR) and at least two experiments in triplicate (mAChR). [a] 7-MEOTA was tested with 30 μmol/l, others with 100 μmol/l; nt, non tested.

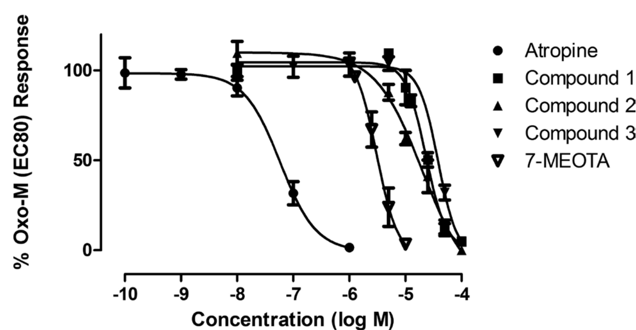

Figure 4. Antagonistic effect of tested compounds on M_1 muscarinic receptors. Inhibition curves were obtained in the presence of EC_{80} Oxo-M (1 μmol/l). Data represent the mean ± SEM of three measurements.

was able to inhibit M_1 muscarinic receptor in a concentration range one order of magnitude lower than the 7-MEOTA-donepezil derivatives (see Tab. 2).

None of the tested compounds showed any potentiating effect on the M1 muscarinic receptor as does BQCA (Fig. 5).

Contraction study

The functional significance of the binding characteristics demonstrated above (i.e. inhibition of AChE and muscarinic receptors) was examined in the rat urinary bladder, a complex model involving both of these targets. Three types of stimulation – EFS and agonist stimulation by MCh and ATP, were applied in order to evoke contractions of the isolated bladder strip preparations on which the effects of 7-MEOTA and one 7-methoxytacrine-donepezil derivative (compound **2**) were examined (Fig. 6). Since EFS leads to the release of all the transmitters within the bladder innerva-

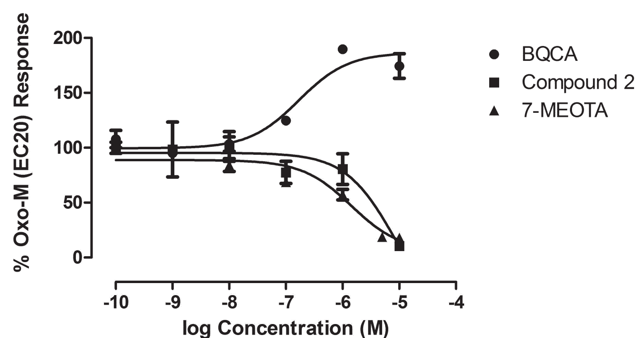

Figure 5. Effect of novel compound **2** and standard BQCA on CHO-M_1WT2 cells stably expressing M_1 mAChR. Increasing concentrations of tested compounds were added to wells and incubated for 10 min. EC_{20} of Oxo-M was then added. Data represent the mean ± SEM of three measurements.

tion, specifically acetylcholine and ATP that are co-stored in the rat parasympathetic neurons (Vesela et al. 2012), MCh and ATP were employed for distinguishing the cholinergic and the purinergic effects (Werner et al. 2007). All three types of stimulation evoked contractions that were enlarged by increasing the intensity of the stimulation. Analyses employing a two-way ANOVA revealed the basal responses (responses in the absence of any derivate in the EFS, MCh and ATP groups) not to be significantly different in any group. EFS thus induced frequency-dependent contractions. The maximum responses occurred in the range of 20–40 Hz and were 17.9 ± 2.7 and 17.0 ± 4.0 mN in the 7-MEOTA and compound **2**-treated groups, respectively. The non-selective muscarinic receptor agonist MCh, which is resistant towards AChE cleavage, evoked concentration-dependent contractile responses showing maximums of 26.0 ± 3.2 and

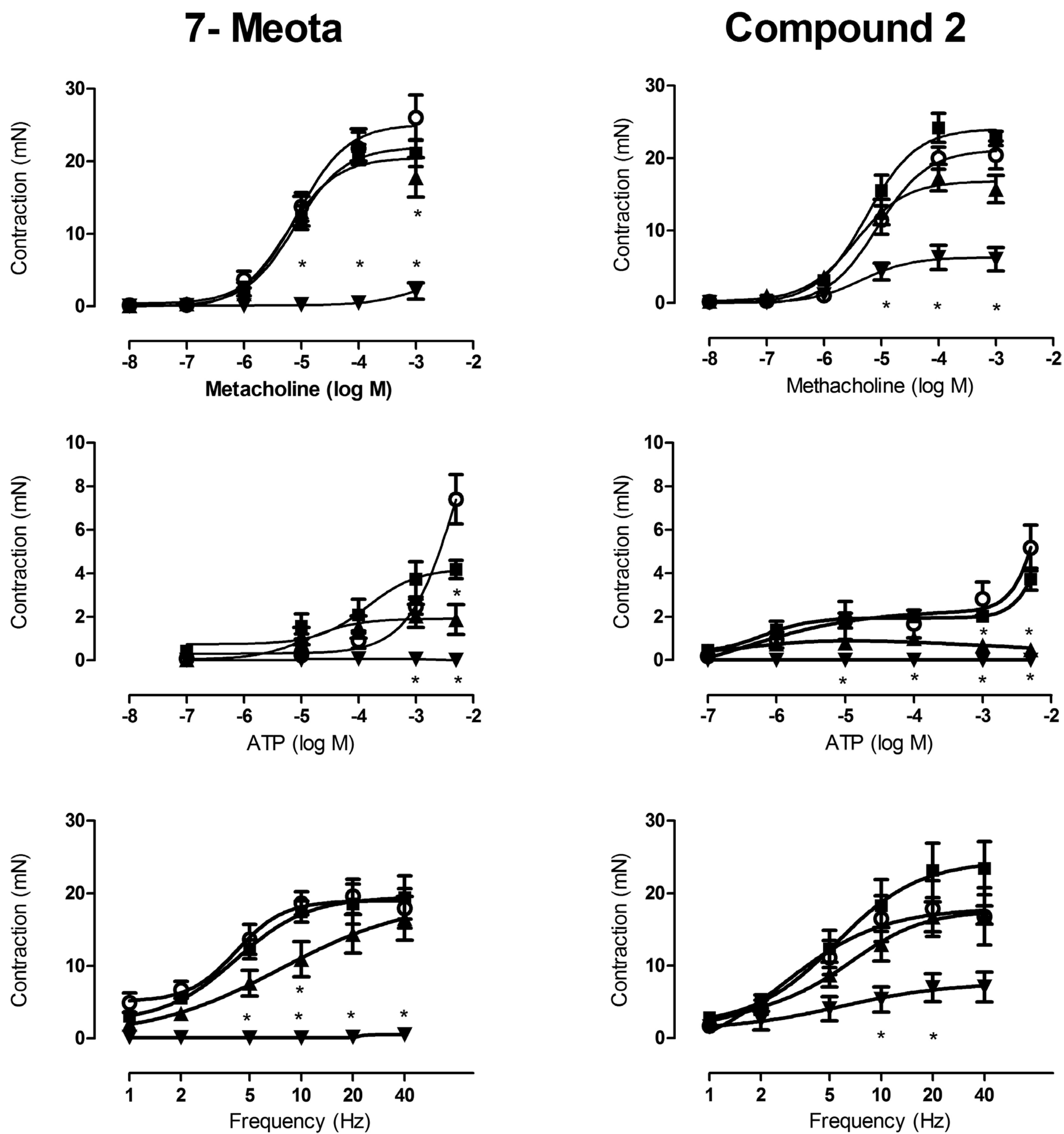

Figure 6. Data from 7-MEOTA (left column) and from compound **2** (right column) in the contraction study. Graph in absence (○) and presence of 10^{-4} mol/l (▼), 10^{-5} mol/l (▲), and 10^{-6} mol/l (■) of tested compounds.

20.4 ± 2.0 mN for the 7-MEOTA- and compound **2**-treated groups, respectively. ATP evoked smaller contractions but no maximum response could be identified at the concentration range used currently. At the largest concentration used, the contractile responses were 7.4 ± 1.1 and 5.2 ± 1.0 mN for the 7-MEOTA- and compound **2**-treated groups, respectively. A tendency to biphasic concentration-dependent responses to ATP was indicated. A first phase showed a pEC50 of 6.5 (merged data from both groups). The second phase of the response appeared at ATP concentrations higher than 10^{-5} mol/l (7.3 ± 1.1 and 5.2 ± 1.0 mN, for the 7-MEOTA- and compound **2**-groups, respectively).

The presence of the compounds (7-MEOTA and compound **2**) at the lowest concentration examined (10^{-6} mol/l) showed no or little inhibition of the ATP-evoked responses. At the higher concentrations, and in particular at the highest concentration (10^{-4} mol/l), significant decreases in the contractions occurred ($p < 0.05$). The same pattern appeared for the largest concentration of the derivatives for the EFS- as well as the MCh-evoked responses, tentatively indicating non-specific effects. A concentration of 10^{-5} mol/l of MEOTA still tended to inhibit the contractions to EFS (18.6 ± 1.6 *vs.* 10.9 ± 2.4 mN at 10 Hz) in the presence and absence of 7-MEOTA, respectively ($p < 0.01$). However, this was the only significant change, if disregarding the 10^{-4} mol/l concentration. For compound **2**, no significant changes occurred regarding EFS with the exception of the 10^{-4} mol/l concentration. 7-MEOTA at 10^{-5} mol/l significantly reduced the methacholine-evoked response. In the absence of 7-MEOTA, the contractile response to methacholine at 10^{-3} mol/l was 26.0 ± 3.1 mN, whereas in its presence it was 17.8 ± 2.7 mN ($p < 0.001$; $n = 7$).

BBB penetration estimation

The ability to penetrate the BBB is a main prerequisite for a centrally-acting drug. The modified PAMPA assay showed that all new compounds readily penetrate across the lipophilic membrane by passive diffusion. Except for compound **2**, the drugs showed similar penetration abilities as the parent compound 7-MEOTA (Tab. 3), which has been proved to cross BBB (Soukup et al. 2013). Donepezil, which was used as a model of a CNS-permeable drug, confirmed an adequate experimental setup by its high penetration rate (87%).

Discussion

The area of prophylaxis against organophosphates still needs further exploration. Different approaches have been investigated but many problems have been found. After administration of stoichiometric or catalytic scavengers (e.g. butyrylcholinesterase or phosphotriesterase) immunologic reactions were invoked (Bajgar 2004; Lenz et al. 2007), and their use is limited to experimental purposes due to high cost. Administration of anticonvulsants shows low prophylactic efficiency. Also, some problems have been reported regarding reactivators (Bajgar 2005), principally regarding the route of administration due to their fast metabolism, but also due to their fast excretion. Recently, prophylaxis against nerve gases has focused predominantly on reversible AChE inhibitors. One of such medicaments, PANPAL, was introduced into the Czech Army in 2002 (Bajgar 2004). Pyridostigmine, which is not effective in the brain at lower doses, was combined with trihexyphenidyl and benactyzine that mitigate peripheral side effects, so that a higher dose of pyridostigmine can be applied. Furthermore, both anticholinergics penetrate to the central compartment, where they are able to protect the cholinergic receptors from the overstimulation of ACh during the poisoning. It has been demonstrated that exposure of pyridostigmine alone can lead to neurobehavioral deficits and region-specific alterations of AChE and AChR (Abou-Donia et al. 2001). Albequerque et al. (1985) presented that physostigmine (co-applied with atropine) combined with a ganglionic blocking drug mecamylamine was the most effective medication for protecting rats against lethal doses of VX.

Accordingly we started to look for a new compound, which would be able to protect AChE and simultaneously AChR against organophosphate intoxication. Inspired by the positive 7-MEOTA results and its efficacy in VX poisoning prophylaxis (Bajgar et al. 1983), and the "PANPAL" approach, we investigated how 7-MEOTA derivatives are able to influence AChE, AChR activity, and the mechanism of action of such hybrid compounds.

The new 7-MEOTA-donepezil derivatives exerted more potent (compound **1**, **2**) or similar (**3**) AChE-inhibitory activity compared to 7-MEOTA. 7-MEOTA has been reported to be a better AChE inhibitor than pyridostigmine (Lorke et

Table 3. AChE inhibitors permeation ability

	Permeation (%)	R^2
7-MEOTA	66	0.9898
Compound **1**	63	0.9974
Compound **2**	44	0.9940
Compound **3**	62	0.9970
Donepezil	87	0.9829

The degree of membrane permeation of the compounds expressed in percentage, i.e. the ratio of the concentration assessed in the acceptor well (as a result of the membrane penetration) and of the concentration applied into the donor well. Linearity of the standard curve was proved by the determination coefficient represented as R^2.

al. 2011). Moreover, as has been said above, pyridostigmine does not penetrate the BBB (Dunn et al. 1997) and it is not able to offer protection against seizures and subsequent neuropathology induced by organophosphates, especially in cases of soman-induced toxicity (Dunn et al. 1997). On the contrary, 7-MEOTA is able to pass the BBB (Kunes et al. 2005). Based on the fact that 7-MEOTA and donepezil alone cross the barrier (Kim et al. 2010), we presumed that 7-MEOTA-donepezil hybrids might penetrate the BBB too. The modified PAMPA assay showed that all new compounds readily penetrate across the lipophilic membrane by passive diffusion. Data have shown that a possible solution for this matter is co-administration of AChEI and the muscarinic antagonist scopolamine, which speaks in favour of our hypothesis.

The M_1 subtype of mAChR is abundantly found in the cortex and hippocampus (Fisher et al. 2002), whilst a small population of M_1 appears in salivary glands and the presynaptic area of the bladder parasympathetic nervous system (Tobin 1995; Abrams et al. 2006). Notably, compound **3** is able to cause 50% inhibition of AChE and M_1 mAChR in the same concentration range, whilst compounds **1** and **2** showed one order of magnitude lower ability to inhibit M_1 mAChR than AChE. In contrast, 7-MEOTA inhibited M_1 receptor at a lower concentration (IC_{50} = 3 μmol/l) than it inhibits AChE (IC_{50} = 10 μmol/l). None of the compounds showed any ability to potentiate the M1 muscarinic receptor effect. From this point of view, compound **3** possesses promising *in vitro* antimuscarinic action on the centrally occurring muscarinic subtype.

Further examination was focused on peripheral receptors. After organophosphate intoxication, the overstimulation of the muscle type of nAChR that occurs at the motor end-plate in respiratory and other muscles (Sungur et al. 2001) is followed by fasciculation and weakness, eventually resulting in asphyxiation. Thus, the hypothesis of using a nAChR antagonist was developed. Sheridan et al. (2005) pointed out the difficulties of administering a dose of a compound that antagonizes the effect of excessive ACh, but which does not cause paralysis of muscle. Turner et al. (2011) introduced a non-competitive antagonist on nAChR, which acts as an open channel blocker and is able to protect guinea pigs against poisoning by nerve agents. We observed an antagonist effect in all tested compounds on the muscle type of nAChR expressed in TE671 cells. Generally, the effect of the tested AChEI was similar (IC_{50} = 1–3 μmol/l). However, compounds **1** and **2** inhibited AChE in comparable concentrations to those acting on the nicotinic receptor; whereas 7-MEOTA and compound **3** needed to be administered at higher concentrations to inhibit AChE. Moreover, 7-MEOTA has been evaluated previously, concerning how it influences the neuronal α4β2 nicotinic receptor (Soukup et al. 2013). 7-MEOTA interacts slightly more effectively with muscular than neuronal nAChR (IC_{50} = 4 μmol/l, respectively 15 μmol/l).

The effect on peripheral mAChR (M_2 and M_3 subtype) was examined employing an organ model (rat urinary bladder). When applying EFS, the antagonistic effect of 7-MEOTA and compound **2** was evident. However, it was not clear whether the cholinergic pathway is the only pathway involved. Furthermore, 7-MEOTA in high concentration completely inhibited the strip contractions. Since an atropine-resistant bladder contraction exists (Andersson and Wein 2004) and since 7-MEOTA completely inhibited the nerve-evoked response, 7-MEOTA is not likely to affect only the cholinergic pathway. Hence we applied MCh (mAChR agonist) and ATP (P2X purinoreceptor agonist) to distinguish the different mechanisms of action influencing the contraction. Even though compound **2** is a derivative of 7-MEOTA, it acts through a different pathway. Compound **2** inhibits contraction induced by MCh through mAChR in a concentration-dependent manner and the highest concentration tested (10^{-4} mol/l) resulted in 3-times weaker contractions than MCh did on its own. On the other hand, 7-MEOTA caused less inhibition of the muscarinic response, and at 10^{-4} mol/l the contraction almost completely vanished. Differences in inhibition of purinoreceptor effects also occurred, but there seems to be an opposite action. 7-MEOTA decreases contractions in a concentration-dependent manner and compound **2** antagonism on P2X receptors is rather subtle. This is confirmed by the EFS stimulation. At low frequencies, the purinergic-mediated force is enhanced (Werner et al. 2007). Thus 7-MEOTA inhibits contraction at low frequencies, and in high concentration (10^{-4} mol/l) completely inhibits bladder contraction to EFS even at higher frequencies (40 Hz). Note that a concentration-dependence can be observed in the ATP-evoked contraction that indicates 7-MEOTA has a higher effect on the purinergic rather than the cholinergic pathway. On the other hand, the contractile reaction is maintained in the presence of compound **2** (at all concentrations) in response to EFS, while a dose-dependence is observed on the MCh-evoked contractions. Thus it seems that antagonism on the purinergic P2X receptors is absent or smaller than the effect on the cholinergic system. However, it cannot be completely ruled out.

In conclusion, this study provides evidence that the newly synthesized 7-MEOTA-donepezil derivatives have anticholinergic effect; *in vitro* and *ex-vivo* experiments showed that they can inhibit AChE, nAChR and mAChR of both peripheral and central types. Furthermore, their mechanisms of interaction were elucidated. Overall, 7-MEOTA- donepezil hybrids represent promising compounds whose features could be an effective way to diminish the effect of overstimulation of cholinergic receptors during organophosphate poisoning. However, their real

prophylactic potency and benefit must be definitively verified *in vivo*.

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