Electronic Supplementary Material (ESI) for Toxicology Research.

Electronic Supplementary Information

7-Methoxytacrine – 4-Pyridinealdoxime Hybrid as Novel Prophylactic Agent with Reactivation Properties in Organophosphate Intoxications

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Contents of Electronic Supplementary Information

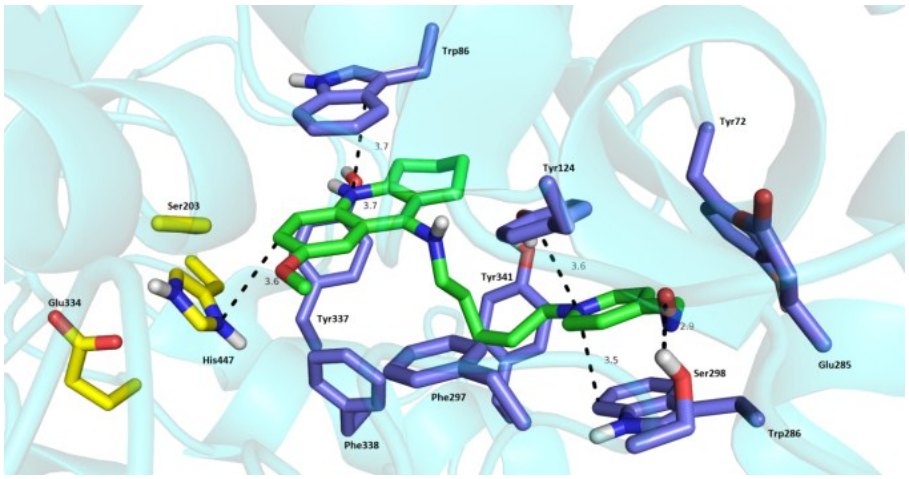

Figure 1. Superimposition of ligand **5** (green carbon atoms) in *h*AChE. The rigid enzyme part is displayed in cartoon representation, relevant amino acids responsible for the enzyme-ligand interactions in dark blue and the catalytic triad in yellow. Figure was created with PyMol viewer (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC).

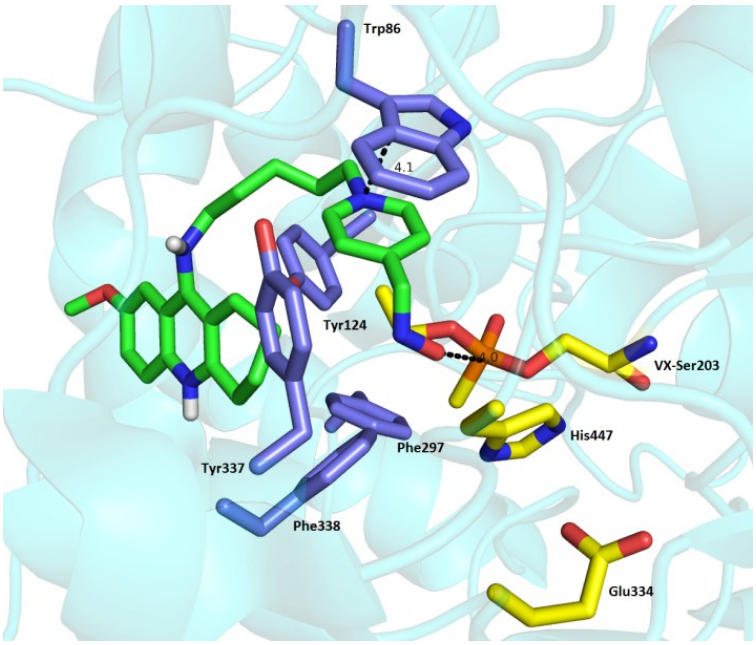

Figure 2. Representation of the binding mode of compound **5** (green carbon atoms) into the VX-inhibited *m*AChE. The rigid enzyme part is displayed in cartoon representation, important amino acids responsible for the enzyme-ligand interactions in dark blue and the catalytic triad in yellow. Figure was created with PyMol viewer (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC).

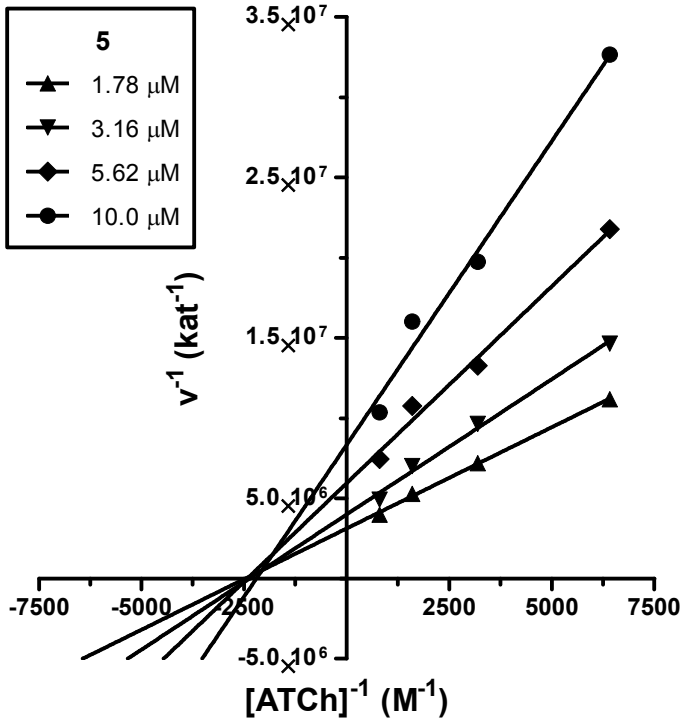

Figure 3. Steady-state inhibition of AChE hydrolysis of acetylthiocholine (ATCh) by compound **5**. Lineweaver–Burk reciprocal plots of initial velocity and increasing substrate concentrations (0.156 – 1.250 mM) are presented. Lines were derived from a weighted least-squares analysis of the data points.

EXPERIMENTAL SECTION

Chemistry

General Chemical Methods

All the chemical reagents used and eluents for UHPLC-MS were purchased from Sigma-Aldrich (Prague, Czech Republic). Solvents for synthesis were obtained from Penta chemicals Co (Prague, Czech Republic). The course of the reactions was monitored by thin layer chromatography (TLC) on aluminium plates precoated with silica gel 60 F254 (Merck, Prague, Czech Republic) and then visualized by UV 254. Melting points were determined on a microheating stage PHMK 05 (VEB Kombinant Nagema, Radebeul, Germany) and are uncorrected. Uncalibrated purity was ascertained by UHPLC-UV using a reverse phase C18 chromatographic column at the wavelength 254 nm. All the compounds exhibited purity in range 95.63 – 100%. NMR spectra were recorded on Varian S500 spectrometer (operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C; Varian Comp. Palo Alto, USA). For ^{1}H δ are given in parts per million (ppm) relative to DMSO (δ = 2.50), $CDCl_3$ (δ = 7.26), CD_3OD (δ = 3.31), and for ^{13}C relative to DMSO (δ = 39.43), $CDCl_3$ (δ = 77.00), CD_3OD (δ = 49.05). Spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), or m (multiplet). The coupling constants (*J*) are reported in Hertz (Hz). High-resolution mass spectra (HRMS) were determined by Q Exactive Plus hybrid quadrupole-orbitrap spectrometer.

7-Methoxy-1,2,3,4,9,10-hexahydroacridin-9-one (1).

4-Methoxyaniline (5.00 g, 40.60 mmol), ethyl 2-oxocyclohexanecarboxylate (7.12 mL, 44.66 mmol) and *p*-toluenesulfonic acid monohydrate (115.84 mg, 0.61 mmol) were treated with toluene (125 mL), stirred and refluxed for 8 hours using a Dean-Stark trap to remove water from reaction mixture. Evaporation of toluene gave crude enaminoester which was thereafter dissolved in warm diphenylether (32.5 mL). Ethanol formed in the course of the reaction was removed with Dean-Stark apparatus. The mixture was heated to 220°C and stirred for approx. 2 hours, until the starting compound had completely disappeared (monitored by TLC using EtOAc/MeOH (95:5) as eluent). The reaction mixture was allowed to cool to room temperature. Subsequently, hexane (100 mL) was added. Formed precipitate was collected by filtration, washed with additional hexane (100 mL) and dried. No further purification was required.

Yield 9.12 g, 98%; mp 257 -259°C. Purity: 100%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.28 (s, 1H), 7.44 (d, *J* = 2.9 Hz, 1H), 7.41 (d, *J* = 9.0 Hz, 1H), 7.20 (dd, *J* = 9.0, 3.0 Hz, 1H), 3.79 (s, 3H), 2.67 (t, *J* = 6.2 Hz, 2H), 2.43 (t, *J* = 6.2 Hz, 2H), 1.77 – 1.65 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 175.42, 154.85, 146.07, 134.15, 124.19, 121.67, 119.29, 114.69, 104.19, 55.39, 27.26, 22.16, 22.02, 21.75. HRMS $[M+H]^+$: 230.1161 (calculated for $[C_{14}H_{16}NO_2]^+$: 230.1176).

9-Chloro-7-methoxy-1,2,3,4-tetrahydroacridine (2).

7-Methoxy-1,2,3,4,9,10-hexahydroacridin-9-one (**1**, 7.50 g, 32.71 mmol) was dissolved in $POCl_3$ (45.73 mL, 0.49 mol) in an ice bath with good stirring as exothermic reaction

occurred. The reaction mixture was refluxed for 2 hours. Excessive phosphoryl chloride was removed by distillation under reduced pressure. The residue was dissolved in $CHCl_3$ (125 mL) and spilled in crashed ice (250 g) alkalized with 25% aqueous ammonium solution. Extracted organic layer was washed with brine, dried over Na_2SO_4 and concentrated to dryness. Crude product was purified by column chromatography using petroleum ether/EtOAc (85:15) as eluent.

Yield 8.10 g, 100%; mp 119.6 – 121.1°C. Purity: 95.63%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ 7.86 (d, *J* = 9.1 Hz, 1H), 7.37 (d, *J* = 2.7 Hz, 1H), 7.30 (dd, *J* = 9.1, 2.7 Hz, 1H), 3.94 (s, 3H), 3.08 (t, *J* = 5.9 Hz, 2H), 3.02 – 2.96 (m, 2H), 2.00 – 1.85 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$-*d*) δ 157.93, 156.54, 142.63, 140.03, 130.14, 128.92, 126.20, 121.99, 101.42, 55.54, 33.78, 27.55, 22.71, 22.64. HRMS $[M+H]^+$: 248.0845 (calculated for $[C_{14}H_{15}ClNO]^+$: 248.0837).

5-[(7-Methoxy-1,2,3,4-tetrahydroacridin-9-yl)amino]pentan-1-ol (3).

To a solution of 9-chloro-7-methoxy-1,2,3,4-tetrahydroacridine (**2**, 4.00 g, 16.15 mmol) in pentan-1-ol (40 mL) 5-amino-1-pentanol (3.33 g, 32.29 mmol) was added. The reaction mixture was stirred at 150 °C for 24 hours. The process of the reaction was monitored by TLC. The mixture was cooled to room temperature, diluted with water (400 mL), extracted with CH_2Cl_2 (3 × 400 mL), brine (400 mL) and finally again with water (400 mL). Collected organic layers were dried over Na_2SO_4 and concentrated in vacuo. Crude product was purified by column chromatography using CH_2Cl_2/MeOH (gradient: 95:5 → 90:10) as eluent.

Yield 3.00 g, 59%; oil. Purity: 99.34%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ 7.82 (dd, *J* = 8.8, 0.7 Hz, 1H), 7.23 – 7.17 (m, 2H), 3.88 (s, 3H), 3.64 (t, *J* = 6.9 Hz, 2H), 3.39 (t, *J* = 6.8 Hz, 2H), 3.04 – 2.98 (m, 2H), 2.72 – 2.65 (m, 2H), 1.91 – 1.84 (m, 4H), 1.71 – 1.55 (m, 4H), 1.51 – 1.44 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$-*d*) δ 155.84, 149.99, 142.84, 129.65, 121.04, 120.33, 117.00, 101.62, 62.06, 55.39, 48.93, 33.38, 32.30, 31.40, 24.63, 23.24, 22.92, 22.64. HRMS $[M+H]^+$: 315.2055 (calculated for $[C_{19}H_{27}N_2O_2]^+$: 315.2067).

***N*-(5-Bromopentyl)-7-methoxy-1,2,3,4-tetrahydroacridin-9-amine (4).**

5-[(7-Methoxy-1,2,3,4-tetrahydroacridin-9-yl)amino]pentan-1-ol (**3**, 2.50 g, 7.95 mmol) and tetrabromomethane (2.90 g, 8.75 mmol) were dissolved in CH_2Cl_2 (50 mL). The reaction mixture was cooled to 0°C. Then, triphenylphosphine (2.29 g, 8.75 mmol) dissolved in CH_2Cl_2 (10 mL) was added dropwise. The mixture was stirred under inert conditions at room temperature for 24 hours. Crude product was extracted with brine (100 mL) and subsequently with water (100 mL). Organic layer was dried over Na_2SO_4 and concentrated to dryness. The residue was purified by column chromatography using CH_2Cl_2/MeOH (gradient: 100:0 → 95:5) as eluent.

Yield 1.23 g, 41%; oil. Purity: 98.52%. ^{1}H NMR (500 MHz, $CDCl_3$-*d*) δ 7.84 (d, *J* = 9.1 Hz, 1H), 7.25 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.21 (d, *J* = 2.7 Hz, 1H), 3.92 (s, 3H), 3.41 (t, *J* = 6.8 Hz, 4H), 3.04 (t, *J* = 6.4 Hz, 2H), 2.74 (t, *J* = 6.3 Hz, 2H), 1.96 – 1.86 (m, 6H), 1.72 – 1.64 (m, 2H), 1.61 – 1.53 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$-*d*) δ 156.31, 155.99, 149.57, 143.51, 130.42, 121.39, 120.28, 117.65, 101.51, 55.50, 48.88, 33.89, 33.56, 32.37, 30.87, 25.60, 24.80, 23.09, 22.88. HRMS $[M+H]^+$: 378.1235 (calculated for $[C_{19}H_{26}BrN_2O]^+$: 377.1223).

4-(Hydroxyimino)methyl-1-{5-[(7-methoxy-1,2,3,4-tetrahydroacridin-9-yl)amino]pentyl}pyridin-1-ium bromide (5).

A mixture of *N*-(5-bromopentyl)-7-methoxy-1,2,3,4-tetrahydroacridin-9-amine (**4**, 1.00 g, 2.65 mmol), 4-pyridinealdoxime (0.97 g, 7.95 mmol) and NaI (0.04 g, 0.27 mmol) in absolute EtOH (5 mL) was refluxed for 48 hours under inert conditions. The product formation was monitored by TLC (R_f = 0.05; CH_2Cl_2/MeOH (9:1) as eluent). The reaction mixture was cooled to room temperature and filtered to remove NaI. Crude product was concentrated to dryness and purified by column chromatography using CH_2Cl_2/MeOH (9:1) as eluent.

As the product contained several contaminants, it became obvious that classical column chromatography was not efficient enough to eliminate all the impurities. Therefore, it turned out to be necessary to purify the product additionally by preparative ultra-high-performance liquid chromatography (UHPLC), which is described below in detail.

Yield 185 mg, 14%; oil. Purity: 99.93%. ^{1}H NMR (500 MHz, CD_3OD-d_4) δ 8.93 (d, *J* = 5.4 Hz, 2H), 8.42 (br s, 1H), 8.28 (s, 1H), 8.18 (d, *J* = 5.5 Hz, 2H), 7.73 (d, *J* = 9.1 Hz, 1H), 7.58 (d, *J* = 2.0 Hz, 1H), 7.47 – 7.39 (m, 1H), 4.63 (t, *J* = 6.9 Hz, 2H), 3.96 – 3.87 (m, 5H), 3.10 – 2.95 (m, 2H), 2.82 – 2.66 (m, 2H), 2.14 – 2.04 (m, 2H), 1.97 – 1.84 (m, 6H), 1.56 – 1.45 (m, 2H). ^{13}C NMR (126 MHz, CD_3OD-d_4) δ 166.63, 157.12, 155.53, 149.73, 149.60, 144.58, 144.11, 133.04, 124.02, 123.80, 120.61, 117.35, 111.59, 103.29, 60.83, 55.26, 46.79, 30.51, 29.96, 27.87, 24.41, 22.93, 21.82, 20.45. HRMS $[M+H]^{2+}$: 210.1258 (calculated for $[C_{25}H_{32}N_4O_2]^{2+}$: 210.1257).

Preparative UHPLC

The analytical method was firstly developed with an analytical column and then transferred to a preparative mode using Synchronis C18, 100.0 x 10.0 (ID), 5 µm preparative column. The crude product (~ 400 mg) was dissolved in minimal amount of methanol (10 mL), sonificated and inserted into an automated UHPLC-UV system built of Dionex Ultimate 3000 and a six-way separator valve for isolation of the fractions. The preparative analytical method was set according to the following parameters:

Column: Synchronis C18, 100.0 x 10.0 (ID), 5 µm. Guard-column: Synchronis C18, 10.0 x 10.0 (ID), 5 µm. Run-time of one analytical cycle: 20 minutes. MFA: ultra-pure water with 0.1% (v/v) HCOOH. MFB: acetonitrile with 0.1% (v/v) HCOOH. Temperature in column compartment: 27°C. Temperature in autosampler: 23°C. Flow-rate: 4.0 mL/min. UV-detection: 210 nm. Injection volume: 50 µL. Separation period for 6-way valve: 7.65 – 8.8 minutes. Elution program: non-linear concave gradient (Table S1).

Table S1. Gradient program for the preparative UV-LC analysis

Time [min]	MFA [%]	MFB [%]	Curve
0	99	1	5

3	99	1	5
15	0	100	7
16	0	100	5
16	99	1	5
20	99	1	5

Curve 5: line

$$B_1 = B_0 + (1-k)(B_2 - B_0)\left(\frac{2^{\frac{-10(t_2-t_1)}{(t_2-t_0)}}}{1-2^{10}}\right) + \frac{k(B_2-B_0)(t_1-t_0)}{t_2-t_0}$$

Curve 7:

For curve 7: k = 0.5; B_0 = concentration of MFB at the previous gradient step [%]; B_1 = current concentration of MFB [%]; B_2 = concentration of MFB at the next gradient step [%]; t_0 = time at the beginning of the gradient step [min]; t_1 = current elapsed time [min]; t_2 = time at the ending of the gradient step [min].

The retention time of compound **5** at this setting was 8.28 ± 0.06 minutes. The injection volume was optimized to avoid overload of the column and collapse of the separation. After complete collection of all fractions, pure product was obtained by concentration in vacuo.

In Vitro Reactivation Screening

Pralidoxime and obidoxime used in this study were synthesized at the Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence or purchased from Leciva (Prague, Czech Republic), Merck (Darmstadt, Germany) or Phoenix Chemicals Ltd. (Bromborough Wirral, United Kingdom). Phenomenex octadecylsilane-bonded silica gel C180 SPE column was obtained from Chromservis s.r.o. (Prague, Czech Republic). Tabun (GA; [(dimethylamino)(ethoxy)phosphoryl]formonitrile), sarin (GB; propan-2-yl fluoro(methyl)phosphinate) and VX (ethyl ({2-[bis(propan-2-yl)amino]ethyl}sulfanyl)(methyl)phosphinate) were obtained from the Military Technical Institute in Brno (Brno, Czech Republic) and their purity was higher than 95%. Paraoxon (POX; diethyl 4-nitrophenyl phosphate) and all other chemicals used were of analytical purity and were purchased from Sigma-Aldrich (Prague, Czech Republic).

Reactivation potencies of tested compounds were evaluated by *in vitro* screening test at two diverse concentrations: 10^{-4} M and 10^{-5} M. Nerve agents (tabun, sarin and VX) and pesticide paraoxon were selected as suitable cholinesterase inhibitors. Human erythrocyte hemolyzate, prepared from blood samples, was applied as a source of AChE. The blood samples were collected from healthy volunteers from vein into disposable

syringes containing 3.8% sodium citrate solution (ratio blood/citrate was 1:10 *w*/*w*). The citrated blood was centrifuged for 20 minutes at 2856 × g and plasma was subsequently removed as supernatant. Erythrocytes were washed three times with phosphate buffer solution (PBS; 0.1 M, pH 7.4), hemolyzed in PBS (0.01 M, pH 7.4) in ratio 1:10 (*w*/*w*), frozen and stored at −80°C until use. Inhibition of AChE was launched by an addition of inhibitor solution in isopropanol (final concentration 10^{-5} M) to the mixture of PBS (0.05 M, pH 7.4) and hemolyzate (activity before inhibition was set up to 10 U/L). Concentration of isopropanol in the sample was 5%. This concentration has no significant effect on the activity of AChE. The enzyme was inhibited for 1 hour. Then, an excess of inhibitor was removed by filtration through C18 SPE column. Originated enzyme was completely inactive. Control sample with uninhibited enzyme was incubated with pure isopropanol at final concentration of 5% for the same time interval. Inhibited enzyme was subsequently incubated for 10 minutes with solution of reactivator in PBS (0.05 M, pH 7.4) at concentrations 10^{-4} M and 10^{-5} M, respectively. After 10 minutes of reactivation, 5,5´-dithiobis(2-nitrobenzoic acid) (Ellman´s reagent; DTNB) in PBS (0.05 M, pH 7.4) was added. The enzymatic reaction was initiated by an addition of AChE substrate – acetylthiocholine (ATCh). Final concentration of DTNB and ATCh in the mixture was 10^{-3} M, respectively. The activity of AChE in hemolyzate was determined by the modified protocol formerly described by Ellman *et al.*.[1–8] Absorbance of the sample was measured spectrophotometrically (Helios Alfa; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 436 nm for 1 minute. AChE activity was evaluated with 2 minutes delay to minimize the interference of DTNB with thiol groups in hemolyzate. Solutions of different cystein concentrations (used instead of hemolyzate) had been measured as well for the absolute calibration of the used method. All results were corrected for oximolysis and inhibition of AChE by reactivator. Reactivation potencies of tested compounds were calculated from obtained data as follows:

$$R = \left(1 - \frac{a_0 - a_r}{a_0 - a_i} \right) \times 100 ,$$

where R indicates percentage of reactivation, a_0 designates activity of intact enzyme, a_i indicates activity of inhibited enzyme and a_r designates activity of reactivated enzyme minus oximolysis. Each measurement was performed at standard laboratory temperature (25°C) and was repeated in triplicate. Calculations were performed using software Microsoft Excel (Redmont, WA, USA) and GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA).

Inhibition of Human AChE

AChE inhibitory activities of tested compounds were determined using modified Ellman's method[1,9] and are expressed as IC_{50}, i.e. concentration of compound required for 50% reduction in enzyme activity. Human red blood cell AChE (RBC-AChE; E.C. 3.1.1.7), 5,5´-dithiobis(2-nitrobenzoic acid) (Ellman´s reagent; DTNB), phosphate buffer solution (PBS, pH 7.4) and acetylthiocholine (ATCh) were purchased from Sigma-Aldrich (Prague, Czech Republic). Polystyrene Nunc 96-well microplates with flat bottom shape (ThermoFisher Scientific, USA) were used for the measuring purposes. All the assays were carried out in 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 7.4. Enzyme solutions were prepared at 2.0 U/mL in 2 mL aliquots. The assay medium (100 μL) consisted of 10 μL of enzyme, 40 μL of 0.1 M PBS

(pH 7.4), 20 μL of 0.01 M DTNB, 10 μL of tested compound and 20 μL of 0.01 M substrate (ATCh iodide solution). Assayed solutions of target compounds (10 μL, 10^{-3} – 10^{-9} M) were preincubated with AChE for 5 minutes. The reaction was initiated by addition of 20 μL of substrate. The activity was determined by measuring of the increase in absorbance at 412 nm at 37°C in 2 minutes intervals using Multi-mode microplate reader Synergy 2 (Vermont, USA). Each concentration was assayed in triplicate. Percentage of inhibition (*I*) was calculated from the measured data as follows:

$$I = \left(1 - \frac{\Delta A_i}{\Delta A_0} \right) \times 100 ,$$

where ΔA_i indicates absorbance change provided by cholinesterase exposed to anticholinesterase compound. ΔA_0 indicates absorbance change caused by intact cholinesterase, where phosphate buffer was applied in the same way as the anticholinesterase compound. Software Microsoft Excel (Redmont, WA, USA) and GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA) were used for the statistical data evaluation.

Kinetic Characterization of AChE Inhibition

The kinetic study of RBC-AChE inhibition was performed by using Ellman's method (described above).[1,9] For the measurements following concentrations of substrate were used: 0.156, 0.313, 0.625 and 1.250 mM. V_{max} and K_m values, respectively, of the Michaelis-Menten kinetics as well as K_i and $K_{i'}$ values were calculated by non-linear regression from the substrate velocity curves. Linear regression was used for calculation of Lineweaver-Burk plots. All calculations were performed using GraphPad Prism software.

Molecular Modeling Studies

From the online RCSB protein databank (www.pdb.org) models of *h*AChE (PDB ID: 4EY7, resolution: 2.35 Å) and *m*AChE (PDB ID: 2Y2U, resolution: 2.60 Å) were downloaded and prepared for flexible molecular docking by MGL Tools utilities. The preparation of these enzymes involved removal of the surplus copies of the enzyme chains and non-bonded inhibitors, addition of polar hydrogens and merging of non-polar ones. Default Gasteiger charges were assigned to all atoms. Flexible parts of the enzymes were determined by a spherical selection of residues approximately around the center of the active site. A grid box of 33 × 33 × 33 Å was positioned to encompass all flexible residues selected for *h*AChE and *m*AChE. The rotatable bonds in the flexible residues were detected automatically by AutoDock Tools 1.5.4 program. Because of the limitation of the program used for flexible molecular docking, water molecules had to be removed from the system. The flexible enzyme parts contained 40 residues for *h*AChE and 27 for *m*AChE. Following xyz coordinates of the grid box centers were applied: *h*AChE (10.698, -58.115, -23.192); *m*AChE (28.21, 15.53, 12.40). The studied ligand **5** was firstly drawn in HyperChem 8.0, then manually protonated as suggested by MarvinSketch 6.2.0. software (http://www.chemaxon.com), geometrically optimized by semi-empirical quantum-chemistry PM3 method and stored as pdb files. The structure of the ligand **5** was processed for docking in a similar way as abovementioned flexible parts of the enzymes

by AutoDock Tools 1.5.4 program. Flexible molecular docking was carried out in AutoDock Vina 1.1.2 program utilizing computer resources of the Czech National Grid Infrastructure MetaCentrum. The parallelized search algorithm of AutoDock Vina efficiently combines a Markov chain Monte Carlo-like method for the global search and a Broyden-Fletcher-Goldfarb-Shano gradient approach for the local search.[10] It is a type of memetic algorithm based on interleaving stochastic and deterministic calculations. Each docking task was repeated 10 times with the exhaustiveness parameter set to 16, employing 16 CPU in parallel multithreading. From the obtained results, the solutions reaching the minimum predicted Gibbs binding energy were taken as the top-scoring modes. The graphic representations of the docked poses were rendered in PyMOL 1.3 (The PyMOL Molecular Graphics System, Version 1.5.0.4, Schrödinger, LLC).

Assessment of Cell Viability

Standard MTT assay (Sigma-Aldrich, Prague, Czech Republic) was applied according to the manufacturer´s protocol on CHO-K1 cells (Chinese hamster ovary, ECACC, Salisbury, UK) in order to compare the cytotoxic effects of compounds of interest. The cells were cultured under ECACC recommended conditions and seeded in a density of 8 000 per well. Tested compounds were dissolved directly in the growth medium (F-12), with the only exception of 7-MEOTA, which was first dissolved in DMSO and subsequently the stock solution was diluted with the growth medium. The cells were exposed to the tested compounds for 24 hours. Then, the growth medium was replaced by a medium containing 10 μM of MTT and the cells were allowed to produce formazan for further 3 hours under surveillance. Thereafter, medium with MTT was sucked out and crystals of formazan were dissolved in DMSO (100 μL). Cell viability was assessed spectrophotometrically by the amount of produced formazan. Absorbance was measured at 570 nm with 650 nm reference wavelength on Synergy HT (BioTek, USA). IC_{50} were subsequently calculated from the control - subtracted triplicates using non-linear regression (four parameters) of GraphPad Prism 5 software for Windows (GraphPad Software, San Diego, CA, USA). Final IC_{50} and SEM values were obtained as a mean of 2-3 independent measurements.

Determination of Cell Viability on HepG2 Cells

Hepatotoxicity of tested compounds was evaluated using cell line HepG2 from human liver hepatocellular carcinoma (ATCC, Virginia, USA). These cells were seeded in 96-well plate at density $17x10^3$ per well in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) with 10% PBS (Gibco, USA) and were left attached overnight. The incubation was performed under conditions of 37°C, 5% CO_2 and 80 – 95% air humidity. Stock solutions of tested compounds were prepared directly in DMEM. Thereafter, they were serially diluted and added to cells in 96-well culture plate. Cell viability was detected using (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described by Mosmann[11] after 24 hours incubation with tested compounds. After 24 hours the medium was sucked out and 100 μL of MTT solution (0.5 mg/mL) in serum free DMEM medium was added to cells. The cells were then incubated for one hour. After that, the medium was sucked out and formed violet crystals of MTT formazan were dissolved in 100 μL of DMSO under shaking. The absorbance was measured with a microplate reader (Beckman Coulter Inc., California, USA) at a test wavelength of 570 nm. The IC_{50} values were

calculated using four parametric non-linear regression with a statistic software GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA). The data were obtained from three independent measurements. The IC_{50} values are expressed as a mean ± SEM.

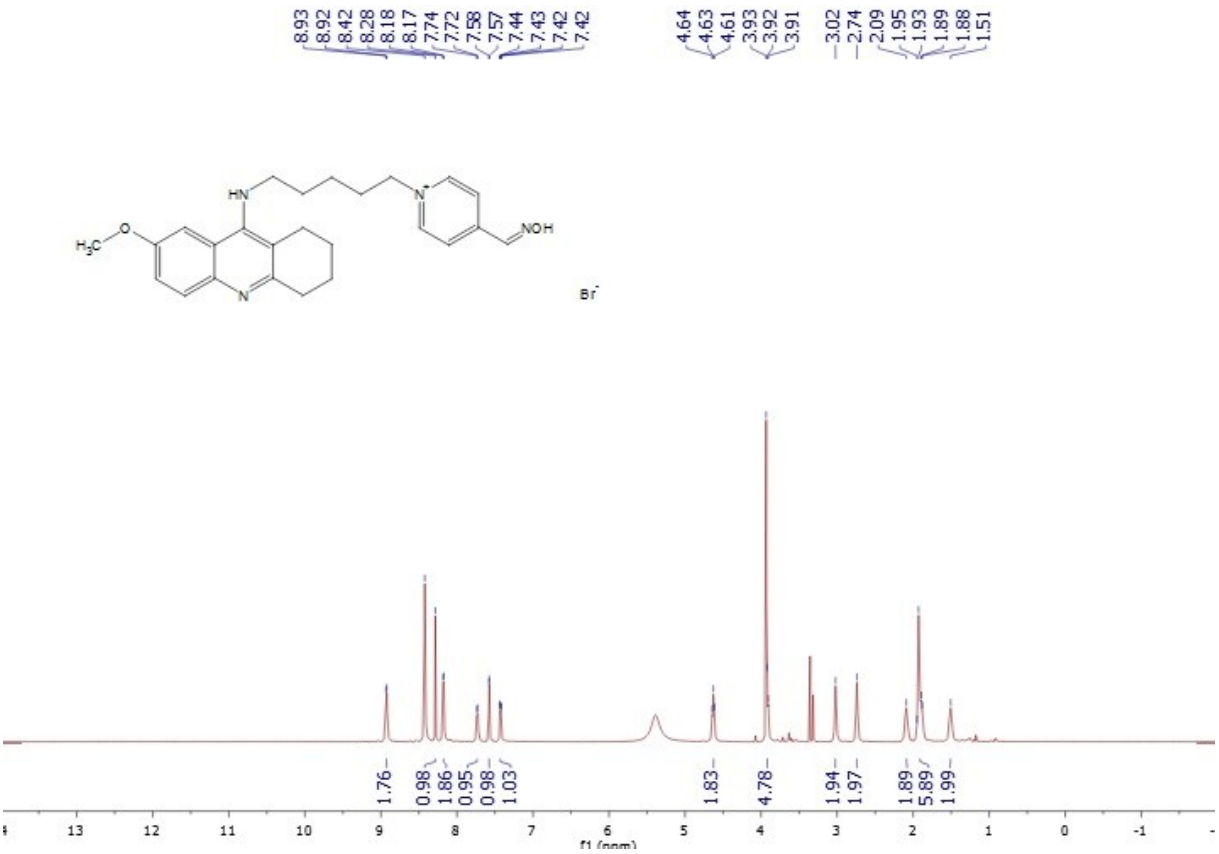

Figure 4. ^{1}H NMR spectrum of compound **5**.

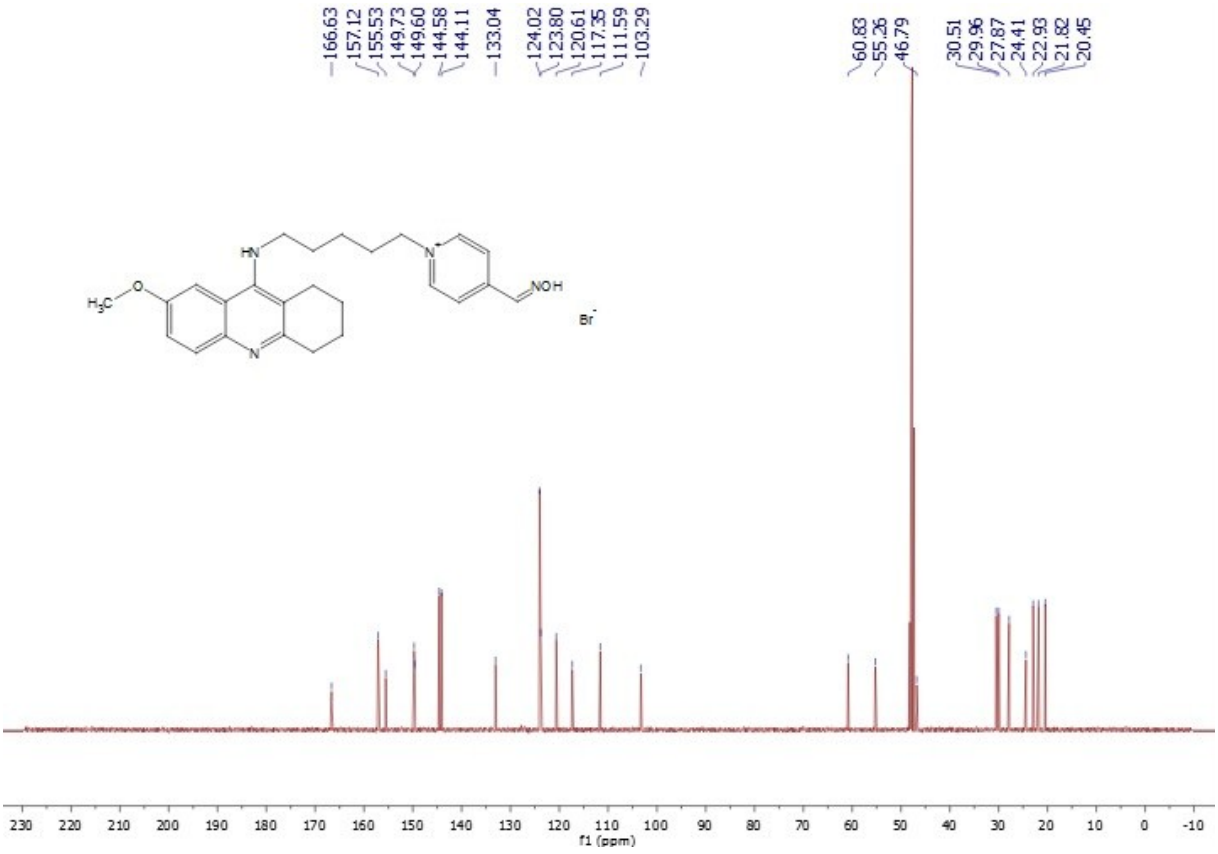

Figure 5. ^{13}C NMR spectrum of compound **5**.

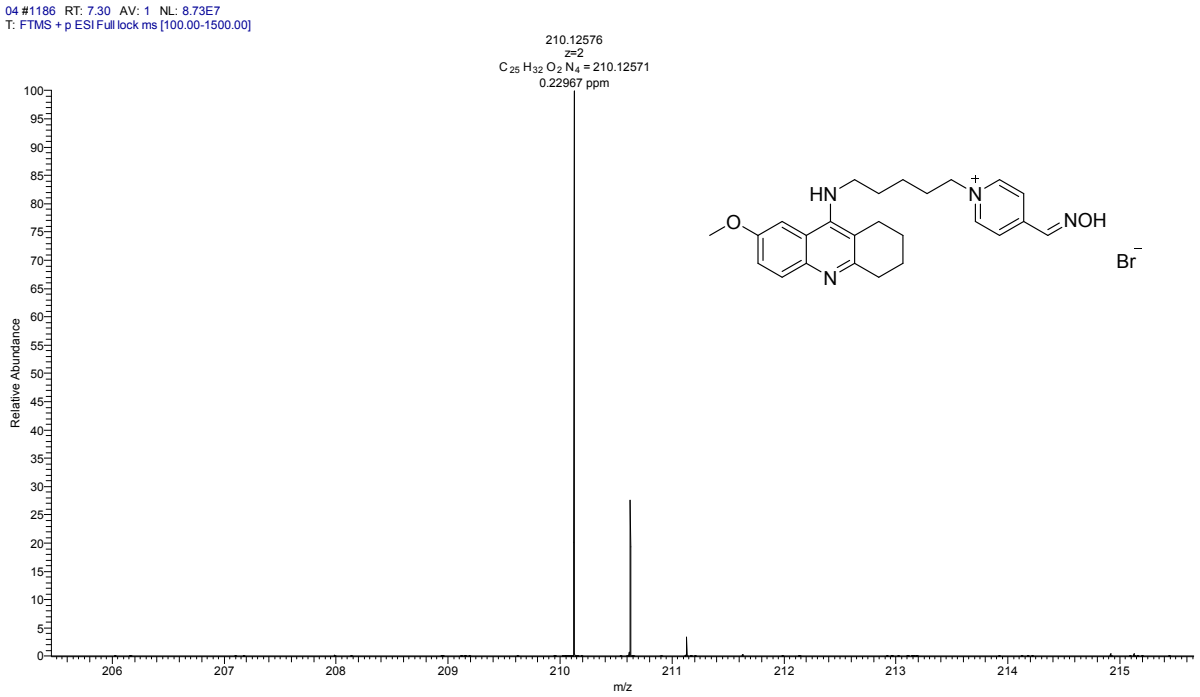

Figure 6. HMRS spectrum of compound **5** (*calculated 210.1257; obtained 210.1258 [M+H]²⁺*).

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