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S02P-2 **Butyrylcholinesterase-proteins interactions in human blood serum**
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S09P-3 **Acetylcholinesterase regulates inflammatory responses in cultured macrophages: a player in cholinergic anti-inflammatory pathway**
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S09P-6 **Indirect effects of dioxin on neuronal AChE expression via astrocytes**
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S09P-8 **Microphthalmia-associated transcription factor regulates acetylcholinesterase expression during melanogenesis of B16F10 cells: a cholinergic regulator in pigmentation**
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S10P-1 **Dual binding site inhibitors of acetylcholinesterase as therapeutic treatments for Alzheimer's disease: any need for an update?**
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S11P-1 **Butyrylcholinesterase inhibitors grafted with antioxidant and neuroprotective activities: novel multifunctional ligands for Alzheimer's disease**
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S11P-2 **7-MEOTA-donepezil hybrids: Potential cholinesterase inhibitors for the treatment of Alzheimer's disease**
Katarina Spilovska *(Czech Republic)*, Eva Mezeiova, Jan Korabecny, Jana Hroudova, Vendula Hepnarova, Martina Hrabinova, Ondrej Soukup, Kamil Musilek, Daniel Jun, Kamil Kuca

ABSTRACTS

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S02P Structure and dynamics of cholinesterases and OP hydrolases

S02P-1 3D structure of natural tetrameric form of human butyrylcholinesterase obtained by Cryo-electron microscopy

Konstantin M. Boyko[1,2], Timur N. Baimukhametov[2], Yury M. Chesnokov[2], Michael Hons[3], Sofya V. Lushchekina[4], Peter Konarev[5], Alexey Lipkin[2], Alexandre L. Vasiliev[2,5], Patrick Masson[6], Vladimir Popov[1,2]

1 Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, Leninsky pr. 33, bld. 2, Moscow, 119071, Russia
2 National Research Center «Kurchatov Institute», Akademika Kurchatova pl. 1, Moscow, 123182, Russia
3 EMBL Grenoble, 71 avenue des Martyrs, CS 90181, 38042 Grenoble Cedex 9, France
4 Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, 4 ul. Kosygina, Moscow 119334, Russian Federation
5 Shubnikov Institute of Crystallography of Federal Scientific Research Centre "Crystallography and Photonics" Russian Academy of Sciences, Leninsky pr. 59, Moscow, 119333, Russia
6 Neuropharmacology Laboratory, Kazan Federal University, 18 Kremlevskaia St., 48000 Kazan, Russian Federation

Human butyrylcholinesterase (BChE) is a stoichiometric bioscavenger of toxic organophosphates. It can be used as an antidote to protect acetylcholinesterase, and is a protein of choice for development of detoxification biocatalysts for clinical applications. Despite the number of different monomeric structures of recombinant human BChE obtained to date, all attempts to obtain an atomic structure of the natural glycosylated tetrameric BChE were unsuccessful.

Here, we present for the first time the 3D structure of the natural tetrameric form of human butyrylcholinesterase, obtained by Cryo-EM technique at a final resolution of 8.8Å. The tetramer has a C2 symmetry, with all subunits arranged as a "propeller-like" tetramer. This is in contrast with previous "flat" model of subunits arrangement in tetramer. Cryo-EM structure shows that the two opposite BChE subunits are placed higher (or lower) the plane of the other two subunits. Despite glycan chains were obscured in the electron density due to their relative disoder, they could be modeled based on the positions of the residues anchoring these glycans. The electron density allowed to distinguish that C-terminal tails of all the subunits interact with each other and form a helix around the PRAD-peptide, supporting rigidity of the tail. The tail is situated in the center of the tetramer and is oriented nearly perpendicular to the tetramer "plane".It was also observed that the subunits in the tetramer have different contacts with neighbouring subunits. This allows to consider the tetramer as a dimer of dimers which is additionally strengthened by the C-terminal tail interactions.

Keywords
butyrylcholinesterase, 3D structure, cryoelectron microscopy, tetramer, native structure

Acknowledgement
The work was supported by the Russian Science Foundation (project 14-24-00172) in part of the structural studies. The tetrameric human BChE was provided by the late B.P. Doctor (WRAIR, Washington DC, USA), father of cholinesterase-based stoichiometric bioscavengers. This work is dedicated to his memory.

S02P-2 Butyrylcholinesterase-proteins interactions in human blood serum

Jacek Jasiecki[1], Krzysztof Waleron[1], Bartosz Wasąg[2,3]

1 Faculty of Pharmacy with Subfaculty of Laboratory Medicine, Medical University of Gdansk, Gdansk, Poland
2 Department of Biology and Medical Genetics, Medical University of Gdańsk, Gdańsk, Poland
3 Laboratory of Clinical Genetics, University Clinical Centre, Gdańsk, Poland

Blood serum proteins serve various functions, including transport of lipids, hormones, vitamins. They are responsible for maintaining acid-base balance, oncotic pressure, plasma viscosity, and functioning of the immune system. There are several hundred different proteins in the blood serum, which total concentration varies within the limits of 6.6-8.7 g/dl, but only a small amount is determined for laboratory diagnostics. One of the serum protein is butyrylcholinesterase (BChE, EC 3.1.1.8), which exists predominantly in the form of a glycosylated tetramer (G4) with a mass of 340 kDa. Four identical subunits assemble into a tetramer by the interaction of a proline-rich peptide with the BChE tetramerization domain at the C-terminus. Our results suggest that BChE interacts with plasma proteins and form much larger complexes than predicted from mass of tetramer. In order to investigate and isolate such complexes we developed a strategy to find protein-protein interactions by combined native size-exclusion chromatography (SEC) with affinity chromatography using resin that binds BChE. Moreover, to confirm specificity of protein complexes we performed also fractionation of blood serum proteins by density gradient ultracentrifugation followed by co-immunoprecipitation using anti-BCHE monoclonal antibodies. The proteins isolated in complexes with BChE were identified by mass spectroscopy.

Acknowledgement
This project was supported by the Polish National Science Center (NCN), grant No. 2017/01/X/NZ4/01522

S02P-3 Dimerization interface of cholinesterases: analysis of crystal structures, free energy molecular dynamics calculations, and *in silico* alanine screening

Novichkova D. A.[1], Lushchekina S. V.[1], Sussman Joel L.[2]

1 Emanuel Institute of Biochemical Physics, RAS, Moscow, Russia
2 Dept. of Structural Biology, Weizmann Institute of Science, Rehovot, Israel

For acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), several oligomeric forms are known. *In vivo*, the monomers in dimers are covalently bound by a C-terminal disulfide bond formed after association of the monomers. Available crystal structures were obtained for truncated forms without disulfide bonds and serve as good models for describing the role of non-covalent interactions in the dimerization of cholinesterases before linking by disulfide bonds.

Here, we analyze the formation of the four-alpha-helix bundle in cholinesterases and differences between AChE and BChE dimers. To identify interactions stabilizing the four-alpha-helix bundle, we counted hydrophobic interactions, solvent accessible surface (SASA), and hydrogen bonds between monomers and estimated electrostatic contributions to dimerization. To reveal the contribution of amino acids in the area of contact to dimerization, we performed free energy perturbation (FEP) alanine screening. Potential of mean force (PMF) calculations of dimerization revealed a difference between acetylcholinesterase and butyrylcholinesterase in the dimerization process and stability of non-covalent dimers.

According to replica exchange molecular dynamics umbrella sampling (REMD-US) calculations, the free energy of BChE dimerization is 20 kcal/mol, which is 15 kcal/mol less than the free energy of hAChE dimerization. BChE has less hydrophobic contacts than hAChE. Electrostatic contribution to oligomerization energy is almost the same for hAChE, mAChE, tcAChE, and BChE. In the case of BChE, contribution from the loops surrounding the helices forming bundles is less significant than that from the helices, whereas in all AChEs, vice versa. The *in silico* alanine screening showed that hydrophobic interactions between the helices are most important for dimerization with stabilization by charged amino acids, mostly lying on surrounding loops.

Keywords
acetylcholinesterase, butyrylcholinesterase, free energy perturbation, in silico alanine screening, replica exchange

Acknowledgement
Supported by the Russian Science Foundation (project #14-13-00124)

S03P Interaction of cholinesterases with substrates, inhibitors and reactivators

S03P-1 4-aminoquinolines as reversible inhibitors of human cholinesterase activity

Anita Bosak[1], Dejan M. Opsenica[2], Goran Šinko[1], Matija Zlatar[2], Zrinka Kovarik[1]

1 Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10000 Zagreb, Croatia
2 Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 12-16, 11000 Beograd, Serbia

We synthesised eight derivatives of 4-aminoquinolines differing in the substituents attached to the C(4)-amino group and C(7) carbon of 4-aminoquinoline, and tested their potency to inhibit human AChE and BChE. All of the compounds reversibly inhibited both enzymes with dissociation inhibition (*Ki*) constants from 0.50 to 50 μM exhibiting selectivity. In other words, for all compounds, AChE exhibited higher affinity than BChE. The most potent inhibitors of AChE were compounds with an octyl chain or adamantane, regardless of the group in position C(7). The shortening of the chain length caused the AChE inhibition decrease by 5-20 times. Docking studies made it clear that the high AChE affinity resulted from simultaneous interactions of the quinoline group with aromatic residues of both the catalytic active site and the peripheral site. In conclusion, the inhibition potency and selectivity classify several novel compounds as leads for further modification and optimization towards the development of new inhibitors of AChE and potential drugs for treatment of neurodegenerative diseases.

Keywords
acetylcholinesterase, butyrylcholinesterase, treatment, 4-aminoquinoline, Alzheimer's disease

Acknowledgment
this study was financed by the Croatian Science Foundation (Grant. No. 4307) and Ministry of Science and Technological Development of Serbia (Grants no. 172008 and 172035)

S03P-2 Synthesis of Nerve Agents' surrogates from Dialkyl Alkylphosphonates for Antidote Screening and Toxicological Studies

Samir F. de A. Cavalcante[1,2,3], Leandro B. Bernardo[1], Kamil Kuča[2], Alessandro B. C. Simas[3]

1 Instituto de Defesa Química, Biológica, Radiológica e Nuclear – IDQBRN, Exército Brasileiro, Centro Tecnológico do Exército, Avenida das Américas 28705, Área 4 – IDQBRN, Guaratiba, Rio de Janeiro, 23020-470, Brazil.
2 Department of Chemistry, Faculty of Science, University of Hradec Kralové, Rokitanskeho, 62, 50003, Hradec Kralové, Czech Republic.
3 Instituto de Pesquisas de Produtos Naturais – IPPN, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco H, Cidade Universitária, Rio de Janeiro, 21941-902, Brazil

Nerve Agents are toxic organophosphorus compounds which inhibit cholinesterases, pivotal enzyme in Parasympathetic Neurotransmission. As they are Schedule 1 compounds in accordance to Chemical Weapons Convention, strict controls are applied and some research groups may have their work hampered due to requirements for synthesis and manipulation. Nerve Agents' surrogates have emerged as affordable substitutes for more realistic approach for development of antidotes and biochemical and toxicity studies, as they are structurally related to Nerve Agents and considered as CWC Schedule 2 compounds, yielding similar enzyme adducts. As Laboratório de Análises Químicas – LAQ (ISO 17025) at IDQBRN have been participated in OPCW Proficiency Tests, striven to obtain the "OPCW Designated Laboratory" status, we have synthesized different dialkyl alkylphosphonates for verification purposes. Therefore, we have proposed synthesis of surrogates for our research on Medicinal Chemistry using them as starting materials. They have proven to be very useful compounds in our research and our syntheses have delivered good yields and purity of final compounds.

Keywords
Nerve Agents' Surrogates, Chemical Weapons Convention, Cholinesterases, Drug Screening, Dialkyl Alkylphosphonates.

Acknowledgments
S.F.A.C. would like to thank OPCW for research grant (L/ICA/ICB/201062/15) and Brazilian Army and University of Hradec Kralové for travel grant.

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S03P-3 Biological evaluation of cysteine targeted insecticides

Hrabinova M.[1,2], Schmidt M.[1,3], Gorecki L.[1,2], Kucera T.[1,2], Psotka M.[1,3], Svobodova B.[1,2], Hrabcova V.[1,3], Hepnarova V.[1,2], Jun D.[1,2], Kuca K.[1,3], Musilek K.[1,3], Korabecny J.[1,2]

1 University Hospital Hradec Kralove, Biomedical Research Centre, Sokolska 581, 500 05 Hradec Kralove, Czech Republic
2 University of Defense, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Trebeska 1575, 500 01 Hradec Kralove, Czech Republic
3 University of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62, 500 03 Hradec Kralove, Czech Republic

According to the World Malaria Report, there were 216 million cases of malaria with 445000 causalties in 2016. Current anticholinesterase insecticides, such as carbamates and organophosphates, act via covalent modification of serine at the bottom of the active site. Traditional chemical insecticides are highly toxic to insect but similarly to mammals. The cysteine-targeting concept of new insecticides is focused on cysteine 447 located in the peripheral site of mosquito acetylcholinesterase. In mammalian enzyme, the cysteine residue is replaced by phenylalanine, whereas honeybees or bumble-bees have this cysteine residue protected. This approach has been proposed to overcome insecticide resistance and to develop promising environmental-friendly insecticides.

The eight cysteine-targeted insecticides (succimides or maleinimides) were prepared via optimised synthetic route. The inhibitory activity of novel compounds and standards (paraoxon, bendiocarb and carbofuran) towards human acetylcholinesterase, human butyrylcholinesterase and mosquito acetylcholinesterase from *Anopheles gambiae* were determined using the modified spectrophotometric Ellman's method. The potentiometric titration using acetylcholine as a substrate was used for validation of Ellman's method. All data showed that the IC_{50} values obtained from both methods were almost similar. Human butyrylcholinesterase was used as common off-target for acetylcholinesterase inhibitors, and no inhibitory effect was determined. The binding mode of the inhibitors was determined using the rapid dilution assay.

Pyridinium maleimides were found with excellent efficacy towards mosquito acetylcholinesterase in contrast to the human enzyme and with significantly improved selectivity index compared to paraoxon. Despite some limitations, we believe that specific optimisation of the structure of molecule connected to maleimide moiety may lead to the development of novel promising insecticides.

This work was supported by Ministry of Health of the Czech Republic (no. 16-34390A) and University of Defense (Long-term organization development plan Medical Aspect of Weapons of Mass Destruction).

Keywords
malaria, acetylcholinesterase, insecticide, cholinesterase inhibitor, cysteine

S03P-4 An alternative substrate for human erythrocyte Acetylcholinesterase activity detection

Sheemona Chowdhary[1], Rajasri Bhattacharyya[1], Dibyajyoti Banerjee[1]

1 Department of Experimental Medicine and Biotechnology, Post Graduate Institute of Medical Education and Research, Chandigarh, India

Acetylcholinesterase (AChE) is the target of pesticides like organophosphates (OP). OP exert their toxic effect by irreversible phosphorylation of the AChE leading to cholinergic crisis and neurotoxicity. Erythrocyte AChE is the surrogate biomarker for the detection of inhibition by OP. There are numerous methods for the detection of AChE activity.[1] Unfortunately, the method popularly used for AChE detection has inherent limitations.[1] To overcome such a problem, we have explored 1-Naphthyl acetate (1-NA) as

an alternative substrate for the assessment of AChE activity using in silico tools and in vitro experiments. The in silico results have shown that 1-NA is a better substrate for AChE. The fluorescence and chromogenic properties of 1-naphthol were studied. The results proved that 1-NA has specificity for AChE similar to Acetylthiocholine. Moreover, it was observed that in terms of Michaelis constant (Km) 1-NA is a better substrate than Acetylthiocholine. We believe that 1-NA is a candidate substrate for development of a method for screening of OP poisoning.

Keywords
1-naphthyl acetate; organophosphorus pesticides; acetylcholinesterase

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S03P-5 Acetylcholinesterase reactivators based on oxime-functionalized biodegradable ionic liquids

Yevgen Karpichev[1], Illia Kapitanov[1], Nicholas Gathergood[1], Ondřej Soukup[2], Vendula Hepnarova[3], Daniel Jun[3], Kamil Kuča[4]

1 Department of Chemistry and Biotechnology, School of Science, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia yevgen.karpichev@ttu.ee
2 Biomedical Research Center, University Hospital Hradec Kralove, Sokolska 581, 500 05 Hradec Kralove, Czech Republic
3 Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defense, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
4 Department of Chemistry, Faculty of Science, University of Hradec Kralove, Rokitanskeho 62, 500 03 Hradec Kralove, Czech Republic

Progress in the development of biodegradable ionic liquids (ILs) [1] allowed finding sustainable fragments to assist the synthesis of sustainable molecules by means of "benign by design" approach. Based on our recent experience in creating micellar catalytic systems for decomposition of organophosphates [2, 3] we have elaborated the following oxime-functionalized low-toxic biodegradable ILs as potential AcChE reactivators: amide/ester linked (amino acid free) IL (I) as well as L-alanine (II) and L-phenylalanine (III) containing compounds with pyridinium aldoxime moiety in cationic part. Variation of amino acid variation (e.g. Me for I and phenyl for II) can help us to analyze a role of hydrophobicity of IL's cation in AcChE reactivation. The reactivation capacity of novel ILs were evaluated towards AcChE inhibited by typical toxic organophosphate agents. The regularities of antidotal activity of studied compounds are to use in the further improvement of their structures.

Keywords
reactivators; oximes; functional ionic liquids

Acknowledgement
The authors acknowledge funding from Estonian Research Council grant PUT1656 and EU 7th Framework Programme for research, technological development and demonstration activities under grant agreement No. 621364 (TUTIC-Green).

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S03P-6 *In silico* screening of novel BChE-reactivators

Tomas Kucera[1], Rafael Dolezal[2], Kamil Musilek[2,3]

1 University of Defence, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic; tomas.kucera2@unob.cz
2 University Hospital, Biomedical Research Centre, Sokolska 581, 500 05 Hradec Kralove, Czech Republic
3 University of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62,500 03 Hradec Kralove, Czech Republic

Several years, there are ideas how to use reactivators of BChE in prophylaxis of OP-poisoning. They could be applied in combination with human BChE as a pseudo-catalytic scavenging system. However, the effective hBChE reactivator is still missing.

The aim of this project is to find highly active and plausibly universal reactivator of hBChE. In the first phase, a database of about 6 mil. structures (ZINC Lead Like) was screened by rigid molecular docking. The receptor (hBChE) was found in the PDB database (pdb code 3DJY, hBChE inhibited by tabun) and prepared for docking. For the second phase, over one hundred molecules were selected. These structures were docked to hBChE with flexible residues within the active site. After manual inspection, over twenty molecules were chosen. Such molecules were modified (e.g. addition of oxime moiety, pK_a optimization) and redocked to hBChE with flexible residues. Finally, two novel compounds were recommended for synthesis. The newly designed compounds will be further synthesized and evaluated on the model of OP-inhibited hBChE and hAChE. They could be used for development of new series of hBChE reactivators.

Keywords
butyrylcholinesterase, BChE, oxime, reactivator, pseudo-catalytic scavenger

Acknowledgment
The work was supported by the Grant Agency of the Czech Republic (no. 18-01734S) and University of Defence (Long-term organization development plan Medical Aspect of Weapons of Mass Destruction). Computational resources were provided by the CESNET LM2015042 and the CERIT Scientific Cloud LM2015085, provided under the programme „Projects of Large Research, Development, and Innovations Infrastructures".

S03P-7 Phenyltetrahydroisoquinoline-based triazole compounds are high-affinity potential reactivators of nerve agent-inhibited human acetylcholinesterase

Nikolina Maček Hrvat[1], Jarosław Kalisiak[2], Antonio Zandona[1], Goran Šinko[1], Zoran Radić[3], K. Barry Sharpless[2], Palmer Taylor[3], Zrinka Kovarik[1]

1 Institute for Medical Research and Occupational Health, HR-10000 Zagreb, Croatia
2 Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037, USA
3 Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, CA 92093-0650, USA

Ten phenyltetrahydroisoquinoline-based compounds synthesized using alkyne+azide [3+2] building block cycloaddition were tested as potential reactivators of human acetylcholinesterase (hAChE) inhibited by different organophosphates. Computational docking indicated molecule phenyltetrahydroisoquinoline moiety association with the hAChE peripheral anionic binding site (Trp286, Tyr337 and Tyr341). Therefore, stabilization near the gorge opening seemed to control the general orientation of the pyridinium ring with its attached aldoxime group inserted into the internal gorge of the hAChE active center. All of the oximes were tested *in vitro* as potential reactivators of sarin-, cyclosarin-, tabun- and VX-conjugated hAChE and potent reactivators were identified, especially with the cyclosarin-hAChE conjugate. Nevertheless, in order to acquire results applicable to reactivation *in vivo*, compounds should be tested at concentrations higher than 10μM, which proved limiting due to the concomitant reversible inhibition of unconjugated hAChE. High oxime affinity was observed for hAChE, but not for human butyrylcholinesterase, where an aromatic peripheral site is absent. Therefore, we tested the oximes as reversible inhibitors of hAChE. All of the compounds potently inhibited hAChE with dissociation inhibition constants in nM range. To further explore potential for safe antidotal activity, we tested oxime cytotoxicity on the human neuroblastoma SH-SY5Y cell line. No cytotoxicity was observed at studied concentrations. In conclusion our study has shown that likely binding poses of an oxime in the hAChE active center do not always ensure enhanced enzyme activity for *in vivo* reactivation. Very high affinity of a candidate oxime for unconjugated hAChE may prove counterproductive for reactivation in tissue.

Keywords
nerve agent, oxime reactivators, cholinesterases, reversible inhibitor, cytotoxicity

Acknowledgement
This work was supported by the Croatian Science Foundation (4307) and the NIH CounterACT Program U01 NS 5058048.

S03P-8 *In silico and In vitro* evaluation of two novel oximes K456 and K733 against paraoxon inhibited human acetylcholinesterase and butyrylcholinesterase

Syed M Nurulain[1], M. Qaiser Fatmi[1], Amna Iqbal[1], Shahrukh Malik[1], Huba Kalasz[2], Kamil Musilek[3], Kamil Kuca[3], Georg Petroianu[4]

1 Department of Biosciences, COMSATS University, Park Road, Tarlai, Islamabad, Pakistan.
2 Semmelweis University, Budapest, Hungary
3 University of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62, 500 03 Hradec Kralove, Czech Republic; kamil.musilek@gmail.com
4 Department of Cellular Biology & Pharmacology, Herbert Wertheim College of Medicine, Florida International University, University Park GL 495 D, 11200 SW 8th St, Miami, FL 33199, Florida, USA. gpetroia@fiu.edu

Organophosphorus compounds (OPs) irreversibly inhibit cholinesterases: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). There is wide variety of applications of OP compounds including warfare chemicals and pesticides. Oxime-type reactivators are used to reactivate the OP inhibited AChE and BChE. Present study was aimed to evaluate the reactivation potency of two novel oximes K456 and K733 against organophosphate inhibited AChE and BChE. Efficacy was compared with K27 and pralidoxime (2-PAM). Molecular mechanism of reactivation by the oximes is predicted by *In silico* method. Intrinsic toxicity of novel oximes in term of IC_{50} and 50 % reactivation of inhibited enzymes (R_{50}) were evaluated by *in vitro* methods using human RBC-AChE and plasma BChE. *In silico* study revealed lower free binding energies, but novel oximes did not bind with catalytic anionic site of enzymes. *In vitro* studies showed higher intrinsic toxicity by K456 and K733 than K27 and pralidoxime. R_{50} for human RBC-AChE were K456=203.59μM±66.96; K733= 405.55μM±67.36; K27=2.68μM ±0.98 and pralidoxime 30.71μM±5.10 (mean±SEM) respectively. No substantial reactivation in BChE was noted by tested concentration of novel oximes. The study concludes that oximes with peripheral binding/far from catalytic anionic site are ineffective reactivators. K27 with central (inside the active gorge) binding was superior to all tested oximes.

Keywords
Paraoxon, oxime, molecular docking, K456, K733, K27, pralidoxime

S03P-9 Facile synthesis of cysteine-acetylcholinesterase targeted insecticides

Miroslav Psotka[1,3], Lukas Gorecki[1,2], Barbora Svobodova[1,2], Kamil Musilek[1,3], Daniel Jun[1,2], Jan Korabecny[1,2], Kamil Kuca[1,3]

1 University Hospital Hradec Kralove, Biomedical Research Centre, Sokolska 581, 500 05 Hradec Kralove, Czech Republic; miroslav.psotka@gmail.com;
2 University of Defence, Faculty of Military Health Science, Department of Toxicology and Military Pharmacy, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic;
3 University of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62, 500 03 Hradec Kralove, Czech Republic

Malaria is annually responsible for more than 400 thousands causalties. The disease is transmitted via infected female *Anopheles*

mosquitoes. Spread of the malaria can be prevented by using either chemical compounds known as insecticides or by genetically engineered plants.[1,2] Mechanism of action of currently deployed insecticide involves inactivating acetylcholinesterase (AChE, EC 3.1.1.7) enzyme by binding to Ser360 (*Anopheles gambiae* numbering). More recently, Cys447 located close to active site entrance was emerged as an alternative target to overcome insecticide resistance and also improving selectivity towards insect AChE over mammalian one.[3] In our contribution, we have developed novel, straightforward and facile synthesis for Cys-targeted insecticides containing either maleimide or succinimide scaffolds. Employment of Grubbs olefin metathesis allowed us to obtain the final compounds in multistep synthesis in relatively high yields. We propose that the described synthetic route might be used in large scale-up for further studies.

This work was supported by Ministry of Health of the Czech Republic (no. 16-34390A) and University of Defence (Long-term organization development plan Medical Aspect of Weapons of Mass Destruction).

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S03P-10 Inhibition of human acetylcholinesterase and butyrylcholinesterase by methylene violet 3RAX

Seda Onder, Kevser Biberoglu, Ozden Tacal

Department of Biochemistry, School of Pharmacy, University of Hacettepe, Ankara, Turkey

Cholinesterases are divided into two classes according to differences in substrate specificity, behaviour in high substrate concentrations, inhibitor sensitivity and tissue distribution: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The both enzymes are sensitive to broad spectrum of molecules and may be inhibited by several compounds, such as organophosphate and carbamate pesticides or nerve agents. In a previous study, a phenazine-derived natural product, geranyl-phenazine-diol was shown to inhibit human AChE with IC50 value of 2.62 µM. Phenazines which are naturally produced by bacteria and archaeal *Methanosarcina* species are nitrogen containing tricyclic molecules with antibiotic, antitumor, and antiparasitic activities. Phenazines are used as electron acceptors-donors in wide range of fields including environmental biosensors.

In this study, the inhibitory effect of a synthetic phenazine dye, methylene violet 3RAX (also known as diethyl safranine) was tested on human erythrocyte AChE and human plasma BChE and its inhibitory mechanism on both enzymes was studied in detail. AChE and BChE activities were assayed spectrophotometrically at 25 oC in 50 mM MOPS buffer pH 8, using 0.05-0.4 mM butyrylthiocholine or 0.025-0.4 mM acetylthiocholine as substrate, 0.125 mM DTNB and 0-80 µM dye. Kinetic analyses showed that methylene violet 3RAX acts as a hyperbolic noncompetitive inhibitor of AChE with K_i value of 1.42±0.09 µM; α=1 β=0.11. On the other hand, it caused linear competitive inhibition of BChE with K_i value of 0.46±0.02 µM; α=∞. In conclusion, methylene violet 3RAX with K_i value in the low micromolar range may be a promising lead candidate for the treatment of Alzheimer's disease.

Keywords
Acetylcholinesterase, butyrylcholinesterase; methylene violet 3RAX; cholinesterase inhibition.

S03P-11 Molecular modeling studies on the interactions of aflatoxin B1 and its metabolites with pheripheral and catalytic anionic sites of human acetylcholinesterase

Joyce S. F. D. de Almeida[1], Rafael Dolezal[2,3], Samir F. de A. Cavalcante[4], Kamil Kuca[2], Kamil Musileck[2], Daniel Jun[5], Tanos C. C. França[1,3]

1 *Laboratory of Molecular Modeling Applied to Chemical and Biological Defense (LMDQB) Military Institute of Engineering, Praça General Tibúrcio 80, 22290-270 – Rio de Janeiro, Brazil;*
2 *Department of Chemistry, Faculty of Science, University of Hradec Králové, Rokitanskeho, 62, 50003 Hradec Králové, Czech Republic;*
3 *Faculty of Informatics and Management, Center for Basic and Applied Research, University of Hradec Králové, Rokitanskeho, 62, 50003 Hradec Králové, Czech Republic;*
4 *Institute of Chemical, Biological, Radiological and Nuclear Defense – ICBRND, Technological Center of the Army – CTEx, Guaratiba, Rio de Janeiro, 23020-470, Brazil;*
5 *Department of Toxicology, Faculty of Military Healthy Sciences, University of Defense, Trebesska 1575, 500 01, Hradec Kralové, Czech Republic*

Aflatoxins are secondary metabolites of the fungi *Aspergillus flavus* and *A. parasiticus*. Among them Aflatoxin B1 (AFB1) is the most frequent type in nature and the most carcinogenic and hepatotoxic for mammals. AFB1 is also inhibitor of the enzyme acetylcholinesterase (AChE) and, therefore, a potential chemical and biological warfare agent, as well as its metabolites. In order to investigate this, we performed inedited theoretical studies on the interactions of AFB1 and its metabolites inside the catalytic and the peripheral anionic sites (CAS and PAS) of human acetylcholinesterase (*Hss*AChE), to verify their stability, suggest the preferential ways of inhibition, and compare their behavior to each other. Molecular docking, molecular dynamics and MM-PBSA calculations for the systems *Hss*AChE/AFB1-metabolites, on both sites were performed. All the metabolites presented negative values of interaction energies in comparison to AFB1. This suggests that they can be better inhibitors of *Hss*AChE. Also, the energy values obtained for the CAS were lower than for the PAS for all metabolites, suggesting that they may preferentially bind in the CAS and come closer to the active site. This behavior is different from the experimentally observed for AFB1, pointing to a different way of inhibition for its metabolites.

Keywords
aflatoxin B1, metabolites, acetylcholinesterase, pheripheral anionic site, catalytic anionic site.

Acknowledgements
The authors wish to thank the Military Institute of Engineering, University of Hradec Kralové, Federal University of Rio de Janeiro, and University of Defense for the infrastructure, the Brazilian financial agencies CNPq (Grant no 306156/2015-6), FAPERJ (Grant no E-02/202.961/2017), for financial support. This work was also supported by the excellence project FIM.

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S03P-12 Chlorinated pyridinium oximes are potent reactivators of acetylcholinesterase inhibited by nerve agents

Tamara Zorbaz[1], Nikola Maraković[1], Kamil Musilek[2], Zrinka Kovarik[1]

1 Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10001 Zagreb, Croatia
2 University of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62,500 03 Hradec Kralove, Czech Republic

Chlorinated bispyridinium aldoximes (Cl-oximes) analogous to previously reported efficient reactivators of inhibited AChE (K027, K048, K203) were designed and synthesized with the premise that the addition of a chlorine atom increases their lipophilicity in comparison to the reference oximes and that they could therefore achieve higher brain concentrations than the ones reported for non-chlorinated analogues. The affinity of hAChE for Cl-oximes was moderate, but higher than for analogous non-chlorinated oximes, as well as higher than the affinity of hBChE for Cl-oximes. Their reactivation efficacy for nerve agent-inhibited AChE was in the following order: cyclosarin>VX>sarin>tabun. Predictably, the electron-withdrawing effect of the chlorine atom led to a lower pK_a value of the oxime groups as confirmed by UV/VIS measurements. Finally, using the molecular modelling approach we attempted to attribute the differences in the predicted binding modes of the tested oximes to their observed reactivity. As molecular docking results suggested, the non-bonding interactions between the chlorine atoms and neighbouring amino acid residues play a significant role in the stabilization of Cl-oximes in a productive conformation in the case of cyclosarin-inhibited AChE.

Keywords
organophosphates, antidotes, HI-6, 2-PAM

Acknowledgment
This work was supported by the Croatian Science Foundation (no. 4307) and the Grant Agency of the Czech Republic (no. 18-01734S).

S03P-13 Bistable dynamic behavior of endogenous butyrylcholinesterase expressed in Expi293 cells

Irina Zueva[1,4], Sofya Lushchekina[2], Oksana Lockridge[3], Lawrence M. Schopfer[3], Patrick Masson[4]

1 A.E. Arbuzov Institute of Organic and Physical Chemistry Subdivision of the Federal State Budgetary Institution of Science „Kazan Scientific Center of Russian Academy of Sciences", 420088, Kazan, Russia
2 N. M. Emanuel Institute of Biochemical Physics of Russian Academy of Sciences, 119334, Moscow, Russia
3 Eppley Institute, University of Nebraska Medical Center, Omaha, NE 68198-5900, USA
4 Neuropharmacology Laboratory, Kazan Federal University, 420021, Kazan, Russia

An endogenous tetrameric wild-type human BChE expressed in Expi293 cells hydrolyzes the neutral substrate N-methylindoxyl acetate (NMIA) with the same K_m as wild-type huBChE (0.14 mM) [1]. For this enzyme, the steady state is preceded by a pre-steady state phase of several minutes in 10 mM Bis-Tris, pH 7 at 25°C.

Thermal inactivation of this BChE is biphasic. Kinetic constants (k_1 and k_2) for thermal inactivation shows differences between this mutant and plasma derived wtBChE: the Expi293 is more stable at 55°C and less stable at 60°C than natural wtBChE [2]. At 55°C half-life times of the first and the second phases are 11 min and 43 min for plasma wtBChE; 14 min and 36 min for the Expi293 wtBChE, respectively. At 60°C, the corresponding values are 6 min and 14 min for natural wtBChE; 3 min and 11 min for Expi293 wtBChE. The endogenous enzyme is more stable in urea: urea-induced denaturation is 10 % slower than for the wtBChE and the urea concentration at the mid-point of denaturation is 4.1M for wtBChE and 4.6M for the endogenous enzyme.

A bistable dynamic behavior of the endogenous BChE is also observed from pre-steady state behavior for hydrolysis of 1 mM NMIA, showing either long *lags* or *bursts* under the same conditions while plasma BChE shows only *lags*. Molecular mechanic simulations have been undertaken to determine the molecular basis of bistability of this wild-typeBChE.

Acknowledgement
This work was supported by the Russian Science Foundation grant #17-14-01097 to PM.

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S03P-14 Expi 293 cells expressing an endogenous wild-type butyrylcholinesterase, and a variety of esterases that self-reactivates after phosphylation by all types of organophosphorus agents

Irina Zueva[1,3], Oksana Lockridge[2], Lawrence M. Schopfer[2], Patrick Masson[3]

1 A.E. Arbuzov Institute of Organic and Physical Chemistry Subdivision of the Federal State Budgetary Institution of Science „Kazan Scientific Center of Russian Academy of Sciences", 420088, Kazan, Russia
2 Eppley Institute, University of Nebraska Medical Center, Omaha, NE 68198-5900, USA
3 Neuropharmacology Laboratory, Kazan Federal University, 420021, Kazan, Russia

A human embryonic kidney cell line (Expi293), adapted for suspension growth in serum-free medium, secretes a tetrameric butyrylcholinesterase (BChE). Expression levels are very low, but are increased 10-fold upon treatment with polyethylenimine. DNA sequencing shows that this enzyme is wild-type BCHE.

This endogenous BChE displays catalytic properties very close to that of natural huBChE with butyrylthiocholine and N-methylindoxyl acetate as substrates [1]. Several endogenous co-secreted esterases self-reactivate after inhibition by echothiophate, paraoxon, cresyl saligenin phosphate (CBDP), racemic coumarin(CM)-soman, CM-tabun and CM-VX. Overall reactivation rate constants, k_r, of diethylphosphorylated enzymes after inhibition by echothiophate and paraoxon are 0.171 min^{-1} and 0.059 min^{-1}, respectively, suggesting multiple OP-hydrolyzing enzymes. After phosphonylation by CM-soman, CM-tabun and CM-VX, k_r values range from 0.0375 min^{-1} to 0.0078 min^{-1}. k_r of CBDP-inhibited enzyme is 0.028 min^{-1}. Interestingly, an apparent aging rate is observed after phosphylation. The aging rate of the soman-phosphonylated enzyme(s) is approximately 2-fold slower than for wtBChE (half-time =16 min against 9 min for wtBChE [2]). The half-time for aging after inhibition by CBDP is 31 min whereas aging of wtBChE-CBDP is almost instantaneous [3]. Diethylphosphorylated enzyme(s) inhibited by paraoxon and echiothiophate age(s) with apparent k_a =0.162 min^{-1} and 0.057 min^{-1}, respectively. This difference also supports the multiple enzyme hypothesis. Further studies are in progress to indentify the different OP-reacting enzymes produced by this Expi293 cell line.

Acknowledgement
This work was supported by the Russian Science Foundation grant #17-14-01097 to PM.

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S04P Reactivators of AChE, OP inhibitors - mechanism of toxicity, detection and analytical methods, diagnosis of exposure, detoxification and therapy; counter-terrorism strategies

S04P-1 Reactivating efficacy of oximes K203 and K027 against a direct acetylcholinesterase inhibitor in rat diaphragm: dose-response modeling

Evica Antonijevic[1], Kamil Musilek[2], Kamil Kuca[3], Danijela Djukic-Cosic[1], Marijana Curcic[1], Zorica Bulat[1], Biljana Antonijevic[1]

1 University of Belgrade, Faculty of Pharmacy, Department of Toxicology "Akademik Danilo Soldatović", Serbia
2 University of Hradec Kralove, Faculty of Science, Department of Chemistry, Czech Republic
3 University Hospital in Hradec Kralove, Biomedical Research Center, Czech Republic

Correspondence: evica.antonijevic@pharmacy.bg.ac.rs

In efficacy testing of experimental oximes, traditionally reactivation of OP-inhibited acethylcholinesterase (AChE) has been analysed by comparing the obtained effects of the single dose with the control [1]. However, quantitative analysis of *in vivo* dose-response data by benchmark dose (BMD) approach would improve both identification and quantification of the effect and it will allow more rigorous comparison of different oximes efficacies [2]. Thus, we have evaluated *in vivo* dose-response relationship for two promising experimental oximes, K203 and K027, concerning reactivation of diaphragmal AChE inhibited by dichlorvos (DDVP). To compare the oximes effects, BMD-covariate method was used to estimate oxime dose (with 90% confidence intervals) that elicits a pre-specified effect size of 50% (1.5-fold increase in AChE activity compared to DDVP-treated group). Wistar rats (5/group) were treated with oxime (0/1.25/2.5/5/25/50% LD_{50} *im*) immediately after DDVP challenge (75% LD_{50} *sc*). Activity of AChE was measured in rat diaphragm homogenates by modified Ellman´s method 60 min after the treatment. Dose-response modeling was done by PROAST software (version 65.5, RIVM, Nederlands). Exponential model m5-ab ($y=a[c-(c-1)\exp(-bx^d)]$) was selected as best estimate with parameters: a_{K203}=0.1525, a_{K027}=0.1498, b_{K203}=0.008472, b_{K027}=0.03941, c=2.117 and d=0.8916. Derived BMD_{50} were K203=117 (56, 209) and K027=21 (10, 37) µmol/kg bw, indicating that oxime K027 induces the same effect size with 5.5-times lower dose compared to oxime K203. Moreover, obtained confidence intervals of BMDs did not overlap allowing the conclusion that more potent dose-response relationship belongs to experimental oxime K027.

Keywords
dichlorvos, benchmark dose, oxime potency, rat diaphragm

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S04P-2 Inhibition of cholinesterases following percutaneous intoxication with V agents in rats

Jiri Bajgar[1,2], Kamil Kuca[1,2,3], Jiri Kassa[3]

1 Department of Chemistry, Faculty of Science, University of Hradec Kralove, Hradec Kralove, Czech Republic.
2 Biomedical Research Center, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic
3 Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic

Female Wistar rats were percutaneously (p.c.) intoxicated (1xLD_{50}) with VX and its two derivatives differing in their substitution on nitrogen (diethyl- and dibutyl- derivatives). Blood cholinesterase activity was continuously monitored; 100 min after the intoxication (or after death), acetylcholinesterase (AChE) activity was determined in diaphragm and brain parts (pontomedullar area - PM, frontal cortex - FC and basal ganglia – BG). Blood ChE activity remains unchanged at very short interval (5 min) after VX administration; this interval was prolonged for diethyl- and dibutyl derivatives. AChE activity was decreased to 20-30% of control values in diaphragm, then in FC (60-70%) and PM (54-74%). AChE activity in BG was relatively resistant (cca 80%). When the AChE activity was compared for all three agents in relationship to survival (11 animals) or death (7 animals), significant differences between the activities in survived (32%) and died (13%) rats were demonstrated in diaphragm but not in the blood. This tendency (higher AChE activity in survived animals) was also observed in PM and FC, however, not statistically significant. It is concluded that substitution on nitrogen atom probably influences penetration through the skin; the rest of agent molecule (phosphorus head) probably influences AChE inhibition. As hypothesis, AChE activity in diaphragm could be important for survival or death in case of p.c. intoxication with these types of V agents.

Keywords
VX, derivatives, blood, rat, diaphragm, brain parts, acetylcholinesterase, percutaneous intoxication

S04P-3 Near attack conformation approach for molecular modeling studies upon the prophylactic agent 7-methoxytacrine-4-pyridinealdoxime hybrid compared with other reactivators of VX-inhibited *Hss*AChE

Jorge Alberto Valle da Silva[1], Eugenie Nepovimova[2], Kamil Kuča[3], Teodorico Castro Ramalho[3,4], **Tanos Celmar Costa França[1,3]**

1 Laboratory of Molecular Modeling Applied to the Chemical and Biological Defense (LMCBD), Military Institute of Engineering, Rio de Janeiro/RJ, Brazil
2 Biomedical Research Centre, University Hospital Hradec Kralove, Czech Republic
3 Center for Basic and Applied Research, Faculty of Informatics and Management, University of Hradec Kralove, Hradec Kralove, Czech Republic
4 Laboratory of Molecular Modeling, Chemistry Department, Federal University of Lavras, Lavras, MG, Brazil

The novel 7-methoxytacrine-4-pyridinealdoxime agent, named hybrid 5C, is a hybrid compound comprised of a linkage between 7-methoxytacrine (7-MEOTA-4-PA) and reactivator 4-pyridinealdoxime (4-PA) moieties through a 5-carbon length-spacer. This compound was formerly designed as a prophylactic agent for intoxication by organophosphates (OP), able to form a complex with acetylcholinesterase (AChE) and reactivate this enzyme in case of OP inhibition. In order to check if the 5 carbons spacer is the ideal to maximize the interactions of this compound inside AChE, we performed in this work docking, molecular dynamics and mmpbsa studies on a series of analogues of hybrid 5C, varying the spacer-length from 1 to 10 carbons long. Our results helped to elucidate the interactions of these compounds with the different binding sites inside human AChE (*Hss*AChE) and pointed to the 4 and 5 carbons long as the best spacers for optimizing these interactions.

Keywords
Acetylcholinesterase; molecular modeling; aldoxime; 7-MEOTA-4-PA.

Acknowledgement
The Authors wish to thank the Brazilian financial agencies CNPq and FAPERJ for financial support, and the Military Institute of Engineering for the physical infrastructure and working space. This work was also supported by the excellence project FIM.

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S04P-4 Design, synthesis and *in vitro* evaluation of a promising new class of bifunctional uncharged hybrid reactivators for nerve agent-inhibited human acetylcholinesterase

José Dias[1], Julien De Sousa[2,4], Yerri Jagadeesh[2], Charlotte Courageux[1], Anne-Julie Gastellier[1], Christopher Timperley[3], Richard Brown[4], Gianluca Santoni[5], Martin Weik[5], Rachid Baati[2], Florian Nachon[1]

1 Département de Toxicologie et Risques Chimiques , Institut de Recherche Biomédicale des Armées, BP73, 91223, Brétigny-sur-Orge, France.
2 Université de Strasbourg, ICPEES, UMR CNRS 7515, 25 rue Becquerel, 67087 Strasbourg, France.
3 Room A117 Building 7, Dstl Porton Down, Salisbury, Wiltshire SP4 0PQ, UK.
4 University of Southampton, Department of Chemistry, Highfield, Southampton, SO17 1BJ, UK.
5 Institut de Biologie Structurale, Campus EPN, 71 Avenue des Martyrs, 38044 Grenoble, France

Acetylcholinesterase (AChE) is a key enzyme of the Central Nervous System (CNS) hydrolyzing the neurotransmitter acetylcholine. By targeting AChE, OPNA and organophosphorus pesticides irreversibly inhibit the cholinergic transmission leading to a certain death if untreated. The current treatment available in the French army consists of an auto-injector containing a methanesulfonate salt of 2-PAM for AChE reactivation, an anticholinergic drug, atropine and avizafone, a prodrug of diazepam for limiting convulsions. However, this treatment displays major drawbacks in terms of CNS bioavailability, restricted spectrum action and effectiveness.

The aim of this project is to develop a new class of more efficient human nerve agent-inhibited acetylcholinesterase. We designed, synthesized and evaluated a new class of bifunctional uncharged hybrid reactivators composed of a 3-hydroxypyridinaldoxime linked to a tacrine derivative. The *in vitro* efficacy of this reactivators has been assessed. We show that this new class of reactivators outperform HI-6 in restoring the human AChE activity inhibited by VX, sarin, tabun and paraoxon. By X-ray crystallography, we have been able to observe some of these new hybrids inside of the catalytic site of hAChE and TcAChE.

Keywords
Acetylcholinesterase, reactivator, organophosphorus.

S04P-5 The early tissue alteration induced by different oximes in rats

Vesna Jaćević[1,2,3], Eugenie Nepovimova[3], Kamil Kuča[3]

1 National Poison Control Centre, Military Medical Academy, Crnotravska 17, 11000 Belgrade, Republic of Serbia
2 Faculty of Medicine of the Military Medical Academy, University of Defense, Pavla Jurišića Šturma 1, Belgrade, Republic of Serbia
3 Department of Chemistry, Faculty of Science, University of Hradec Kralove, Rokitanskeho 62, 500 03 Hradec Kralove, Czech Republic

Newly developed oximes, when taken in overdoses and sometimes even when introduced within therapeutic ranges, may injure the different organs. In this work, we focused our attention on an investigation of morphological lesions produced by increasing doses of asoxime, obidoxime, K027, K048, and K075 were selected as experimental reactivators. The whole experiment was conducted on Wistar rats. All rats were sacrificed 24 hrs and 7 days after single *im* application of 0.1 LD_{50}, 0.5 LD_{50} and 1.0 LD_{50} of each reactivator. Tissue alterations were carefully quantified by semiquantitative grading scales - cardiac, diaphragm, muscular, pulmonary, gastric, hepatic and splenic damage score, respectively. Morfological structure of different tissues treated with of 0.1 LD_{50} of all reactivators were similar to those evaluated in the control groups. Focal and reversible degenerative and vascular changes, were established in tissue samples after treatment with 0.5 LD_{50} of asoxime, obidoxime and K027 ($p < 0.01$ vs. control group). Acute alterations were developed in tissue samples within 7 days following treatment with 0.5 LD_{50} and 1.0 LD_{50} of all reactivators. The most severe tissue alterations were found in rats treated with .0 LD50 of K048 and K075 ($p < 0.001$ vs. control and asoxime group, respectively). Our results showed that all AChE reactivators given by a single, high, unitary dose regimen, have adverse effect not only on the main visceral and muscular tissues, but on the whole rat as well, but the exact cause-effect relationship causing cellular injury remains to be established in further investigation.

Keywords
reactivator; oximes; toxicity; pathohistology; tissue injury.

S04P-6 Cytotoxicity study of oxime@CB7 complexes for central nervous system penetration of quaternary acetylcholinesterase reactivators

Petr Jost, Lubica Muckova, Jana Zdarova Karasova

Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence in Brno, Trebesska 1575, 50001 Hradec Kralove, Czech Republic

Acetylcholinesterase (AChE, E.C. 3.1.1.7) reactivators (also known as oximes) represent a class of antidotes that are used as therapeutics in cases of organophosphate (pesticide or nerve agent) poisoning. The AChE reactivators are highly hydrophilic compounds due to their quaternary nitrogen/s and hydrophilic oxime groups included in the structure. The absorption and distribution of such antidotes is limited by these structural factors. Their delivery may be improved through their encapsulation into macrocycles. Use of these vehicles may provide some retention effect or better targeting into the central nervous system via enhanced biological barriers´ permeability.

Cucurbit[n]urils are a family of rigid macrocycles provided by the acid condensation of glycoluril and formaldehyde. Encapsulation of oximes K048 and asoxime by cucurbit[7]uril (CB[7]) might provide controlled/delayed drug release from a depot or enhanced biological barriers permeability.

In our work we compared the cytotoxicity of oximes K048 and asoxime with their encapsulated forms using 3-(4, 5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Panel of five different cell lines was used . The cytotoxicity was calculated for 24 h interval after the treatment. Our results show, that oxime@CB7 complexes decrease the cytotoxic effect of oximes used individually.

The work was supported by the institutional support “Long-Term Development Plan (DZRO- ZHN)” and Czech Science Foundation (GA CR) project No. 18-08937S.

S04P-7 Brain Exposure of bis-Pyridinium Oxime KR-26256

Hyun Myung Lee[1], Sunjoo Ahn[1], Sung Hee Cho[1], Soo Bong Han[1], Hyejin Kim[1], Sang Ho Lee[1], Gyeunghaeng Hur[2], **Young-Sik Jung[1]**

1 Korea Research Institute of Chemical Technology, Daejeon 34114, South Korea.
2 Agency for Defense Development, Daejeon 34188, South Korea

A number of strategies through structural modification of pyridinium oximes have been developed to circumvent the Blood-Brain Barrier (BBB). Some of the attempted examples are (1) enhancement of lipophilicity by introduction of a fluorine atom into pyridinium ring, (2) facilitation of glucose transporters introduction of glucose moiety on the pyridinium nitrogen, (3) use of a prodrug by uncharged dihydropyridyl moiety, etc

One of the strategies that our group tried was the introduction of fluorine atoms into the heterocyclic ring of pyridinium oximes to increase their lipophilicity.[1] In our continuing effort towards the development of new oxime reactivators, we were interested in monoquaternary pyridinium oximes with *N*-alkyl side chains, because oximes with hydrophobic side chains may penetrate the BBB more easily than 2-PAM with an *N*-methyl side chain. We also investigated bis-pyridinium oximes with diethyl ether linker between two pyridine rings.

The synthesized pyridinium oximes were evaluated their inhibitory activities on AChE, as well as their potency to reactivate AChE inhibited by paraoxon organophosphorus agent. The plasma and brain disposition of oximes were evaluated in male ICR mice, and the oximes concentrations in the plasma and brain were measured by LC-MSMS analysis. Therefore, KR-26256 which is a bis-pyridinium oxime showed higher brain concentration as well as better brain/plasma ratio compared with HI-6.

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S04P-8 Analysis of bis-pyridinium mono-aldoximes in serum and organs using a high-throughput high performance liquid chromatography approach

Gellert Karvaly[1], Kornélia Tekes[2], Huba Kalász[3]

1 Department of Laboratory Medicine, Semmelweis University, Budapest, Hungary
2 Department of Pharmacodynamics, Faculty of Pharmacy, Semmelweis University, Budapest, Hungary
3 Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, Semmelweis University, Budapest, Hungary

Bis-pyridinium mono-aldoximes (BPMA) are established first-line antidotes against anticholinergic toxicants. Several novel BPMA substances are even more promising candidates, yet their pharmacological properties have not been elucidated. The most prominent candidate compounds are K-117, K-127 and K-269.

A bioanalytical method employing high-performance liquid chromatography with ultraviolet absorbance detection is presented for the quantitative assay of K-117, K-127 and K-269. Following brief sample preparation consisting of extraction or dilution with 0.3 mol/L perchloric acid, the substances were determined in serum, cerebrospinal fluid, kidney, liver, eye and cochlea. The analytes were baseline-separated on a Phenomenex Kinetex EVO-C18 100x3mm (5 µm) column using reversed phase ion-pair chromatography in an isocratic run lasting 4 min and were detected at 275 nm. The employed internal standard was K-117 and K-127 for the evaluation of K-127, and K-117 and K-269, respectively.

The method was validated according to the effective guideline of the European Medicines Agency. The approach has been applied for determining the pharmacokinetic properties of K-117, K-127 and K-269 in rats following intramuscular administration.

The project has been financially sponsored by grant NN126968 of the Hungarian National Office of Research, Development and Innovation (Budapest, Hungary).

S04P-9 A comparison of the reactivating and therapeutic efficacy of a novel bispyridinium oxime K870 with commonly used pralidoxime and the oxime HI-6 in sarin-poisoned rats and mice

Jiri Kassa[1], Vendula Hepnarova[1], Kamil Musilek[1,2], Daniel Jun[1]

1 Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
2 Department of Chemistry, Faculty of Science, University Hradec Kralove, Rokitanskeho 62, 500 03 Hradec Kralove, Czech Republic

The ability of a novel bispyridinium oxime K870 and currently available oximes (pralidoxime, HI-6) to reactivate sarin-inhibited acetylcholinesterase and to reduce acute toxicity of sarin was evaluated. *In vivo* determined percentage of reactivation of sarin-inhibited rat blood, diaphragm and brain acetylcholinesterase showed that the potency of newly developed oxime K870 to reactivate sarin-inhibited acetylcholinesterase roughly corresponds to the reactivating efficacy of pralidoxime with the exception of diaphragm where the oxime K870 was more effective than pralidoxime. However, the oxime HI-6 was found to be the most efficient reactivator of sarin-inhibited acetylcholinesterase. While the oxime HI-6 was able to reduce the acute toxicity of sarin

more than five times, the novel oxime K870 and pralidoxime decreased the acute toxicity of sarin less than three times. Based on the results, we can conclude that the reactivating and therapeutic efficacy of newly developed oxime K870 is significantly lower compared to the oxime HI-6 and, therefore, it is not suitable for the replacement of the oxime HI-6 for the antidotal treatment of acute sarin poisoning.

Keywords
sarin; acetylcholinesterase; K870, pralidoxime, HI-6

The study was supported by the grant of Ministry of Defence – „Long-term organization development plan – Medical Aspects of Weapons of Mass Destruction".

S04P-10 The monoquarternary reactivators for the treatment of organophoshorous intoxication

Gorecki L.[1,2], **Korabecny J.[1,2],** Svobodova B.[1,2], Kucera T.[1], Malinak D.[2,3], Junova L.[1,2], Hepnarova V.[1,2], Hrabinova M.[1,2], Soukup O.[1,2], Jun D.[1,2], Musilek K.[2,3], Kobrlova T.[1,2], Konecny J.[1,2], Psotka M.[1,3], Dolezal R.[2,3], Honegr J.[2], Kuca K.[2,3]

1 Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Czech Republic
2 Biomedical Research Centre, University Hospital Hradec Kralove, Czech Republic
3 Department of Chemistry; Faculty of Science; University of Hradec Kralove; Hradec Kralove; Czech Republic

Mono- and bis-pyridinium aldoximes are the only causal antidotes that are designated for the treatment of organophosphate (OP) poisoning. Intoxication by OPs is caused either by pesticides or by the nerve agents, the latter belong to group of chemical warfare agents. These compounds irreversibly inhibit enzyme acetylcholinesterase (AChE) that is no more able to fulfill its physiological function. Mono- and bis-pyridinium aldoximes are able to restore catalytic function of AChE. The reactivating ability of aldoximes is limited by several drawbacks like low blood-brain barrier permeation, low reactivation potency against specific nerve agents etc. In order to obtain efficient treatment of OP, the introduction of novel AChE reactivators raised as an important issue. For over 60 years of intensive research, none of the reactivators reached sufficient activity. Herein, we present novel mono quaternary reactivators that possess excellent in vitro activity to restore AChE activity after intoxication with different nerve agents as well as pesticides. The molecular docking simulations, total synthesis and biological evaluation will be discussed.

The study was supported by Long-term development plan, by Ministry of Health of the Czech Republic, grant No. 17-32801A, by SV/FVZ201803 and SV/FVZ201708.

S04P-11 Decontamination of warfare agent

Jan Marek[1], Marketa Benkova[1], Jan Misik[2], Ondrej Soukup[1,2], Marek Matula[2], Daniel Jun[2]

1 Biomedical Research Center, Faculty Hospital Hradec Kralove, Czechia
2 Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czechia

Project is aimed at the development of new combined micellar decontamination systems based on quaternary nitrogen compounds having detergent and active decontamination properties, which will cause faster hydrolysis of chemical warfare agents. In the case of biological agents, these molecules are strong disinfectants, able to destabilize pathogen membrane structures. Several decontamination mixtures will be prepared and tested both in vitro and in vivo for their decontamination and disinfection properties against selected chemical and biological agents. The expected result of the project is efficient decontamination solution for personal skin decontamination with good tolerability.

S04P-12 *In vitro* characterization of the standard acetylcholinesterase reactivators

Muckova L.[1], Hepnarova V.[1], Misik J.[1], Jun D.[1], Soukup O.[1]

1 Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defense, Hradec Kralove, Czech Republic

Acetylcholinesterase (AChE; 3.1.1.7) reactivators play a key role in the treatment of organophosphate poisoning. The main mechanism of reactivators is disruption of the covalent bond between organophosphorus compounds and AChE and restore the physiological function of this enzyme. On the other hand, there are some evidence, other mechanisms not related to reactivation, which may lead to survival.

Thus, their effect on muscarinic (M_1 subtype), nicotinic (α_7 subtype) and N-methyl-D-aspartat (NMDA; 2B subtype) receptor was studied. They are able to significantly modulate the receptors at higher concentration (100 μM) and for this reason, their toxicities were tested. Cytotoxicity of standard oximes was evaluated using neuroblastoma cell line SH-SY5Y. MTT assay and real-time cell viability assay were used to measure cytotoxicity of selected compounds.

The tested reactivators showed different cytotoxicity. Methoxime was the most and K027 was the least toxic. Reactivators had no influence on NMDA receptor in tested concertation. The nicotinic receptor was the most inhibit by K027. However, trimedoxime and obidoxime showed the highest inhibition of muscarinic receptor.

Keywords
reactivator, cytotoxicity, muscarinic receptor, nicotinic receptor, NMDA receptor

Acknowledgements
This work was supported by the Ministry of Education, Youth, and Sport, Czech Republic- SV/FVZ201508 and by the Long-term organization development plan Medical Aspects of Weapons of Mass Destruction of the Faculty of Military Health Sciences, University of Defence.

S04P-13 Uncharged Reactivators of Cholinesterases Inhibited by Organophosphorus Nerve Agents

N. Probst[1], A. Braïki[1], P. Warnault[1], J. Renou[1], C. Gomez[1], G. Mercey[1], T. Verdelet[1], R. Baati[2], J. Dias[3], G. Calas[3], F. Nachon[3], M. Weik[4], L. Jean[1], P.Y. Renard[1]

1 Normandie University, COBRA, UMR 6014 and FR 3038; University of Rouen; INSA of Rouen; CNRS, 1 rue Tesniere F-76821 Mont-Saint-Aignan, Cedex, France
2 Université de Strasbourg, UMR CNRS 7515 ICPEES, 25 Rue Becquerel, 67087 Strasbourg, France
3 Département de Toxicologie, Institut de Recherche Biomédicale des Armées BP7391993 Brétigny/s/Orge, France
4 Commissariat à l'Energie Atomique, Institut de Biologie Structurale, F-38054 Grenoble; CNRS, UMR5075, F-38027 Grenoble; Université Joseph Fourier, F-38000, Grenoble, France

The acute toxicity of OPNA results from irreversible inhibition of AChE (EC 3.1.1.7), a key enzyme in neurotransmission, via the formation of a covalent P–O bond at the catalytic serine. Inhibition of AChE leads to the accumulation of acetylcholine neurotransmitter (ACh) in the synaptic cleft causing among other symptoms, seizures and respiratory arrest leading to death.

The current urgency treatment of OPNA poisoning is based on the administration of a cocktail of three components: an antimuscarinic agent (e.g. atropine), an anticonvulsant drug (e.g. diazepam) and mono or bispyridinium AChE reactivator (e.g. pralidoxime, obidoxime, trimedoxime). The high nucleophilicity of these alpha-nucleophiles allows the displacement of the phosphyl group from the catalytic serine, yielding to the restoration of AChE activity.

However, reactivation of central AChE is inefficient due to the fact that positively charged pyridiniums poorly cross the brain blood barrier (BBB). Moreover pyridinium(s) oximes exhibit a quite narrow spectrum of reactivation. Despite decades of research in this field, there are no efficient and general broad-spectrum reactivators for OP-inhibited AChE.

In this context, we have developed families of new uncharged reactivators of OP-inhibited acetylcholinesterase and/or OP-inhibited butyrylcholinesterase with the potential to cross the BBB. Three new families of uncharged reactivators display in vitro reactivation potencies towards VX-, tabun- and paraoxon-inhibited human AChE that are superior to those of the mono- and bis-pyridinium aldoximes (e.g. 2-PAM, HI-6, obidoxime, HLö-7, TMB-4) which include those currently used in the armed forces.

Keywords
organophosphorus, AChE, reactivator, aldoxime, uncharged.

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S04P-14 In vitro determination of oxidative stress induced by oxime reactivators using chromatographic methods

Váňova N., Mišík J., Múčková L., Herman D., Pejchal J.

Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence in Brno, Hradec Králove, Czech Republic
e-mail: nela.vanova@unob.cz

Even though reactive oxygen/nitrogen species (ROS/RNS) are physiologically generated in biological systems, their excessive production may cause severe damage of cellular components. Excessive production of ROS/RNS can occur in response to various stressors such as xenobiotics, radiation or pathological processes. Oxidative stress has also been reported to cause adverse effects of some therapeutic drugs including acetylcholinesterase (AChE) oxime reactivators which are used in therapy of organophosphate poisoning.

In this study, we determined the effect of obidoxime, methoxime, asoxime, pralidoxime and trimedoxime on redox homeostasis in cultured human hepatoma (HepG2) cells. The cells were incubated with oximes at concentration corresponding with their IC_{50} for 1, 4 and 24 hours. Intracellular ROS levels were determined using two fluorescent probes (2',7' dichlorodihydrofluorescein diacetate and dihydroethidium). Malondialdehyde and 3 nitrotyrosine were measured using LC-MS/MS. Additionally, non-protein thiols and non-protein disulfides were evaluated to reflect antioxidant capacity. Individual reactivators displayed distinct quantitative and/or qualitative changes in redox homeostasis reflecting different role of oxidative stress in their intrinsic toxicity. Future perspectives are to test new AChE reactivators synthetized at Department of Toxicology and Military Pharmacy in order to minimalize their unwanted side effect related to oxidative stress.

This work was supported by the Ministry of Defence of the Czech Republic through a Long-term organization development plan 1011.

S04P-15 *In vitro* evaluation of quinuclidinium oximes as reactivators of human cholinesterases inhibited by organophosphorus compounds

Antonio Zandona[1], Ines Primožič[2], Maja Katalinić[1], Zrinka Kovarik[1]

1 Institute for Medical Research and Occupational Health, HR-10000 Zagreb, Croatia
2 Faculty of Science, University of Zagreb, HR-10000 Zagreb, Croatia
E-mail address of presenting author: azandona@imi.hr

This study focused on the evaluation of the use of quinuclidinium oximes as potential antidotes in organophosphorus compound (OPs) poisoning. We determined the reversible inhibition of human red blood cell acetylcholinesterase (AChE) and human plasma butyrylcholinesterase (BChE) by 14 quinuclidinium oximes as well as the reactivation of tabun-, VX-, paraoxon-, sarin- and cyclosarin-inhibited enzymes. Reversible inhibition constants were within 3 µM to 4 mM, depending on the oxime structure. The highest inhibition was observed for Q5, which has a long aliphatic chain on the quinuclidinium ring quaternary nitrogen. It seems that AChE is selective toward oximes that have groups in *meta* position on the benzene ring and BChE to those with a group in *para* position. Quinuclidinium potency to reactivate organophosphorus-inhibited cholinesterases *in vitro* proved promising in restoring cholinesterase activity. VX- and paraoxon-inhibited AChE was reactivated by several candidates at up to 90 - 100 % within 1-4 hours. Oximes with a group in *para* position showed reactivation potency for cyclosarin-inhibited BChE with reactivation up to 90-100 %. Furthermore, at the very beginning of antidote development, we investigated if quinuclidinium oximes are cytotoxic to selected cell lines. As results indicate, quinuclidinium oximes did not show cytotoxic profiles up to 800 µM. An exception was observed only for Q5, an oxime with a long aliphatic chain in the structure, influencing cell vitality at concentrations significant for reactivation of cholinesterases.

Keywords
quinuclidinium, organophosphorus, oximes, reactivation

Acknowledgment
This work was supported by the Croatian Science Foundation grant no. 4307 and UIP-2017-05-7260.

S05P Enzymes and proteins other than ChEs interacting with OP

S05P-1 Determination of BChE Activity by Mass Spectrometric Analysis of Its Adduct with Russian Vx

Murashko E.A., Dubrovskii Ya. A., Beltyukov P.P., Radilov A.S., Babakov V.N.

Research Institute of Hygiene, Occupational Pathology and Human Ecology, Federal Medical Biological Agency, build. 93, Kapitolovo Station, G/P Kuzmolovsky, Vsevolozhsky District, Leningrad Region, 188663, Russia; e-mail: kate.murashko@gmail.com

Phosphonylated butyrylcholiesterase (BChE) is a marker of exposure to organophosphorus compounds, including nerve agents and pesticides. In cases of poisoning with nerve agents, it is important not only to establish the fact of poisoning, but also to give a quantitative estimate. The most common quantitative characteristic is BChE inhibition. We developed a highly sensitive method for the quantification of BChE inhibition by Russian Vx (VR) by mass spectrometry. For model experiments we used donor human blood plasma exposed to VR at concentrations of 1–100 ng/ml.

Butyrylcholinesterase was selectively isolated from plasma by immunoprecipitation and then subjected to enzymatic hydrolysis with pepsin. The hydrolysate was analyzed by HPLC-MS/MS using MRM mode, which allowed determination of the VR-modified nonapeptide FGESAGAAS (m/z 930 Da) at a very low level of VR (1 ng/mL). To measure the inhibition of BChE, an excess of VR is added to one sample, and the nonapeptide peak area is considered to correspond to 100% inhibition. The inhibition of BChE in samples containing different concentrations of VR are determined by ratio of the nonapeptide peak area in each specific sample to that at 100% inhibition.

BChE inhibition,% = (S_{930}/S_{930}100%)*100

It was found that the VR-modified nonapeptide peak area is linearly related to VR concentration. The BChE inhibition measured by mass spectrometry was consistent with the results of Ellman's assay (R^2≥0.98). The advantages of the proposed approach over Ellman's assay include the possibility of quantification of inhibition at low doses of nerve agent and lack of necessity to construct calibration plots.

Keywords
butyrylcholiesterase, nerve agents, VR, adduct, inhibition

S05P-2 An *in-vitro* induction of Paraoxonase 3 activity in Hepatocytes by Resveratrol

Kumari Priyanka[1,3], Kiran Dip Gill[1], Surjit Singh[2], Indu Verma[1]

1 Departments of Biochemistry, Post Graduate Institute of Medical Education and Research Chandigarh-160012, India
2 Departments of Internal Medicine, Post Graduate Institute of Medical Education and Research Chandigarh-160012, India
3 Departments of Immunopathology, Post Graduate Institute of Medical Education and Research Chandigarh-160012, India

ku.priyanka@gmail.com

Background: Managing burden of Coronary Artery Disease (CAD) is a battle for researchers over the globe as disease seems to be multifactorial. Duet concert of genetics and environmental factors over oxidative stress and inflammation accounts for disease progression. Human Paraoxonase 3 an HDL associated endogenous antioxidant enzyme, has been identified as antiatherogenic entity. Modifiable risk factors like diet and lifestyle play a supreme role in regulating paraoxonase activity.

Rationale: To understand how the activity of Paraoxonase 3 can be modulated by using various pharmacological agents to derive the therapeutic benefit in CAD patients.

Methodology: After approval of Institutional review board (No.55/IAEC/293), Hepatoma derived cell line (HepG2) was exposed to resveratrol, tempol, quercetin, simvastatin and nicotine in varying doses. MTT based optimum dose was selected and measured the PON3 enzymatic activity (Spectrophotometry/ HPLC), concentration (ELISA), cellular ROS (using H2DCFH-DA), NOS (Griess assay) and protein expression (western blot) in cell lysates and supernatants.

Results: Resveratrol treatment led to significant increase in PON3 activity ($p \leq 0.001$) in HepG2 cells whereas other pharmacological agents had no major significant effect on PON3 activity, expression and concentration.

Conclusion: PON3 induction by resveratrol translates new avenues in cardio-therapeutics.

Keywords
Paraoxonase , Resveratrol, PON3 activity

Acknowledgements
ICMR, New Delhi

S06P Stoichiometric bioscavanger, biotechnology and therapeutical aspects

S06P-1 Smart & Innovative Tools for Cholinesterases Related Applications

Emilie David[1], Benoît Roubinet[1]

1 CHEMFORASE, Bât. IRCOF n° 40, 1 rue Lucien Tesnière, 76130 Mont-Saint-Aignan, France

CHEMFORASE[1] is a french innovative biotechnological start-up, built in 2016, and specialized in the manufacturing and marketing of affinity resins for purification of cholinesterases. The lead product, Hupresin®, makes it possible to purify efficiently both plasmatic and recombinant human butyrylcholinesterase (BChE). This innovative Hupresin® technology has perfect characteristics to specifically bind cholinesterases, providing the best performances on the market. CHEMFORASE also developed a fast flow affinity resin, Hupresin® AC, efficient for the purification of plasmatic BChE. A new affinity resin with better capacity is under development. These new chromatographic supports should facilitate the large-scale production of BChE and reduce the costs associated for the production of BChE-based drugs such as nerve agents bioscavengers.

Hupresin® Magnet is the magnetic version of Hupresin® that is compatible with the efficient extraction of BChE from small samples. This technology might facilitate the development of diagnosis tool useful for proving exposure to nerve agents and for identifying the type of poison involved.

Based on its know-how, CHEMFORASE offers his knowledge for your research. The company has expertise in organic chemistry synthesis and has laboratory equipment to manage gram scale synthesis: organophosphorus nerve agents simulants, organic fluorophores, fluorescent probes, heterocyclic molecules.

As part of its research and development program, CHEMFORASE is continuously seeking for new academic and industrial collaborations in order to develop innovative tools for cholinesterases applications.

Keywords
butyrylcholinesterase, Hupresin®, affinity resins

[1] www.chemforase.com

S06P-2 *In Vitro* Evaluation of Standard Acetylcholinesterase Reactivators as Reactivators of Human Plasma Butyrylcholinesterase

Lucie Junova[1], Vendula Sepsova[1], Kamil Musilek[1,2], Daniel Jun[1]

1 Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Trebesska 1575, 500 01, Hradec Kralove, Czech Republic
2 Department of Chemistry, Faculty of Science, University of Hradec Kralove, Rokitanskeho 62, 500 03 Hradec Kralove, Czech Republic

Bioscavengers are considered to be a promising approach in the prophylaxis or treatment of poisoning by organophosphorus inhibitors (OPI; nerve agents and organophosphate pesticides). They can efficiently neutralize diverse OPIs in the bloodstream before they reach their natural targets - cholinesterases. Antidotal efficacy of administered butyrylcholinesterase (BChE; EC 3.1.1.8), one of the possible bioscavengers, could be further increased when it is co-administered with an oxime reactivator of a sufficient reactivation potency. Therefore, the activity of BChE, inhibited by OPI, could be continuously renewed (pseudo-catalytic bioscavenger).

In this study, we evaluated the ability of standard reactivators (pralidoxime, obidoxime, HI-6, methoxime and trimedoxime) and newly developed ones (K027, K048 and K203) to reactivate human plasma BChE inhibited by nerve agents (sarin, cyclosarin, VX and tabun) and dimethoxy and diethoxy pesticide (dichlorvos and paraoxon). Overall reactivation potency was decreased as follows: cyclosarin > sarin > VX > paraoxon > dichlorvos > tabun. HI-6 was the most efficient reactivator of cyclosarin- and sarin -inhibited BChE, whereas pralidoxime achieved highest potency for VX. Obidoxime was the most active in the case of pesticide inhibited enzyme. Reactivation of tabun-inhibited BChE was negligible for all tested compounds. Generally, reactivation ability of examined standard reactivators was deficient and uneven as they were designed for the reactivation of acetylcholinesterase. Therefore, there is a need for development of both more balanced and potent reactivators, suitable for pseudo-catalytic bioscavengers. Assayed oximes will serve for further standardization of our in vitro testing method and subsequent evaluation of newly synthesized BChE reactivators.

Keywords
bioscavengers, butyrylcholinesterase, nerve agents, organophosphates, oxime reactivators

Acknowledgement
This work was supported by the Ministry of Defence of the Czech Republic- "Long-term organization development plan Medical Aspects of Weapons of Mass Destruction".

S06P-3 Novel bisquaternary heteroaromatic compounds as potential reactivators of human butyrylcholinesterase

David Malinak[1,2], Eugenie Nepovimova[1], Marketa Neugebauerova[1], Miroslava Hozova[2], Vendula Hepnarova[3], Daniel Jun[3], Rafael Dolezal[1,2], Kamil Musilek[1,2], Kamil Kuca[1]

1 University of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62, 500 03 Hradec Kralove, Czech Republic david.malinak@uhk.cz; eugenie.nepovimova@uhk.cz
2 University Hospital Hradec Kralove, Biomedical Research Centre, Sokolska 581, 500 05 Hradec Kralove, Czech Republic
3 University of Defence, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic

Human butyrylcholinesterase (*h*BChE) is well-known stoichiometric scavenger in case of organophosphorus (OP) intoxication. However, its major limitation lies in binding of only one OP moiety per *h*BChE molecule and thus necessity of its very high dosage prior or post intoxication. This issue might be resolved by use of *h*BChE reactivators that could cleave irreversibly bound OP moiety from the enzyme active site and restore its scavenging function. This concept has been called pseudo-catalytic scavenger. Within our contribution, we would like to present bisquaternary heteroaromatic compounds that are butyrylcholinesterase reactivators and might act as potential pseudo-catalytic bioscavengers. Recently, we have prepared and evaluated over 20 novel compounds that displayed better *h*BChE reactivation activity than clinically used reactivators.

This work was supported by the Czech Science Foundation (No. 18-01734S).

S09P Biological functions, development and non-cholinergic function of cholinesterases

S09P-1 Butyrylcholinesterase and its variants (rs3495 & rs1803274) association with Major Depressive Disorder

Sliha Awan[1], Syed M Nurulain[1], Sadaf Munir[1], Sania Ghafoor[1], Rabia Habib[2], Maleeha Azam[3]

1 Department of Biosciences, COMSATS University, Islamabad, Pakistan
2 Functional Proteomics lab, Department of Biosciences, COMSATS University, Islamabad, Pakistan
3 Functional Genomics lab, Department of Biosciences, COMSATS University, Islamabad, Pakistan

Major Depressive Disorder (MDD) is a psychiatric condition. Globally, it is known to be the fourth leading source of ill health. Butyrylcholinesterase is a cholinergic enzyme with diversified reported functions. Objectives of the present study was to find the status of BChE in depressive individuals and to investigate the association of two SNPs of BCHE (rs3495; c.*189G<A) and (rs1803274; c.*1699G>A). Study was conducted with the approval from Ethical Review Board of the Department of Biosciences and consents from participants. Seventy six MDD patients and fifty four healthy controls were recruited for the study. Depressive individuals were diagnosed by the consultant psychiatrist. BChE activity was measured using plasma by Ellman's method. The blood samples were genotyped for rs3495 using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and rs1803274 by allele refractory mutation system-polymerase chain reaction (tetra-primer ARMS-PCR). Biochemical estimation of BChE showed a significant decrease activity in MDD patients (0.020 µmol/L/min; n=54) than healthy controls (0.028 µmol/L/min; n=76). Genetic analysis revealed no significant association for rs3495. However, the statistical analysis of the genotyped data of rs1803274 showed statistically significant association under dominant model (OR: 2.32; 95% CI: 1.09-4.96; p-value =0.025). Homozygous GG genotype was higher in control (p-value=0.01) as compared to the cases. Significant result was also noted in allele frequency distribution (p-value =0.01). The study concludes that BChE may have a tentative role in pathophysiology of MDD. Genetic association of rs1803274 with the disease is also evident. A further study with different ethnic groups is suggested.

Keywords
Butyrylcholinesterase; rs3495; rs1803274; Major depressive disorder

S09P-2 Alkaloids derived from traditional Chinese medicine are inhibitors for inflammation and acetylcholinesterase

Xiang P. Kong[1,3], Miranda L. Xu[1,2], Etta Y. L. Liu[1,2], Qiyun Wu[1,2], Tina T. X. Dong[1,2], David C. C. Wan[4], Karl W. K. Tsim[1,2]

1 Shenzhen Key Laboratory of Edible and Medicinal Bioresources, SRI, The Hong Kong University of Science and Technology, Shenzhen, China;
2 Division of Life Science, Center for Chinese Medicine, The Hong Kong University of Science and Technology, Hong Kong, China;
3 College of Chinese Medicine, Shanxi University of Traditional Chinese Medicine, Taiyuan 030024, China;
4 School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China

The inhibitors for acetylcholinesterase (AChE), an enzyme hydrolyzing acetylcholine in cholinergic synapses, have been used for the treatment of Alzheimer's disease (AD). Alkaloids inhibiting AChE activity are commonly found in traditional Chinese medicine (TCM), e.g. gelantamine from *Lycoris radiata*, berberine from *Coptis chinensis*, huperzine A from *Huperzia serrata*. Many of these alkaloids also show regulatory role on inflammation, including the suppression on neuro-inflammation. Here, we aimed to reveal the possible relationship of these alkaloids in having both anti-inflammation and anti-AChE properties, in particular the role of which in "cholinergic anti-inflammatory pathway (CAP)". A compound database containing 1,500 alkaloids from 113 kinds of TCM was developed. By molecular docking, the database was probed for AChE-inhibitory effect. Over 200 alkaloids showing AChE binding effect were further tested by its activities in inhibition of AChE, as well as in LPS-induced inflammatory responses. Thus, the current results could provide a good foundation for further research and development of TCM alkaloids on AD treatment.

Acknowledgements
This work was supported by Shenzhen Science and Technology Committee Research Grant (JCYJ20160229205726699, JCYJ20160229205812004, JCYJ20160229210027564, CKFW2016 082916015476, JCYJ20170413173747440, ZDSYS201707281432317 and 20170326). Xiang P. Kong hold a postdoctoral funding from Shenzhen municipal government ([2017]1225).

S09P-3 Acetylcholinesterase regulates inflammatory responses in cultured macrophages: a player in cholinergic anti-inflammatory pathway

Etta Y. L. Liu[1,2], Miranda L. Xu[1,2], Xiang P. Kong[1], Qiyun Wu[1,2], Tina T. X. Dong[1,2], Karl W. K. Tsim[1,2]

1 Shenzhen Key Laboratory of Edible and Medicinal Bioresources, SRI, The Hong Kong University of Science and Technology, Shenzhen, China;
2 Division of Life Science, Center for Chinese Medicine, The Hong Kong University of Science and Technology, Hong Kong, China

Acetylcholine (ACh), the primary neurotransmitter released by vagus nerve, suppresses the levels of pro-inflammatory cytokines and tissue damage via the α7-nicotinic ACh receptor (α7-nAChR); this connection is being known as "cholinergic anti-inflammatory pathway (CAP)", a communication between immune and nervous systems. Acetylcholinesterase (AChE) is responsible for rapid elimination of ACh in vertebrate. In the treatment of Alzheimer's disease (AD), AChE inhibitors are commonly employed. The modulatory role of AChE inhibitors in inflammation have been reported. Here, the expression profile of AChE was determined in cultured macrophages. The tetramic form of PRiMA-linked AChE was found to be the predominant form, and its glycosylation pattern was similar to that of brain AChE. The challenge of LPS induced the rate of transcription of AChE, and this induction was shown to be triggered by NFκB, a key transcription factor in regulating immune responses. In LPS-treated macrophages, the release of cytokines was inhibited by co-applied galantamine, or other AChE inhibitors, in a dose-dependent manner: this LPS-induced inflammation was also altered by over expression of PRiMA-linked AChE. In cultured macrophages, the LPS-induced cell migration, confirmed by Transwell® motility assay, was suppressed by applied ACh, and this suppression was further enhanced by the co-applied galantamine, or other AChE inhibitors. In parallel, the levels of MMP2 and CDC42, two pro-migratory genes, were suppressed in the present of galantamine. Thus, the role of AChE in CAP needs to be elucidated.

Acknowledgments
This work was supported by Shenzhen Science and Technology Committee Research Grant (JCYJ20160229205726699, JCYJ20160229205812004, JCYJ20160229210027564, CKFW2016 082916015476, JCYJ20170413173747440, ZDSYS201707281432317 and 20170326). Etta Y. L. Liu hold a Lee's Pharmaceutical-Kanya Lee Postgraduate Scholarship.

S09P-4 Genistein, a phytoestrogen in soybean, induces the expression of acetylcholinesterase via G protein-coupled receptor 30 in PC12 cells

Etta Y.L. Liu[1,2], Miranda L. Xu[1,2], Qiyun Wu[1,2], Tina T.X. Dong[1,2], Sibao Chen[3], Karl W. K. Tsim[1,2]

1 Shenzhen Key Laboratory of Edible and Medicinal Bioresources, SRI, The Hong Kong University of Science and Technology, Shenzhen, China;
2 Division of Life Science, Center for Chinese Medicine, The Hong Kong University of Science and Technology, Hong Kong, China;
3 State Key Laboratory of Chinese Medicine and Molecular Pharmacology (Incubation), The Hong Kong Polytechnic University, Shenzhen Research Institute, Shenzhen, China

Several flavonoids have been identified to induce the expression of AChE in PC12 cells, e.g. daidzin, irisflorentin, cardamonin and genistein. Among them, genistein is the most robust inducer for AChE activity. Genistein, 4',5,7-trihydroxyisoflavone, is a major isoflavone in soybean, which is known as phytoestrogen having known benefit to brain functions. Being a common phytoestrogen, the possible role of genistein in the brain protection needs to be further explored. In PC12 cells, application of genistein significantly induced the expression of neurofilaments, markers for neuronal differentiation. In parallel, the expression of tetrameric

form of proline-rich membrane anchor (PRiMA)-linked acetyl-cholinesterase (G4 AChE), a key enzyme to hydrolyze acetylcholine in cholinergic synapses, was induced in a dose-dependent manner: this induction included the associated protein PRiMA. Genistein-induced AChE expression was fully blocked by the pre-treatment of H89 (an inhibitor of protein kinase A) and G15 (a selective G protein-coupled receptor 30 (GPR30) antagonist), which suggested a direct involvement of a membrane-bound estrogen receptor-GPR30-in the cultures. In parallel, the estrogen-induced activation of GPR30 induced AChE expression in a dose-dependent manner. The genistein/estrogen-induced AChE expression was triggered by a cyclic AMP responding element (CRE) located on the *ACHE* gene promoter. The binding of this CRE site by cAMP response element-binding protein (CREB) induced *ACHE* gene transcription. We have shown for the first time the activation of GPR30 could be one way for estrogen or flavonoids, possessing estrogenic properties, to enhance cholinergic functions in the brain, which could be a good candidate for possible treatment of neurodegenerative diseases.

Acknowledgments

This work was supported by Shenzhen Science and Technology Committee Research Grant (JCYJ20160229205726699, JCYJ20160229205812004, JCYJ20160229210027564,CKFW2016082916015476,JCYJ20170413173747440,JCYJ20151030164022389,20170326andZDSYS201707281432317). Etta Y. L. Liu hold a Lee's Pharmaceutical-Kanya Lee Postgraduate Scholarship.

S09P-5 Status of Cholinesterases in Heroin, Hashish (Cannabis) and Polydrug Addicts

Syed M Nurulain[1], Sliha Awan[1], Sadaf Munir[1], Tahira Javed[1], Sania Ghafoor[1], Rabia Habib[1]

1 Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan

Drug addiction is strongly influenced by biochemical, neuromodulator and genetics. It has been established that cholinergic system acts as neuromodulator with dopaminergic system, a major player in addiction. Putative role of cholinergic enzymes other than cocaine is hardly addressed. Present study was designed to evaluate the status of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in heroin, hashish (cannabis) and polydrug users. Study was conducted with the approval from Ethical Review Board of the Department of Biosciences and consent from participants. Twenty healthy non-addict age and sex matched individuals and eighteen male substance abusers from each group were included who fulfilled the inclusion criteria. Exclusions criteria include no chronic diseases of any kinds, no other neuronal disorders and used drugs for three or more months. Age group of non-addicts was 29.50±2.17. Age groups of the addicts were; heroin, 28.44±1.32, hashish 27.00±1.38 and polydrug users 26.06±2.27. Cholinergic enzymes were measured by Worek et al.1999 method based on Ellman's principal. AChE was measured from whole blood and BChE from plasma. Results are expressed as (µmol/L/min; Mean±SEM). Results showed statistically significant increased activity of AChE in heroin addicts (0.029 ±0.003) than non-addicts (0.021±0.002). AChE activity in hashish and polydrug users were 0.017±0.001 and 0.016±0.033 respectively and were not statistically significant. BChE measurement showed higher enzyme activity in all three groups; 0.031±0.007, 0.027±0.006, 0.027±0.006 for heroin, hashish and polydrug users respectively. The study concluded that butyrylcholinesterase have tentative physiological roles in addiction. Further studies in this direction may lead to novel approaches in therapy.

Keywords

Acetylcholinesterase, butyrylcholinesterase, heroin, hashish, polydrug

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S09P-6 Indirect effects of dioxin on neuronal AChE expression via astrocytes

Rui Sha[1,2], Yangsheng Chen[1,2], Yiyun Liu[1,2], Li Xu[1,2], Heidi Qunhui Xie[1,2*], Bin Zhao[1,2*]

1 State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center of Eco-Environment Sciences, Chinese Academy of Sciences, Beijing 100085 China

2 University of Chinese Academy of Sciences, Beijing, China

Acetylcholinesterase (EC3.1.1.7; AChE) is one of the most important enzymes in the cholinergic system. Our previous works showed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a notorious persistent organic pollutants, suppressed neuronal AChE activity by both transcriptional and post-transcriptional regulations via aryl hydrocarbon receptor pathway in SK-N-SH human neuroblastoma cells [1, 2]. In the nervous system, the most abundant cell type, astrocyte is regarded to play vital roles in protecting neurons from various kinds of insults, including environmental pollutants. Astrocytes have been considered as one of the target cells of dioxin in the nervous system. However, whether astrocytes are able to mediate indirect effect of dioxin on neuronal AChE is still unknown. In the present study, we aimed to reveal the potential indirect effect by using conditional medium derived from dioxin-treated astrocytes. Rat primary astrocytes were employed which were exposed to TCDD at 0.01 to 1 nM directly for 4 days. After the treatment, the astrocyte conditioned medium (ACM) was collected and administrated to the primary neurons on DIV (day *in vitro*) 2 for 4 days. Meanwhile, the primary neurons (DIV 2) from the same bench were exposed to TCDD directly at same concentrations for 4 days. The results showed that the enzymatic activity and mRNA expression of AChE was suppressed in TCDD-ACM-treated neurons compared to those of solvent-ACM-treated neurons. The effective concentrations of TCDD were 0.01 and 0.03 nM, which are close to the average serum TCDD concentration in exposed population from different areas of the world. However, AChE was less sensitive in the primary neurons directly exposed to TCDD. These results suggested that astrocytes play roles in mediating the indirect effect of TCDD on neuronal AChE expression.

Keywords
Dioxin; Astrocyte; Neuron; astrocyte conditioned medium; Acetylcholinesterase

Acknowledgments
This work was supported by Natural Science Foundation of China (Nos. 21377160, 21525730), and the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDB14030400).

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S09P-7 Non-neuronal cholinergic system in rat aorta

Szmicseková K.[1,2], Kiliánová Z.[1], Krajčovičová K.[1], Krejci E.[2], Paul Hrabovská A.[1]

1 Faculty of Pharmacy, Department of Pharmacology and Toxicology, Comenius University in Bratislava, Slovak Republic
2 Cognition and Action Group, Université Paris Descartes, France

We have showed before that both cholinesterases are present in rat aorta, while inhibition of butyrylcholinesterase impairs physiology of the isolated organ. The endothelium-dependent vasodilatory effect of acetylcholine (ACh) on vessels is well known, but physiological or pathological importance of this effect in live animals is questionable, and origin of possibly acting ACh unclear. Hypothesizing that aorta is a non-neuronal cholinergic tissue, the main aim of this project was to examine the presence of the proteins involved in the synthesis, storage, release, and degradation of ACh. Target-specific primers were used in RT-qPCR for determination of relative expressions and proteins were visualized by immunohistochemistry using commercially available antibodies. We confirmed the presence of high-affinity transporter and vesicular acetylcholine transporter in aorta, but no choline acetyltransferase was detected. Instead, relatively high levels of carnitine acetyltransferase were observed thus we assume this enzyme to be responsible for ACh synthesis in aorta. Additionally, present organic cation transporters OCT2 and OCT3 (but not OCT1) suggest possible involvement in ACh transmembrane transport. We confirmed the presence of both cholinesterases in rat aorta, more precisely in the smooth muscle, while no protein or activity was detected in the endothelium. Our results confirm aorta to be a non-neuronal cholinergic tissue carrying a full machinery for synthesis, storage and release and degradation of ACh.

This project was supported by grant from Faculty of Pharmacy of Comenius University (FaF/2/2018) and Bourse du Gouvernement Français.

S09P-8 Microphthalmia-associated transcription factor regulates acetylcholinesterase expression during melanogenesis of B16F10 cells: a cholinergic regulator in pigmentation

Qiyun Wu[1,2], Aster H. Y. Fung[2], Miranda L. Xu[1,2], Etta Y. L. Liu[1,2], Ran Duan[1,2], Ping Yao[2], Tina T. X. Dong[1,2], Karl W. K. Tsim[1,2]

1 Shenzhen Key Laboratory of Edible and Medicinal Bioresources, SRI, The Hong Kong University of Science and Technology, Shenzhen, China;
2 Division of Life Science, Center for Chinese Medicine, The Hong Kong University of Science and Technology, Hong Kong, China

Acetylcholinesterase (AChE) hydrolyses acetylcholine that functions as a neurotransmitter in neurons. The non-neuronal functions of AChE have been proposed in different cell types. Here, we revealed the expression of AChE in melanocyte and melanoma, in which the tetrameric (G4) form was the major isoform. In the melanogenesis of cultured B16F10 murine melanoma, the amount of AChE was markedly decreased. The differentiation of melanoma led to: (i) increase of melanin and its synthesis enzyme tyrosinase; (ii) change of intracellular cAMP level; and (iii) decrease of microphthalmia-associated transcription factor (MITF). The regulation of AChE during melanogenesis was hypothesized to be mediated by two transcriptional factors: cAMP responsive element binding protein (CREB) and MITF. In cultured melanoma, application of cAMP suppressed the expression of AChE, as well as the promoter activity of human ACHE gene. This suppression was shown to be mediated by a cAMP responsive element (CRE) located on the *ACHE* promoter, and mutation of this site eliminated the suppression. In melanoma, over expression of MITF induced the transcription of *ACHE* gene, and mutation of E-box site of the promoter blocked the induction. In parallel, application of an AChE inhibitor *in vitro* greatly enhanced acetylcholine-mediated responses of melanogenic gene expressions; but the enhancement was not revealed in the present of agonists of muscarinic acetylcholine receptor. Therefore, our results indicated that AChE transcription is specifically regulated by cAMP-dependent signaling pathway during melanogenesis of B16F10 cells, suggesting a potential role of AChE being played in this differentiation process.

Acknowledgements
This work was supported by Shenzhen Science and Technology Committee Research Grant (JCYJ20160229205726699, JCYJ20160229205812004, JCYJ20160229210027564, CKFW2016 082916015476, JCYJ20170413173747440, ZDSYS201707281432317 and 20170326). Qiyun Wu hold a Prof. SD Kung Scholarship.

S10P Alzheimer's disease and diseases related to cholinesterases

S10P-1 Dual binding site inhibitors of acetylcholinesterase as therapeutic treatments for Alzheimer's disease: any need for an update?

K. Petrov[1], I. Zueva[1], J. Dias[2], S. Lushchekina[3], V. Semenov[1], F. Nachon[2], E. Nikolsky[4,5], P. Masson[6]

1 Arbuzov Institute of Organic and Physical Chemistry, FRC Kazan Scientific Center of RAS, Arbuzov str. 8, Kazan, 420088, Russia
2 Institut de Recherche Biomédicale des Armées, 91223 Brétigny-sur-Orge, France
3 N.M. Emanuel Institute of Biochemical Physics of Russian Academy of Sciences, Kosygina str. 4, Moscow 119334, Russia
4 Kazan State Medical University, 49 Butlerova Street, Kazan 420012, Russia
5 Kazan Institute of Biochemistry and Biophysics, FRC Kazan Scientific Center of RAS, Lobachevsky str. 2/31, Kazan, 420111, Russia
6 Kazan Federal University, 18 Kremlyovskaya str, Kazan, 420008, Russia

Alzheimer's disease (AD) is a broadly spread neurodegenerative disorder of ageing population manifesting itself in progressing loss of cognitive functions down to total demolition of intellect and disability. Profound synaptic dysfunction contributes to early loss of short-term memory in Alzheimer's disease. Here we show the protective effects against amyloid-induced synaptic toxicity of C-35, a potent reversible inhibitor of acetylcholinesterase (AChE).

Crystal structure of the complex between human AChE and C-35 revealed tight contacts of ligand along the enzyme active site gorge. Molecular dynamics simulations indicated that the external flexible part of the ligand establishes multiple transient interactions with the enzyme peripheral anionic site. Thus, C-35 is a dual binding site inhibitor of AChE.

In amyloid-transgenic mice, C-35, when administered after disease onset, reversed synapse loss, decreased the number of amyloid plaques and restored learning and memory. When administration of C-35 and the clinically relevant AChE dual inhibitor donepezil was terminated three weeks after the trial started, animals, that were receiving C-35 showed a much better ability to learn than those who received physiological saline or donepezil. Our results provide evidence that C-35 has a more pronounced Alzheimer's disease-modifying action than donepezil.

Keywords
Alzheimer's disease, inhibitors of cholinesterase, methyluracil derivatives, β-amyloid

Acknowledgement
This research has been supported by RSF grant 14-50-00014 awarded to PK.

S10P-2 Detection of Alzheimer's drug candidate by Surface-Enhanced Raman Spectroscopy

Jan Proska[1], Lucie Maresova[2], Marek Prochazka[3], Eugenie Nepovimova[1], Kamil Kuca[1]

1 Department of Chemistry, Faculty of Science, University of Hradec Kralove, Rokitanského 62, 500 03 Hradec Kralove, Czech Republic
2 Faculty of Nuclear Sciences and Physical Engineering, Technical University in Prague, Břehová 7, Prague 1 115 19, Czech Republic
3 Charles University, Faculty of Mathematics and Physics, Institute of Physics, Ke Karlovu 5, Prague 2 121 16, Czech Republic

Drug candidate 1-EN-142 was designed and synthesized as a multipotent therapeutic agent to treat Alzheimer´s disease. In its molecule it combines tacrine moiety with naphthoquinone scaffold. For the study of centrally-active molecules in biological samples it is necessary to develop appropriate detection methodology that would determine such compounds in low concentration. Spectroscopy based on Surface-Enhanced Raman Scattering (SERS) was chosen as a comparative method for the electronic detection of compound 1-EN-142 by interdigitated impedance sensor decorated with gold nanoparticles. Since spectroscopic data were not available for this new drug candidate, it was necessary, as well, to acquire its classical Raman spectra in the solution and the solid state. SERS-active substrates were prepared by straightforward procedure so that 20 nm thick layer of gold was deposited by fast magnetron sputtering on silicon wafer. The substrates with roughened gold surface were immersed in solution of 1-EN-142 in methanol for 30 min and dried in the stream of nitrogen. SERS spectra of 1-EN-142 were obtained as an average of 100 spectra measured from an array of 20 x 5 points with 2 µm spacing. Subsequently, the reference spectrum, obtained by the same procedure from a SERS substrate unexposed to 1-EN-142, was subtracted, and the spectrum baseline was corrected using cubic splines. SERS spectra were recorded with a Raman microspectrometer using excitation wavelengths of 633 nm and 785 nm, respectively. Raman spectrum of 1-EN-142 solution in methanol in the range of 390 – 1741 cm^{-1} was collected with laser excitation of 532 nm. SERS *has* proved to be a suitable method of detecting compound 1-EN-142.

Acknowledgements
This work was supported by the grants GA17-19968S and 18-10897S from the Czech Science Foundation.

S11P Multi-target-directed ligands in Alzheimer's disease primarily targeting cholinesterases

S11P-1 Butyrylcholinesterase inhibitors grafted with antioxidant and neuroprotective activities: novel multi-functional ligands for Alzheimer's disease

Damijan Knez[1], Nicolas Coquelle[2], Anja Pišlar[1], Simon Žakelj[1], Marko Jukič[1], Matej Sova[1], Janez Mravljak[1], Florian Nachon[3], Xavier Brazzolotto[3], Janko Kos[1], Jacques-Philippe Colletier[2], Stanislav Gobec[1]

1 Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia
2 University Grenoble Alpes, IBS, F-38044 Grenoble, France; CNRS, IBS, F-38044 Grenoble, France; CEA, IBS, F-38044 Grenoble, France
3 Institut de Recherche Biomédicale des Armées, 91223 Brétigny sur Orge, France

Current symptomatic treatment has only limited clinical efficacy and minute effect on progression of Alzheimer's disease. The research focus has thus shifted from single targets towards multifunctional ligands targeting several pathological processes of the disease [1, 2].

A potent picomolar selective inhibitor of human butyrylcholinesterase [3] was used as the starting point to develop a new series of multifunctional ligands. A focused library of derivatives was designed and synthesized that showed both butyrylcholinesterase inhibition and good antioxidant activity comparable to natural antioxidants. The crystal structure of compound **11** in complex with butyrylcholinesterase revealed the molecular basis for its low nanomolar inhibition of butyrylcholinesterase (K_i = 1.09 ±0.12 nM). In addition, compounds **8** and **11** show metal-chelating properties as determined by the UV-Vis titrations, and reduce the redox activity of chelated Cu^{2+} ions in a Cu-ascorbate redox system. Compounds **8** and **11** decrease intracellular levels of reactive oxygen species, and are not substrates of the active efflux transport system, as determined in Caco2 cells. Compound **11** also protects neuroblastoma SH-SY5Y cells from toxic $A\beta_{1-42}$ species. These data indicate that compounds **8** and **11** are promising multifunctional lead ligands for treatment of Alzheimer's disease.

Keywords
Alzheimer's disease, butyrylcholinesterase, multifunctional ligands, 8-hydroxyquinoline

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S11P-2 7-MEOTA-donepezil hybrids: Potential cholinesterase inhibitors for the treatment of Alzheimer's disease

Katarina Spilovska[1,2,3], Eva Mezeiova[1,2,4], Jan Korabecny[2,4,5], Jana Hroudova[1], Vendula Hepnarova[4,5], Martina Hrabinova[4,5], Ondrej Soukup[2,4,5], Kamil Musilek[3], Daniel Jun[5], Kamil Kuca[3]

1 Department of Psychiatry, First Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic
2 National Institute of Mental Health, Klecany, Czech Republic
3 University of Hradec Kralove, Faculty of Sciences, Department of Chemistry, Hradec Kralove, Czech Republic
4 Biomedical Research Centre, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic
5 University of Defence, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Hradec Kralove, Czech Republic

e-mail: katarina.spilovska@nudz.cz

Alzheimer's disease (AD) is a devastating neurodegenerative disorder characterized by a severe, progressive loss of memory. Currently, AD therapy is limited on the administration of cholinesterase inhibitors (ChEIs) and the N-methyl-D-aspartate (NMDA) antagonist, memantine. Tacrine as the first registered acetylcholinesterase (AChE, E.C. 3.1.1.7) inhibitor was withdraw due to its adverse effects. 7-Methoxytacrine (7-MEOTA) was prepared as a pharmacologically equal active compound with lower toxicity compared to THA. Donepezil as a highly selective inhibitor for AChE was connected with 7-MEOTA scaffold due to the ability to interact within calatytic anionic site (CAS) as well as peripheral anionic site (PAS) regions of AChE [1].

Recent research has been focused on studying the association between the intracellular amyloid beta (Aβ) cascade and the dysfunction of subcellular organelles, especially mitochondria. Mitochondrial enzyme amyloid beta binding alcohol dehydrogenase (ABAD) might contribute to the neuronal dysfunction associated with AD by interacting with intracellular Aβ [2].

These derivatives embodying 7-MEOTA and donepezil moieties [3] could be effective in the treatment of AD with the respect of their ability to interact with the multiple targets. Within our contribution, synthesis, *in vitro* biological evaluation including cholinesterase inhibitory activity and effects on mitochondrial function of 7-MEOTA-donepezil series will be reported.

Acknowledgement
This work was supported by grant No. 17-07585Y given by Grant Agency CR.

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