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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

CHRONIC ILLNESS FROM ORGANOPHOSPHORUS TOXICANT EXPOSURE

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The mechanism of toxicity from acute exposure to organophosphorus toxicants (OP) is understood. Thousands of publications have confirmed that AChE inhibition results in muscle weakness and respiratory failure. AChE activity returns to normal levels within one month, but symptoms can persist for a lifetime. For example, people exposed to sarin in the 1995 Tokyo subway attack still have adverse symptoms 23 years later. Farmers and sheep dippers exposed to OP pesticides have an elevated risk of psychiatric disorders and suicidal behavior. Epidemiology studies show an association between OP exposure and Alzheimer's disease and Parkinson's disease. We propose a mechanism to rationalize these observations independent of cholinesterase inhibition. Mass spectrometry analysis of OP-treated proteins shows that OP make stable adducts on tyrosine and lysine. Furthermore, we have mass spectrometry evidence that OP-lysines promote crosslinks between proteins. The crosslinked proteins are visualized as protein aggregates on SDS gels and Western blots. Mass spectrometry has identified γ-glutamyl-ε-lysine and aspartyl-ε-lysine isopeptide bonds between crosslinked peptides. We propose, but have not yet proven, that isopeptide crosslinked proteins form stable, insoluble aggregates in the brain, similar to the protein aggregates found in Alzheimer's, Parkinson's, and prion diseases. In summary, we propose that chronic neurotoxicity from OP exposure is initiated by OP-lysine formation followed by protein aggregation. Our proposed mechanism could apply to a variety of compounds and lead to an understanding of neurotoxicity induced by many chemicals.

Keywords: crosslinking; protein aggregates; mass spectrometry

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MEETING ABSTRACTS

THE CATALYTIC POWER OF PHOSPHOTRIESTERASES FOR THE HYDROLYSIS AND DESTRUCTION OF ORGANOPHOSPHORUS NERVE AGENTS

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Phosphotriesterase (PTE), an enzyme originally isolated from *Pseudomonas diminuta*, is capable of catalyzing the hydrolysis of many organophosphorus nerve agents. The turnover number for the enzymatic hydrolysis of paraoxon (diethyl p-nitrophenyl phosphate) by PTE is ~500,000 min^{-1}. The protein adopts a distorted $(\beta/\alpha)_8$-barrel structural fold and the active site is perched at the C-terminal end of the β-barrel. The water used for nucleophilic attack of the substrate bridges two divalent metal ions in the active site and is further activated by the side chain carboxylate from an aspartate residue that resides at the end of β-strand 8. Upon binding to the active site, substrates are further activated for hydrolysis by a direct interaction of the phosphoryl oxygen with the β-metal ion. The chemical reaction is initiated via the direct attack of the bridging water/hydroxide at the phosphorus center of the substrate and proceeds with an inversion of stereochemistry. Wild-type PTE is stereoselective for the hydrolysis of chiral substrates. However, the catalytic preferences for the hydrolysis of chiral substrates can be enhanced, relaxed, or inverted by selective mutation of key residues in the active site that dictate the size and shape of the substrate-binding cavity. The extreme toxicity and persistence of the G-type (sarin and soman) and V-type (VX and VR) organophosphorus nerve agents makes the detoxification of these compounds of significant interest. A rational and random mutagenesis strategy has been developed and implemented for the evolution of mutant forms of PTE that are more fully optimized for the catalytic destruction and detoxification of the most toxic organophosphorus nerve agents.

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ISSN 2571-113X (Online)

MEETING ABSTRACTS

RECENT BREAKTHROUGHS IN THE STRUCTURE/FUNCTION STUDIES OF ACETYLCHOLINESTERASE

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The synaptic enzyme acetylcholinesterase (AChE) terminates transmission at cholinergic synapses by rapidly hydrolysing acetylcholine. Examination of the 3D structure of AChE[1] shows that the active site is located at the bottom of a deep and narrow gorge, lined largely by aromatic residues, with its peripheral anionic site located at the top, near the entrance to of the gorge. 3D structures of AChE have been determined for the *Torpedo*, *Electrophorus*, mouse, *Drosophila* and human enzymes. Overall, more than a hundred crystal structures of AChEs, and of covalent conjugates and reversible complexes with various inhibitors and substrate analogues have been determined. Although the 3D structure of the enzyme itself, and of its molecular dimer, are highly conserved, subtle structural differences are seen to occur upon the binding of certain inhibitors. These changes are well correlated with molecular dynamics data, and appear to be of functional significance.

Unfortunately, upon heterologous overexpression, many proteins misfold or aggregate, thus resulting in low functional yields. Human AChE is a typical case of a human protein that necessitates mammalian systems to obtain functional expression. Using a novel computational strategy, we designed an AChE variant bearing 51 mutations that improved core packing, surface polarity, and backbone rigidity. This variant expressed at ~2,000-fold higher levels in *E. coli* compared to wild-type hAChE, and exhibited 20°C higher thermostability with no change in enzymatic properties or in the active-site configuration as determined by crystallography[2,3].

Keywords: acetylcholinesterase; improved stability; improved expression; Proteopedia; 3D structure

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ISSN 2571-113X (Online)

MEETING ABSTRACTS

COUPLING OF ACETYLCHOLINESTERASE TO THE INTERFACIAL PHASE STATE

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The state of the lipid interface is known to influence activity of membrane-bound enzymes. Indeed, many enzymes exhibit changes in activity at phase transitions in the membrane to which they are attached. We utilized a Langmuir trough in which detergent-soluble *Torpedo californica* acetylcholinesterase (DS-*Tc*AChE)[1] was anchored to the solvent face of a phospholipid monolayer in order to study this phenomenon. A peak in activity was observed at the compressibility maximum accompanying the transition between the ordered and fluid phases. Neither molecular nor physical alterations affected this correlation qualitatively, as shown by varying lipid type, pH over 2 units, temperature over 20°C, and lateral pressure over 10 mN/m. Thus the only consistent correlation is between the thermodynamic state of the interface and the measured activity. Our data are consistent with a theory in which the interface state and its corresponding fluctuations control catalytic activity[2]. It was earlier demonstrated that pH-pulses initiated by local acidification of the monolayer propagate, in analogy to sound, at velocities up to 1.4 m/s[3]. We have now shown that such a pulse, by transiently modifying compressibility, can concomitantly and reversibly enhance the activity of DS-*Tc*AChE attached to the monolayer. Our data demonstrate a feasible mechanism for signaling between widely separated biological entities that differs fundamentally from the molecular mechanisms currently accepted, and is also very much faster.

Keywords: acetylcholinesterase; Langmuir trough; monolayer; compressibility; acoustic propagation

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MEETING ABSTRACTS

THE OTHER SIDE OF AChE: ALLOSTERIC SITES AND MODULATORS

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The best-known function of acetylcholinesterase (AChE) is the hydrolysis of the neurotransmitter acetylcholine, however we are increasingly aware of the multifunctionality of this enzyme [1]. The non-hydrolytic functions of AChE are driven by allosteric sites as the peripheral allosteric site (PAS) responsible for amyloidosis in Alzheimer's disease through interaction with β-amyloid peptide.

We would like to show our work about the identification and characterization of new allosteric sites in AChE, using computational tools. This study has allowed us to identify allosteric inhibitors by virtual screening using our in-house MBC chemical library [2] guided by structure-based and fragment hotspot strategies. The identified compounds were also screened for in vitro inhibition of AChE and three of them were observed to be active. Further experimental (kinetic) and computational (molecular dynamics) studies have been performed to verify the allosteric activity. Thus, new compounds have been developed as allosteric modulators that may be valuable pharmacological tools in the study of non-cholinergic functions of AChE.

Keywords: allosteric sites; Alzheimer's disease; molecular dynamics; allosteric inhibitor

Acknowledgement

Financial support of this research has been provided by Ministerio de Economía y Competitividad (CTQ2015-66313-R). TLB & SM thank the MRC Newton Fund and CR thanks the BBSRC and UCB for a CASE studentship.

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MEETING ABSTRACTS

PARTIAL UNFOLDING OF INSECT ACETYLCHOLINESTERASE: STEPS TOWARD CYSTEINE-TARGETING INSECTICIDES

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To obtain insight into the development of thermally stable insect acetylcholinesterases, 200 distinct, independent, unrestricted, unbiased, isobaric–isothermal, 316-ns molecular dynamics simulations of a substrate-bound mosquito acetylcholinesterase responsible for cholinergic functions (AP-agAChE)[1] were performed using forcefield FF12MC[2] and PMEMD of AMBER 11 with a periodic boundary condition at 1 atm and 340 K. In-depth conformational analysis of these simulations with an aggregated simulation time of 63.2 microseconds revealed partially unfolded regions of AP-agAChE that could be stabilized with mutations for developing thermally stable AP-agAChE variants and thereby enabling rigorous characterization[3] of cysteine-targeting anticholinesterases as potential insecticides that are effective and environmentally safe and also spare beneficial insects[1].

Keywords: insect acetylcholinesterase; protein unfolding; protein engineering; anticholinesterase; cysteine-targeting insecticide

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ISSN 2571-113X (Online)

MEETING ABSTRACTS

THE PROTONATION STATE OF Glu197 AND ITS IMPORTANT ROLE IN STABILIZING CATALYTIC TRIAD OF BUTYRYLCHOLINESTERASE

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The Glu197 of butyrylcholinesterase (BChE) has been long considered as deprotonated in various studies, e.g. discovering the dynamical characters, interpreting the binding properties of inhibitors, and proposing hypotheses for BChE-catalyzed reaction mechanism. By performing a series of 100 ns molecular dynamics simulations, we accidently discovered that Glu197 needed to be protonated to have the structures simulated appropriately, whereas the deprotonated Glu197 eventually caused the collapse of catalytic triad with long enough simulation time.[1] we found that a highly conserved water molecule required Glu197 to be protonated in order to form an important hydrogen bond network, which supported His438 to be preserved within the catalytic triad. Interestingly, catalytic triad and Glu197 have been long recognized for possibly deviating largely from their crystal structure positions, which could be catalytic deficient and is generally considered as the result from difference between crystal and aqueous environment. Here, our results suggest that the large deviations of catalytic triad and Glu197 from crystal structure are caused by inappropriate protonation state of Glu197. This finding of the unexpected protonation state of Glu197 shall provide an important clue that has been long missing for the better understanding of BChE related puzzles or even reconsideration of some BChE-catalyzed reaction mechanisms.

Keywords: protonation state; Glu197; butyrylcholinesterase; catalytic triad

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ISSN 2571-113X (Online)

MEETING ABSTRACTS

RECIPES TO DESIGN SPECIFIC LIGANDS OF HUMAN BUTYRYLCHOLINESTERASE

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Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) hydrolyze the neurotransmitter acetylcholine and function thereby as regulators of cholinergic neurotransmission. Recently, interest has greatly increased in BChE. Firstly, BChE is a good broad spectrum bioscavenger of nerve agent and its efficiency could be significantly increased by the mean of specific reactivators. Secondly, BChE activity in the brain increases with the progression of Alzheimer's disease, thus classifying BChE as a promising drug target in the advanced phase of the disease. AChE and BChE display specificities for substrates and ligands that only partially overlap. This disparity is largely due to differences in the number of aromatic residues lining the active site gorge, which leads to large differences in the shape of the gorge and potentially to distinct interactions with an individual ligand. Considerable structural information is available for the binding of a wide diversity of ligands to AChE. In contrast, structural data on the binding of reversible ligands to BChE was lacking. In the recent years, we solved the X-ray structures of multiple BChE-ligand complexes. Here we will present BChE structures with various ligands, some recently synthesized, to highlight the structural elements leading to their BChE affinity and specificity. These structural data will help to design specific reversible ligands that behave as inhibitors or reactivators.

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ISSN 2571-113X (Online)

MEETING ABSTRACTS

EXPLORING THE EVOLUTIONARY POTENTIAL OF THE αE7 CARBOXYLESTERASE

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The evolution of insecticide resistance is a model system for studying enzyme evolution. Three insect species have independently evolved catalytic organophosphate (OP) detoxification through a single active-site mutation in the αE7 carboxylesterase. To explore the evolutionary potential of αE7, we subjected αE7 from the sheep blowfly to nine rounds of mutation and screening. The final variant contained 11 mutations which increased the rate of OP-hydrolysis more than 1000-fold. Atomic resolution X-ray crystal structures of the evolutionary intermediates reveal the changes in structure and dynamics at each step in the evolutionary trajectory, and hint at the molecular basis for the increased rate of OP hydrolysis. This work explores the potential for the development of αE7 as an enzyme therapeutic for OP poisoning, and worryingly for insecticide resistance, this work suggests that more efficient OP detoxification could be readily acquired by insect pests.

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MEETING ABSTRACTS

PROTEIN DYNAMICS OF PHOSPHOTRIESTERASE: TWO CATIONS REQUIRED FOR ENZYME CATALYSIS

Yuan-Ping Pang
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To investigate how protein dynamics facilitates substrate entering and product exiting the phosphotriesterase active site, over 60 distinct, independent, unrestricted, unbiased, isobaric–isothermal, microsecond molecular dynamics simulations of zinc-containing phosphotriesterase in complex with a substrate analog[1] were performed using the second-generation cationic dummy atom model for the zinc divalent cation, forcefield FF12MC[2], and PMEMD of AMBER 16 with a periodic boundary condition at 1 atm and 277 K, 300 K, and 340 K. In-depth conformational analysis of these simulations with an aggregated simulation time of over 76 microseconds revealed atomic and dynamic details on the phosphtriesterase catalysis and its requirement of two cations, which offers insight into re-engineering of phosphotriesterase to develop an improved scavenger against phosphorous-containing inhibitors of acetylcholinesterase.

Keywords: phosphotriesterase; protein dynamics; zinc; scavenger; protein engineering

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MEETING ABSTRACTS

NEW PROGRESS IN DRUG DESIGN, DISCOVERY AND DEVELOPMENT INVOLVING CHOLINESTERASES

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This talk will briefly discuss our newest progress in drug design, discovery and development involving cholinesterases, particularly in three major therapeutic areas.

(1) On the basis of our previous design and discovery of cocaine hydrolases (CocHs) engineered from human butyrylcholinesterase (BChE), we have further developed a novel, long-acting CocH form, and demonstrated the promising clinical potential of CocHs for therapeutic treatment of cocaine overdose and addiction in clinically relevant animal models. One of the long-acting CocHs is currently in the large-scale protein drug manufacturing process development.

(2) It has been demonstrated that a long-acting CocH (enzyme) is capable of both completely blocking cocaine-induced physiological effects and producing the desirable anti-obesity effects. Mice on a high-fat diet gained significantly less body weight when treated weekly with 1 mg/kg enzyme compared to control mice.

(3) Most recently, we have also designed and tested a new therapeutic strategy for heroin detoxification based on a detailed analysis of the cholinesterases-involved chemical transformation and functional change of heroin in the body. It has been demonstrated in our animal models that a carefully selected cholinesterase inhibitor attenuated acute toxicity and physiological effects of heroin, whereas some other cholinesterase inhibitors may actually enhance the acute toxicity and physiological effects of heroin.

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MEETING ABSTRACTS

IN SEARCHING FOR THE MECHANISM OF BUTYRYLCHOLINESTERASE ACTIVATORS

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It is known that cholinesterases show homotropic pseudocooperative effects: their activity at millimolar substrate concentrations is higher than expected by simple saturation kinetics and they are strongly inhibited at the submolar concentrations. However, we have reported that the anionic site directed inhibitors tetramethylammonium and tetraethylamonium too, increase the activity of human butyrylcholinesterase. At that time, the same phenomenon could not be shown for the horse counterpart. Here, it was searched for other putative activators among often used compounds in cholinesterase research. Indeed, imidazole significantly increase the activity of human enzyme, but also its atypical form and the horse enzyme. On the other hand, 2-PAM shows a certain degree of activation with both human enzymes, but inhibits the horse BChE in a classical competitive manner. To avoid substrate activation, the experiments were performed at around 50 micromolar starting substrate concentrations and were followed by its completion in the presence of different modulator(s) concentrations. Subsequently, the effect of 2-PAM on the phosphorylation by DFP was studied, since the bottom of the active site does not differ in these three enzymes. It seems that the distinctive action of activating agents on the wild type, the atypical human and horse BChE is a consequence of differences in the dynamics of the acylation loop at the active site entrance, rather then the composition of the enzyme's peripheral anionic site.

Keywords: reaction mechanism; butyrylcholinesterase activation; kinetics

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MEETING ABSTRACTS

COMPUTATIONAL ANALYSIS OF REACTION MECHANISMS FOR OPTIMIZATION OF BUTYRYLCHOLINESTERASE-BASED CATALYTIC BIOSCAVENGERS AGAINST ORGANOPHOSPHORUS AGENTS

Sofya Lushchekina [1], Bella Grigorenko [1,2], Alexander Nemukhin [1,2], Sergei Varfolomeev [1,2], Patrick Masson [3]
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Catalytic bioscavengers are second generation bioscavengers. These biopharmaceuticals can be used to degrade toxic organophosphorus agents (OPs) on the skin for decontamination or in the bloodstream for pre-treatment and post-exposure treatment of OP poisoning. Because degradation has to be fast, their catalytic efficiency has be as high as possible (k_{cat}/K_m>106 $M^{-1}min^{-1}$). To be of interest, the catalytic activity of certain enzymes, in particular self-reactivating ChEs, has to be increased by several orders of magnitude. This can be reached by computer-redesign, directed evolution of existing enzymes, and combinational strategies.

Rational design of novel ChE-based catalytic bioscavengers requires a better understanding of chemical mechanisms of inhibition, aging of conjugate, and spontaneous reactivation. Kinetic studies, X-ray crystallography and molecular modeling, in particular QM/MM calculations, present valuable insights into specific reaction routes, role of specific amino acids and obstacles against effective reactivation of phosphylated ChEs.

Introducing new functional groups surrounding the phosphylated serine should create a stable H-bonded network susceptible to activate and orient water molecule, stabilize transition states, and intermediates. Direction of nucleophilic attack of water molecule on phosphorus atom may determine whether dephosphylation is favored over aging. Mutations of key residues surrounding human BChE active site, creating new reaction pathways, have been considered. QM/MM calculations suggest that introduction of a histidine, directing attack of water molecule from apical position competes with the aging reaction, while axial direction of water attack does not. Secondary mutations for stabilizing imidazolium upon activation of water molecule lead to lower energy barrier of reactivation reaction [1].

Keywords: catalytic bioscavengers; organophosphorus compound; butyrylcholinesterase; reaction mechanism

Acknowledgement

Supported by Russian Science Foundation (project #14-13-00124 to SL, BG, AN and #17-14-01097 to PM)

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MEETING ABSTRACTS

ENHANCEMENT IN PYRIDINIUM OXIME-ASSISTED REACTIVATION OF TABUN-INHIBITED ACETYLCHOLINESTERASE ACHIEVED BY ACTIVE SITE MUTATIONS

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Tabun represents a phosphoramide class of organophosphosphates that are covalent inhibitors of acetylcholinesterase (AChE), an essential enzyme in neurotransmission. The currently used therapy in excessive cholinergic stimulation consists of the muscarinic antagonist of acetylcholine stimulation, an anti-seizure drug when indicated and an oxime as the reactivator of inhibited AChE. Since common oximes are particularly ineffective in tabun exposure, we probed the reactivation of phosphoramidate conjugates in more depth by using mutants of AChE and pyridinium oximes to reveal the structural subtleties and yield more information on the architecture of the active centre gorge needed for the reactivation of phosphoramidate agents used in terrorism and as pesticides. Our results indicated that the replacement of aromatic residues with aliphatic ones at the acyl pocket and choline binding site mostly interfered with the stabilization of the oxime's pyridinium ring(s) in the proper orientation of the oxime group toward the phosphorylated active site serine. The peripheral binding site mutation resulted in a 2-5 fold increase in the reactivation rates by bis-pyridinium oximes when compared to the AChE wild type. In the case of mono-pyridinium oximes, we reported a 150-fold enhancement of the maximal reactivation rate for the choline binding site mutation, while the molecular recognition seemed to remain preserved. Therefore, our results emphasized the positive effect of several mutations on oxime embedding and orientation into a position for productive interactions with the tabun-phosphorylated active site serine indicating a future potential for further development of pseudo-catalytic bioscavengers based on AChE mutants.

Keywords: nerve agents; antidotes; 2-PAM; HI-6

Acknowledgement

This work was supported by the Croatian Science Foundation (no. 4307).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

THE SEARCH FOR RESISTANCE-BREAKING AND SPECIES-SELECTIVE MOSQUITOCIDAL INHIBITORS OF *Anopheles gambiae* AChE

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The widespread deployment of insecticide-treated bednets (ITNs) in sub-Saharan Africa has led to a dramatic decline in malaria mortality. However, wide-spread and growing resistance of *Anopheles gambiae* mosquitoes to the pyrethroid class of voltage-gated Na+ channel modulators used on these nets jeopardizes this achievement, and has prompted the search for suitable insecticidal AChE inhibitors to replace pyrethroids. Such compounds would have three favorable characteristics: excellent contact toxicity towards susceptible adult *An. gambiae*, good contact toxicity to those that bear the G119S resistance mutation of AChE, and very weak inhibition of human AChE.[1] We will review our work on the development of aromatic and heterocyclic core methyl and dimethylcarbamate AChE inhibitors,[2] and including both enzymatic inhibition potencies and mosquito contact toxicities. Finally, the inhibition selectivities of particular compounds will be rationalized in the context of our recently obtained high resolution X-ray structures of G119S *An. gambiae* AChE.[3]

Keywords: mosquito; malaria; carbamate; resistance; G119S

Acknowledgement

We thank the NIH (AI082581) for financial support.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

STERIC EFFECTS IN THE DECARBAMOYLATION OF CARBAMOYLATED ACETYLCHOLINESTERASE

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Carbamates are esters of substituted carbamic acids that react with acetylcholinesterase (AChE) in a two-step process, with initial transfer of the carbamoyl acyl group to a serine residue of AChE accompanied by loss of the carbamate leaving group followed by hydrolysis of the carbamoyl enzyme. This hydrolysis, or decarbamoylation, is relatively slow, and half-lives of carbamoylated AChEs range from 4 min to more than 30 days. Since carbamates are poor, slowly reversible AChE substrates, they are effective AChE inhibitors that have been developed as insecticides and therapeutic agents. We show that decarbamoylation rates are independent of the leaving group for a series of carbamates with the same carbamoyl group. For a given leaving group, when the alkyl substituents on the carbamoyl group increased in size from *N*-monomethyl- to *N,N*-dimethyl-, *N*-ethyl-*N*-methyl-, or *N,N*-diethyl-, the decarbamoylation rates decreased by 4-, 70-, and 1000-fold, respectively. Thus the larger the size of the alkyl groups, the slower the rate of decarbamoylation due to active site distortion. Furthermore, solvent deuterium oxide isotope effects for decarbamoylation decreased from 2.8 for *N*-monomethylcarbamoyl AChE to 1.3 for *N,N* -diethylcarbamoyl AChE, indicating a shift in the rate-limiting step from general acid-base catalysis to a likely conformational change.

Keywords: Acetylcholinesterase; Decarbamoylation; N,N-diethyl carbamates

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

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ASSESSMENT OF SCORING FUNCTIONS FOR AChE-LIGAND INTERACTIONS

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Computer-aided drug design is based on molecular modelling which includes two steps; molecular docking accompanied by scoring docked poses. Molecular docking fits the right molecular "key" to a known receptor "lock" by optimizing the atomic coordinates of a ligand to adapt its 3D structure in such a way to accommodate the binding into the receptor. The second step is the determination of a good fit between the ligand "key" and receptor "lock" using a function that correctly prioritizes the docked ligand poses and predicts their binding affinities by taking into account molecular interactions between the ligand, protein and solvent.

The 68 crystal structures of complexes between acetylcholinesterase (AChE, EC 3.1.1.7) and its ligands, deposited in PDB, were analysed by scoring the functions: LigScore1, LigScore2, PLP1, PLP2, Jain, PMF and PMF04. The scores derived from scoring functions were correlated with an inhibition constant for each ligand (Ki or IC_{50}) in a broad range 10^{-3} – 10^{-12} M. Scores were also correlated with other computational properties as the number of rotational bonds, number of H-bond donor or acceptor atoms, molecular complexity index and topological polar surface area. The linear correlation between the scores derived from the scoring function and matching pKi data resulted in the highest r value for the PLP2 function, r = 0.77, with 10% of the slope error. The LigScore1 function resulted in the lowest r value of 0.47 with 23% of the slope error. The PLP2 scoring function is a good candidate in drug discovery related to AChE, although with a higher number of crystal structures of AChE complexes and reliable kinetic data, a better scoring function could be developed.

Keywords: AChE; drug discovery; scoring function; inhibition constant

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

PHENYL VALERATE ESTERASE ACTIVITY OF HUMAN CHOLINESTERASES

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University Miguel Hernandez of Elche, Elche, Spain

The toxicity of organophosphorus compounds (OPs) cannot be explained only by action on acetylcholinesterase or neuropathy target esterase (NTE). A fraction of the membrane bound phenylvalerate esterase activity (PVase) is associated to NTE, the key initiating molecular event in the OP-induced delayed neuropathy (OPIDN). An enzymatic fraction in chicken brain soluble PVase has been reported to be due to a butyrylcholinesterase protein, and we suggested that this enzymatic fraction could be related to the mode of action of the potentiation/promotion phenomenon of the OPIDN. We showed that human butyrylcholinesterase (hBuChE) shows PVase activity. Mipafox, iso-OMPA or PMSF inhibited both activities with similar kinetic constants for both activities. Moreover, the substrates acethylthiocholine and phenyl valerate showed competition in their activities. The results suggest that both activities are related to the same active center.

This work studies in depth the kinetic interactions between phenyl valerate and acetylthiocholine in human butyrylcholinesterase, showing that the interactions are different to the competitive model of substrates according to the Michaelis-Menten reaction. The approach introduced in this work suggests that other site could be involved in the interaction with phenyl valerate.

In addition, we have observed that human acetylcholinesterase has also phenyl valerate esterase activity, but with lower activity than human butyrylcholinesterase. The level of phenylvalerate esterase activity in cholinesterases depends on the species and the type of cholinesterase. Further evaluation of the molecular interactions is under study.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

EFFECTS OF MEMANTINE AND ITS METABOLITE Mrz 2/373 ON SOMAN-INDUCED INHIBITION OF BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE *IN VITRO*

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Background: Memantine is the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, used in the treatment of Alzheimer's disease. Memantine pretreatment assured protection of skeletal muscles from poisoning with nerve agents and an interaction between memantine and AChE was proposed [1].

Aim: Memantine and its main metabolite (1-amino-3-hydroxymethyl-5-methyl adamantine, Mrz 2/373) were used to ascertain their interaction with erythrocyte acetylcholinesterase (AChE) *in vitro*. The effect of these two compounds on the kinetics of the soman-induced AChE inhibition and on the aging of the soman-AChE complex was also investigated.

Methods: Bovine AChE activity was measured titrimetrically and the effect on aging of the soman-AChE complex was studied [2].

Results: Memantine and Mrz 2/373 exerted concentration-dependent inhibition of AChE, with Mrz 2/373 being a more potent inhibitor than the parent compound.

Addition of soman $2.5x10^{-8}$ mol/l induced gradual AChE inhibition that became almost 100% after 20 min. Memantine (0.1, 0.5 and 1 mmol/l) and Mrz 2/373 (0.1 and 1 mmol/l) concentration-dependently slowed down the AChE inhibition.

Neither memantine nor Mrz 2/373 prevented the aging of the soman-AChE complex. After 5 min incubation with AChE and soman, AChE activity was 11%, 36% and 30% in control medium and after adding of 1 mmol/l of memantine and Mrz 2/373, respectively.

Conclusion: Since high micromolar and low millimolar concentrations of memantine can be achieved in rats [3], it is quite possible that memantine and Mrz 2/373 can prevent AChE from inhibition by soman, which could, along with known memantine's neuroprotective activity, explain its potent antidotal effect in soman poisoning.

Keywords: acetylcholinesterase; memantine; Mrz 2/373; soman; pretreatment

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

OXIMES WITH ORTHO-POSITIONED CHLORINE MOIETY EXHIBIT IMPROVED PHYSICAL-CHEMICAL PROPERTIES, EFFICIENT REACTIVATION OF INHIBITED HUMAN ACETYLCHOLINESTERASE AND REDUCED *IN VIVO* TOXICITY

David Malinak [1,2], Tamara Zorbaz [3], Adam Skarka [2], Martina Hrabinova [1,4], Nikola Marakovic [3], Jana Janockova [1,2], Ondrej Soukup [1,4], Jan Misik [1,4], Daniel Jun [1,4], Kamil Kuca [1,2], Zrinka Kovarik [3*], Kamil Musilek [1,2*]
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The series of bisquaternary oximes with ortho-positioned chlorine moiety was designed, prepared and evaluated. The novel compounds exhibited valuable pK_a properties [1] with improved *in vitro* reactivation ability of sarin, cyclosarin, VX, paraoxon- and dichlorvos-inhibited human AChE exceeding the standard monoquaternary or bisquaternary reactivators (pralidoxime, methoxime, trimedoxime, obidoxime and asoxime syn. HI-6). Additionally, some chlorinated compounds presented *in vitro* reactivation ability of tabun-inhibited human AChE similar to the efficiency of trimedoxime. The *in vitro* results were further explained by molecular docking study. The *in vitro* non-cytotoxic properties of novel compounds were determined with miscellaneous results. However, assessment of maximum tolerated dose highlighted that the selected chlorinated reactivator is well tolerated by mice on the level similar to the clinically or experimentally used oxime reactivators [2]. The *in vivo* reactivation study is in progress.

Keywords: organophosphate; antidote; oxime; chlorinated oxime; pK_a

Acknowledgement

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

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Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DESIGN AND SYNTHESIS OF BIFUNCTIONAL FLUOROPYRIDINALDOXIME REACTIVATORS FOR NERVE AGENT-INHIBITED *HUMAN* ACETYLCHOLINESTERASE

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Acetylcholinesterase (AChE) is a key enzyme of the Central Nervous System (CNS), which hydrolyzes the neurotransmitter acetylcholine.[1] By targeting AChE, organophosphorus nerve agents (OPNA) and organophosphorus pesticides irreversibly inhibit the cholinergic transmission, which is leading to death if untreated.[2] Over several years, our group and colleagues have been concentrating on the development a new class of non-permanently charged bifunctional reactivators, that display higher affinity for AChE and high *in vitro* and *in vivo* efficiencies compared to 2-PAM and Hi6.[3] By analogy, recently, we designed bifunctional reactivators that comprise a peripheral site ligand (PSL) connected to a fluorinated reactivator function using a covalent linker. On the basis of our previous work on the synthesis of central hybrid reactivators bearing 6-alkanyl-3-hydroxy-2-pyridinadoxime moiety, and with the goal to develop reactivator with greater lipophilicity and enhanced blood brain barrier (BBB) permeability, we decided to substitute the 3-hydroxy group, initially designed to decrease the oxime pka, with a more electronegative and electron-withdrawing group such as fluorine. Fluorine is known to modulate the pka of the proximal oxime, the conformational bias and the binding properties via molecular interactions. This structural change, compared to the known 6-substituted 3-hydroxy-2-pyridinadoxime scaffold, appeared valuable for both practical and fundamental reasons, eventually providing reactivators with increased reactivation potency and better pharmacological profiles.

Keywords: Acetylcholinesterase (AChE); Central Nervous System (CNS); Organophosph-orus nerve agents (OPNA); 2-Pyridine Aldoxime Methyl Chloride (2-PAM) and Bifunctional reactivators

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

COMBINATION OF OXIMES WITH OVERLAPPING REACTIVATION SPECTRA: OBIDOXIME AND HI-6

Timo Wille, Horst Thiermann, Franz Worek
Presenting author: Timo Wille

Despite extensive oxime research in the last 60 years pralidoxime is still the standard oxime in e.g. United States, British and French forces and obidoxime standard therapy for OP poisoning in several European countries. Oxime research focusses on highly potent oximes with activity against selected nerve agents, broad-spectrum oximes with activity against relevant nerve agents and centrally active (non-)oximes but virtually no compound brought significant improvements compared to the established obidoxime and pralidoxime. In the US MMB-4 is sought to replace pralidoxime and in Germany, France, UK, Canada and other European countries HI-6 is in advanced development for use as nerve agent antidote. Yet, both compounds are not considered as broad-spectrum antidotes and as a mid-term solution combinations of oximes in service with overlapping reactivation potency e.g. obidoxime and HI-6 have been proposed. We here set out to analyze the combination of obidoxime and HI-6 in both a static and dynamic model against poisoning with nerve agents and organophosphorus compound pesticides *in vitro*. In a cuvette based system the combination of HI-6 and obidoxime both 30 µM for sarin-, cyclosarin-, tabun-, VX- and paraoxon-inhibited human AChE did not result in an impaired reactivation compared to the sole use of both oximes but in a broadened spectrum. Similar results were gained with a dynamic model allowing simulation of nerve agent and pesticide toxicokinetics and oxime pharmacokinetics resembling *in vivo* conditions. Additional experiments in species closely related to humans e.g. swine are necessary to analyse a potential benefit *in vivo*.

Keywords: HI-6; obidoxime; reactivation; nerve agents; pesticides

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DESIGN OF BROAD SPECTRUM ANTIDOTES

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The design of reactive molecules such as nerve agent antidotes is inherently challenging due to two intertwined processes imperative for their efficiency: The reversible binding of the initial non-covalent complex in a low energy conformation and the chemical reaction that proceeds *via* a transition state of high(er) energy. Furthermore, a structural and chemical diversity among different nerve agents and their corresponding complex with AChE complicates the design of broad-spectrum antidotes. The development of broad spectrum antidotes has proven challenging and although progress has been made, no new drugs with improved properties have been launched in several decades. Herein, we report a rational, structure-based approach for the development of broad-spectrum antidotes. Based on a hit molecule identified in a high throughput screening targeting the non-inhibited species of AChE, 18 new analogous molecules were designed and synthesized. This resulted in a set of compounds with a diversity in their potency, as desired for subsequent (quantitative) structure-activity relationship ((Q)SAR) modeling. The 18 compounds were investigated for their ability to bind to four different phosphonylated forms of AChE (*i.e.* human AChE inhibited by the nerve agents VX, VR, and tabun, or the substance DFP). The QSAR model was subsequently used to guide the development of a novel set of pyridinium-oxime based broad spectrum antidotes. The mechanism of reactivation of the developed antidotes has been investigated using a combination of X-ray crystallography and molecular modelling.

Acknowledgement

Acknowledgements are not mandatory.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DEMONSTRATION OF THE FIRST SMALL MOLECULE THERAPEUTICS FOR RESURRECTION OF THE AGED FORM OF ACETYLCHOLINESTERASE AFTER EXPOSURE TO ORGANOPHOSPHORUS CHEMICAL NERVE AGENTS AND PESTICIDES

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Organophosphorus (OP) compounds are potent acetylcholinesterase (AChE) inhibitors that have found use as both chemical warfare agents (CWAs) and as pesticides. Following inhibition of AChE by OP compounds, a competitive dealkylation reaction of the phosphylated serine residue occurs – a process referred to as aging. Current therapeutic reactivators of OP-inhibited AChE, mainly oximes, are not effective once aging has occurred. For the first time, we have demonstrated *in vitro* conversion of the aged AChE to the native form using small drug-like molecular therapeutics. As part of this effort, a diverse library of small molecule therapeutics have been developed to both recover the activity of aged-AChE, termed resurrection, as well as the activity of inhibited-AChE, referred to as reactivation. The structure of such therapeutics is derived from pyridyl-based quinone methide precursors (QMPs), sharing structural similarities to known therapeutic oximes. A structure-activity relationship study of synthesized QMP therapeutics was conducted to determine the effect electron-donating and electron-withdrawing groups have on the efficiency of both processes and to design optimized small molecule therapeutics for *in vivo* biological efficacy. Our successes will be presented.

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ISSN 2571-113X (Online)

MEETING ABSTRACTS

NANOTECHNOLOGY STRATEGIES USING OXIMES-LOADED LIPID NANOPARTICLES FOR BRAIN PROTECTION AGAINST ORGANOPHOSPHORUS POISONING

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Nanotechnological "two-in-one" approach using nanoparticles for packaging two oximes in single carriers and nose-to-brain delivery for brain protection against poisoning by organophosphorus agents have been developed. Strategies for designing nanocarriers for drug delivery to the CNS and crossing the BBB showed that nanoparticles based on natural and biodegradable materials are promising. Solid lipid nanoparticles (SLNs) are biocompatible, biodegradable and have very low toxicity, thereby fulfilling the requirements of preclinical safety [1]. 2-PAM and a novel reactivator of VX-, paraoxon-, and tabun-phosphylated AChE [2] a poorly water soluble 6-(5-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)pentyl)-3-hydroxypicolinaldehyde oxime (3-HPA), were loaded in SLNs to offer distinct release profile and half-life for both oximes. To increase the therapeutic time window of both oximes, SLNs with two different compartments were designed. Oxime-loaded SLNs of hydrodynamic diameter 100-160 nm and zeta potential (from -30 to -25 mV) were stable for a period of 10 months at 4ºC. SLNs displayed longer circulation time in the bloodstream compared to free 3-HPA and free 2-PAM. Oxime-loaded SLNs were suitable for intravenous administration. Paraoxon-poisoned rats ($0.8\times LD_{50}$) were treated with 5mg/kg of 3-HPA-loaded SLNs and 2-PAM+3-HPA-loaded SLNs. Brain AChE reactivation up to 30% was slowly achieved in 5 h after administration of 3-HPA-SLNs. Synergistic effect and increased reactivation up to 35% was observed with combination of both oximes.

In addition, new cationic liposomes based on L-α-phosphatidylcholine and cationic surfactant were administered via the intranasal route. These liposomes were found to reach directly central AChEs. This last approach provides evidence that reactivation of central AChEs can be achieved by a non-invasive approach that bypasses the BBB.

Keywords: Solid-Lipid Nanoparticles; Blood-brain barrier; Acetylcholinesterase; Oxime; Paraoxon

Acknowledgement

The work was supported by Russian Science Foundation, grant No. 14-50-00014.

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MEETING ABSTRACTS

UTILIZING STRUCTURE-ACTIVITY RELATIONSHIPS AND MECHANISTIC INSIGHTS TO DESIGN NON-OXIME REACTIVATORS

C. Linn Cadieux, Zachary Canter*, Kevin Martin*, Keith Morgan*, Michael Hepperle

Organophosphorus nerve agents are highly toxic compounds which pose a threat worldwide. These compounds induce toxicity by covalently binding to the active site serine of acetylcholinesterase, which results in inhibition of the enzyme. Without functional acetylcholinesterase, the levels of the neurotransmitter acetylcholine in neuromuscular junctions rise quickly, causing overstimulation of the nervous system, which will culminate in death if not treated. Current treatments rely on small molecules to interact with inhibited enzyme to disrupt the covalently bound phosphorus moiety at the active site. The most effective molecules incorporate a pyridinium oxime which acts via direct nucleophilic attack on the phosphorus to achieve reactivation of the enzyme. These compounds have limited effectiveness because the charged portion of the molecule does not allow them to cross into the central nervous system where acetylcholinesterase inhibition is most harmful. The results of studies that characterized a small molecule reactivator (4-amino-2-((diethylamino)methyl)phenol [ADOC]) that does not incorporate an oxime but is capable of reactivating nerve agent-inhibited enzyme as well as or better than current treatments have been used to inform the design of additional novel compounds. This study describes the *in vitro* characterization of these novel compounds as reactivators of phosphonylated human acetylcholinesterase.

*This research was supported in part by an appointment to the Postgraduate Research Participation Program at the U.S. Army Medical Research Institute of Chemical Defense administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U. S. Army Medical Research and Materiel Command.

This research was supported by the Defense Threat Reduction Agency – Joint Science and Technology Office, Medical S&T Division.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

NEW NON-OXIME REACTIVATORS OF ORGANOPHOSPHATE INHIBITED ACETYLCHOLINESTERASE WITH PROMISING REACTIVATION POTENCY

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Organophosphate (OP) compounds inhibit the enzyme acetylcholinesterase (AChE) resulting in severe symptoms and ultimately death. OP intoxications are currently treated by administration of atropine and certain oxime compounds (Obidoxime, HI-6 or 2-PAM). The latter compounds contain nucleophilic oximes that reactivate OP-inhibited AChE by liberating the phosphylated serine. However, these oximes have several drawbacks such as their intrinsic toxicity, their permanent charge which thwarts penetration of brain tissues and their inability to effectively reactivate all types of nerve agent inhibited AChEs. Therefore, the search for new (non-ionic) antidotes of nerve agent poisoning is of great importance. Recently, several papers reported on the discovery of non-oxime compounds as a result of the in vitro or in silico screening of libraries of bioactive compounds and approved drugs. For instance, Katz et al reported[1] a novel class of compounds in which the 4-amino-2-(diethylamino)phenol (ADOC) appeared to be a key motif responsible for reactivation of OP-inhibited AChE.[2] In addition, several structural derivatives of ADOC were synthesized and evaluated for OP-AChE reactivation by Cadieux et al.[3] That study provided valuable information on key structural features of ADOC with respect to reactivation potency and enzyme inhibition, but unfortunately, none of the reported derivatives performed equal or better than the ADOC parent. We here report the design and synthesis of a new series of ADOC derivatives. We report that one of the compounds synthesized so far showed a remarkably improved in vitro performance compared to ADOC towards VX-, sarin-, cyclosarin- and paraoxon-inhibited human AChE.

Keywords: reactivator; non-oxime; acetylcholinesterase

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

IDENTIFYING AXONAL TRANSPORT-RELATED TARGETS FOR REVERSING THE ADVERSE EFFECTS OF ORGANOPHOSPHATE EXPOSURE

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The chemicals known as the organophosphates (OPs) are found in hundreds of useful agricultural, industrial, and commercial products; however, they have also been associated with a variety of adverse health effects in humans and other non-target organisms. The acute toxicity of OPs is attributed to the inhibition of the enzyme acetylcholinesterase; however, this mechanism is inadequate to explain all of the long-term adverse effects of OPs. In both live imaging studies in primary neuronal culture as well as in manganese-enhanced magnetic resonance imaging (MEMRI) studies of the brains of living rats, we have observed impairments in axonal transport (AXT) associated with both the insecticide OP chlorpyrifos and the nerve agent OP diisopropylfluorophosphate. These observations may be important since AXT is an essential process that is responsible for the movement of a variety of important macromolecules to and from a neuron's cell body. In this presentation, a brief overview of the results of these neuronal culture (trafficking) and MEMRI experiments will be provided. In addition, the results of experiments conducted to date to identify specific molecular targets of OPs that might negatively influence axonal transport will be summarized. These targets include post-translational modifications of structural proteins that affect AXT through the regulation of microtubule dynamics and stability (e.g., Tau phosphorylation, Tubulin Acetylation), and specific signaling kinases (e.g., ERK GSKIIIβ) that are known to regulate various components of the AXT process. These experiments are expected to help us begin to develop novel therapeutic strategies to improve the neuronal deficits associated with OPs.

Keywords: Pesticide; Nerve Agent; Agriculture; Gulf War Illness

Acknowledgement

Funding source: Congressionally Directed Medical Research Programs (CDMRP) and The Medical College of Georgia Foundation, Augusta University

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DIAGNOSIS OF POISONING WITH *O*-ISOBUTYL-*S*-[2-(DIETHYLAMINO)ETHYL]METHYLPHOSPHONOTHIOATE (VR) UNDER ANTIDOTAL THERAPY WITH CARBOXIM

Nadezhda L. Koryagina, Elena I. Savelieva, Anton I. Ukolov, Darya S. Prokofieva, Nataliia S. Khlebnikova, Tatiana I. Aliushina, Elena S. Ukolova, Andrey S. Radilov, Nikolay V. Goncharov
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The choice of biomarkers for establishment of exposure to organophosphorus compounds (OPs) is made based on the results of assessment of the real situation with account for such factors as the required timeframe for providing the results of expert examination, nature and volume of biosamples, available equipment, and the degree of confidence of information on the influencing factor (substance, dose, way of entry, use of antidote).

We estimated the efficiency of express methods of diagnosis of exposure to OPs, specifically, Ellman's cholinesterase activity assay, as well as GC-MS/MS and HPLC/MS/MS determination of OPs fluoride-regenerated from protein adducts and low-molecular hydrolytic metabolites of OPs, respectively. The objects of study were blood and urine samples of rats exposed to VR in a dose of 2×0.4LD50 under conditions of antidotal therapy with Carboxim {5-[[[2-[benzyl(diemthyl)ammonio]ethyl]amino]carbonyl]-2-[(hydroxyimino)methyl]-1-methylpyridinium dichloride}.

Carboxim therapy led to AChE reactivation 3 h after exposure to VR, while in the absence of the therapy the AChE activity recovered within 3 days.

Fluoride regeneration of VR from its blood plasma protein adducts was possible within 7 days after poisoning irrespective of whether the therapy was applied or not

O-isobutyl methylphosphonate was detected in urine 24 h after exposure in the urine samples of animals both subjected and not subjected to antidotal therapy, whereas after 3 days it was detected exclusively in the urine samples of animals not given the antidote.

It was also found that blood plasma levels of free and esterified fatty acids can serve as an additional toxicodynamic parameter of VR poisoning.

Keywords: nerve agents; VR; markers; Carboxim; antidotal therapy

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

COPPER-DEPENDENT HYDROLYSIS OF TRICHLORONATE BY TURKEY SERUM AND ALBUMIN

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Trichloronate is a racemic organophosphatioate insecticide. It induced delayed neuropathic in hens and human. The avian are species with greater susceptibility to organophosphorus poisoning due to their low levels of A-esterases. However, a copper-dependent hydrolyzing activity of hexyl dichlorophenyl phosphoramidate (HDCP), known as "antogonistic stereoselectivity" was recently identified in chicken serum. This study shows the activating effect of copper on the hydrolysis of trichloronate enantiomers by turkey serum and albumin (TSA) using chiral chromatography with CHIRALCEL OD column and heptane HPLC as mobile phase. The trichloronate hydrolysis levels (μM remaining concentration of each isomer) quantified at 37 °C, pH 7.4 and 60 minutes of turkey serum (10 μL) incubated with 300 μM of copper were statistically higher $p<0.05$) for (-)-trichloronate (65 %) than (+)-trichloronate (32%). This estereoselective hydrolysis observed in turkey serum was confirmed by the incubation of 200 μg of turkey serum albumin (amount of this protein estimated in the 10 mL of turkey serum) with 400 μM of racemic trichloronate and 300 μM of copper at physiological condition during 60 minutes; hydrolysis values of 90% and 72% were obtained for (-)-trichloronate and (+)-tricholoronate. In conclusion, the present study evidences the hydrolysis of an organophosphatioate racemic for an A-esterase activity in turkey serum and identifies albumin as the cuproprotein responsible of this Cu^{2+}-dependent stereoselective hydrolysis of this chiral insecticide in the turkey serum.

Keywords: trichloronate; chiral organophosphatioate; hydrolysis; turkey; albumin; serum

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

MASS SPECTRAL DETECTION OF DIETHOXYPHOSPHO-TYROSINE ADDUCTS ON PROTEINS FROM HEK293 CELLS USING MONOCLONAL ANTIBODY DEPY FOR ENRICHMENT

Seda Onder [1,2 *], Lawrence M. Schopfer [2], Ozden Tacal [1], Thomas A. Blake [3], Rudolph C. Johnson [3], Oksana Lockridge [2*]
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Chronic illness from exposure to organophosphorus toxicants is hypothesized to involve modification of unknown proteins. Tyrosine readily reacts with organophosphorus toxicants in proteins that have no active site serine. We developed a monoclonal antibody, depY, that specifically recognizes diethoxyphospho-tyrosine in proteins and peptides, independent of the surrounding amino acid sequence [1]. Our goal was to identify diethoxyphosphorylated proteins in human HEK293 cell lysate treated with chlorpyrifos oxon. Cell lysates treated with chlorpyrifos oxon were examined by ELISA and capillary electrophoresis Western blot. Tryptic peptides were analyzed by liquid chromatography-tandem mass spectrometry. The depY antibody recognized diethoxyphospho-tyrosine containing proteins by ELISA and Western blotting. Mass spectrometry identified 40 diethoxyphospho-tyrosine peptides from 24 proteins in immunopurified samples, but found only 9 diethoxyphospho-tyrosine peptides from 6 proteins when the same sample was not immunopurified on depY. The most abundant proteins in the cell lysate, Histone H4, Heat shock 70 kDa protein 1A/1B, Heat shock protein HSP 90 beta, and Alpha-enolase, were represented by several diethoxyphospho-tyrosine peptides. It was concluded that use of immobilized depY improved the number of diethoxyphospho-tyrosine peptides identified in a complex mixture. The mass spectrometry results confirmed the specificity of depY for diethoxyphospho-tyrosine peptides independent of the context of the modified tyrosine, which means depY could be used to analyze modified proteins in any species.

Keywords: chlorpyrifos oxon; diethoxyphospho-tyrosine antibody; mass spectrometry; ELISA; Western blot

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

INNOVATIVE BIOCATALYSTS AS TOOLS TO DETECT AND INACTIVATE NERVE AGENTS

Elena Porzio [1*], Francesca Bettazzi [2*], Luigi Mandrich [1], Immacolata Del Giudice [1], Odile F. Restaino [3], Serena Laschi [4], Ferdinando Febbraio [1], Valentina De Luca [1], Maria G. Borzacchiello [3], Teresa M. Carusone [1], Franz Worek [5], Antonio Pisanti [6], Piero Porcaro [6], Chiara Schiraldi [3], Mario De Rosa [3], Ilaria Palchetti [2], Giuseppe Manco [1#]

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Pesticides and warfare nerve agents are frequently organophosphates (OPs) or related compounds. Their acute toxicity highlighted more than ever the need to explore applicable strategies for the sensing, decontamination and/or detoxification of these compounds. Herein, we report the use of two different thermostable enzyme families capable to detect and inactivate OPs. In particular, mutants of carboxylesterase-2 from *Alicyclobacillus acidocaldarius* and of phosphotriesterase-like lactonases from *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*, have been selected and assembled in an optimized format for the development of an electrochemical biosensor and a decontamination formulation, respectively. The features of the developed tools have been tested in an *ad-hoc* fabricated chamber, to mimic an alarming situation of exposure to a nerve agent. Choosing ethyl-paraoxon as nerve agent simulant, a limit of detection (LOD) of 0.4 nM, after 5 s of exposure time was obtained. Furthermore, an optimized enzymatic formulation was used for a fast and efficient environmental detoxification (>99%) of the nebulized nerve agent simulants in the air and on surfaces. Crucial, large-scale experiments have been possible thanks to production of grams amounts of pure (>90%) enzymes.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

IONIZABLE, ZWITTERIONIC OXIMES AS COUNTERMEASURES TO VOLATILE ORGANOPHOSPHATE (OP) EXPOSURE

Palmer Taylor [1], William C. Hou [1], Jeremiah Momper [1], Yan-Jye Shyong [1], Zoran Radic [1], John McDonough [2], Zrinka Kovarik [3], Yvonne Rosenberg [4], K. Barry Sharpless [5]

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Small ionizable, zwitterionic oximes of limited toxicity show successful outcomes in non-human primates upon intramuscular post-treatment of exposures to OP's, that enter via the respiratory tract. Along with their inherent limitations, we consider the bases for success in post-exposure treatment of OP toxicity and reversal of OP-induced sequelae of symptoms[1]. (1) High vapor pressure OPs carry the largest acute exposure risk in mass terrorism. Toxic OPs released from explosive devices or into controlled ventilation environments are governed by partial pressure and Fick's Second Law of Diffusion (inverse square of the distance); (2) Low molecular weight, zwitterionic oximes confer optimal nucleophile orientation and activity within the confines of the OP-impacted, active center gorge of human acetylcholinesterase (AChE). (3) We emphasize features of ionizable neutral oximes of low toxicity that allow facile passage of membranes to peripheral and central AChE targets and optimal attack angles in the AChE active center. Hence, for volatile OP's, antidotes must rapidly enter the circulation, post-exposure, to chase the offending OP. Following entry, antidotes should then hastily equilibrate between tissue compartments and cross the blood-brain barrier. Accordingly, we examine the ionization states of zwitterionic oximes and other cationic and anionic (F-) nucleophiles in relation to their kinetic parameters of reactivation[2]. Toxicities, both realized and potential, of nucleophilic antidotes in different ionization states, and pharmacokinetics in mice and macaques, under control and exposure conditions, emerge as critical factors for determining in vivo antidote efficacy. Data will be presented on multiple OP's and their enzyme conjugates[1-3], comparator oximes and in three animal species/strains.

Keywords: zwitterionic oximes; reactivation; organophosphate conjugates; CNS permeability; antidotes

Acknowledgement

Collaborators at TSRI, USAMR-ICD, IMROH, & PlantVax are gratefully acknowledged.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DEVELOPMENT OF PRE- AND POST-COUNTERMEASURES AGAINST OP TOXINS IN MACAQUES

Yvonne Rosenberg [1], James Fink [2], Lingjun Mao [1], Xiaoming Jiang [1], Jonathan Lees [1], Jerry Wang [3], Tara Ooms [3], Narayanan Rajendra [3], Zoran Radic [4], Palmer Taylor [4]
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Deliberate sarin releases in Syria with large numbers of fatalities emphasize the need for OP countermeasures for both military and civilian populations. Therapeutic countermeasures involve several strategies: (i) preventing OP poisoning through administering pre-exposure treatments that scavenge OPs before they inhibit their physiological AChE targets in the brain and in the periphery (ii) post-exposure oxime that can rapidly reactivate OP-inhibited AChE or (iii) a combination of both. In terms of a pretreatment, our recent studies have demonstrated that administration of an aerosolized (aer)-rHuBChE employing a user friendly nebulizer, forms a protective pulmonary bioshield in the lungs of macaques which to date remains intact for at least 4 days. Thus 8 mg/kg of aer-rHuBChE deposited in the lung can prevent symptoms and inhibition of RBC-AChE and plasma BChE following a high (55ug/kg) inhaled dose of aer-paraoxon (Px) 4 days later; an amount known to inhibit circulating ChEs by >95% and cause tremors. In terms of oxime efficacy, macaque studies have demonstrated that a single IM post-exposure injection of the zwitterionic, centrally acting oxime RS194B (62-80ug/kg) plus low-dose atropine rapidly reactivates OP-inhibited RBC-AChE and circulating BChE and dramatically reverse both early and advanced clinical OP symptoms following lethal inhalation exposure to both sarin vapor (49.6ug/kg) and lethal aerosolized paraoxon (100ug/kg).

The increased efficacy of nebulizers in humans and the known synergy between aer-rHuBChE pretreatment with IM RS194B post exposure bodes well for a prophylactic or combination treatment which can protect against potent inhaled OP agents for >6 days without multiple injections.

Keywords: aer-human butyrylcholinesterase; sarin; paraoxon; oxime; reactivation; macaques.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

HUMAN PLASMA-DERIVED BUTYRYLCHOLINESTERASE IS BEHAVIORALLY SAFE AND EFFECTIVE IN CYNOMOLGUS MACAQUES (*Macaca Fascicularis*) CHALLENGED WITH SOMAN

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Organophosphorus compounds (OP) pose a significant threat. Administration of human butyrylcholinesterase (Hu BChE) may reduce or prevent OP toxicity. Thus, we evaluated the safety and efficacy of Hu BChE in monkeys using sensitive neurobehavioral tests while concurrently characterizing absorption and elimination in the presence and absence of high-dose soman exposure to predict time course and degree of protection. Eight young adult male cynomolgus macaques were trained on two distinct automated tests of neurobehavioral functioning. Hu BChE purified under current-Good-Manufacturing Practices (CGMP) was injected intramuscularly at 13.1 mg/kg, producing an average peak plasma value (C_{max}) of 28 Units/ml. The apparent time to maximum concentration (T_{max}) approximated 12 hours and the elimination half-life approximated 80 hours, returning to pre-administration (baseline) levels by 14 days. No behavioral disruptions following Hu BChE administration were observed on either neurobehavioral test, even in monkeys injected 24 hours later with an otherwise lethal dose of soman. Thus, Hu BChE provided complete neurobehavioral protection from soman challenge. These data replicate and extend previous results that used a different route of administration (intravenous), a different species (rhesus macaque), and a different BChE product (non-CGMP material). The addition of two sensitive neurobehavioral tests coupled with the PK/PD results convincingly demonstrates the neurobehavioral safety of plasma-derived Hu BChE at therapeutic levels. Protection against an otherwise-lethal dose of soman by a pre-exposure treatment dose that is devoid of side effects establishes a foundation for additional testing using other exposure routes and treatment times, other challenge agents/routes, or other classes of organophosphate scavengers.

Acknowledgement and disclaimers

Opinions, interpretations, conclusions, and recommendations are those of the author(s) and are not necessarily endorsed by the US Army.

This research complied with the Animal Welfare Act and implementing Animal Welfare Regulations and adhered to the principles noted in The Guide for the Care and Use of Laboratory Animals.

This work was funded by the Defense Threat Reduction Agency, Medical S&T Division.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DESIGN OF A COMBINED APTAMER FOR PARAOXON AND ACETYLCHOLINESTERASE BY IN SILICO APPROACH

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Poisoning by organophosphates (OPs) takes one of the leading places in the total number of exotoxicoses. Detoxication of OPs at the first stage of poisoning could be achieved with the help of aptamers, which are able to bind poisons in the bloodstream [1]. The effectiveness of the aptamers for OPs could be strengthened by their possibility to bind non-covalently with the peripheral anionic site (PAS) of acetylcholinesterase (AChE) defending the active site gorge from OPs molecules. In the present work, we have applied for the first time the *in silico* design of a combined aptamer for paraoxon and PAS of AChE. Based on the published sequence of an aptamer binding organophosphorus pesticides [2], its three-dimensional model was constructed. The most probable binding site for paraoxon was determined by molecular docking and molecular dynamics (MD) methods. Then the nucleotides of the binding site were mutated consequently and the values of free binding energy were calculated using MD trajectories and MM-PBSA approach [3]. On the basis of the energy values, the sequences that bind paraoxon most efficiently have been selected. Molecular docking of sixteen possible nucleotide pairs into PAS of AChE was performed and the pairs that bind with PAS most efficiently have been selected. The 5'-end of the aptamers for paraoxon was modified based on the results of molecular docking. The calculations have shown that the final aptamers interact with paraoxon and PAS of AChE more efficiently than AChE interacts with paraoxon.

Keywords: acetylcholinesterase; aptamer; molecular modeling; paraoxon

Acknowledgement

These studies were supported by the Russian Science Foundation (grant 16-15-00199).

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ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

PROKARYOTIC EXPRESSION OF HUMAN BUTYRYLCHOLINESTERASE AS A TOOL FOR CATALYTIC BIOSCAVENGER DEVELOPMENT

Xavier Brazzolotto [1], Alexandre Igert [1], Virginia Guillon [1], Gianluca Santoni [2], Florian Nachon [1]

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Currently, the best bioscavenger candidate against nerve agent intoxication is human butyrylcholinesterase (BChE). However, the effective dose cost, estimated to about 200 milligrams of pure enzyme, remains challenging despite the production and purification progresses realized these last years. A strategy for reducing dosage and cost would be to turn this scavenging protein into a nerve agent hydrolyzing enzyme, a catalytic bioscavenger. Up to now, screening of large mutant libraries has been hindered by the restricted eukaryotic expression of active BChE. Here we present the successful prokaryotic expression of an active human BChE variant designed with *PROSS*, a sequence- and structure-based algorithm for the soluble prokaryotic expression of difficult proteins. The protein is easily purified with two simple chromatographic steps. Despite 47 point mutations, the enzyme presents similar enzymatic parameters than the wild-type enzyme and its active site gorge structure is identical to that of the native enzyme produced in eukaryotic systems as determined by X-ray crystallography. These data validate the prokaryotic expression of human BChE which will greatly facilitate the screening of variants with nerve agent hydrolytic properties. We have initiated animal studies to assess the protein potency (immunogenicity, pharmacokinetic and bioscavenger efficiency) and will study the production of the tetramer form. On the other hand, we are currently developing high-throughput protocols for the prokaryotic expression, purification and screening of nerve agent hydrolysis.

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Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

BIOSCAVENGERS AND THE MEDICAL MANAGEMENT CHAIN

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Survival and recovery from nerve agent poisoning requires a continuum of medical care, starting with a rapid initial response followed by continued support though the medical chain. In a military context, research into counter-measures to nerve agent poisoning has traditionally focussed on first-aid, pretreatment and prophylaxis; however, there are many opportunities to optimise the management of nerve agent-poisoned casualties.

We have previously demonstrated the efficacy of bioscavenger as a post-exposure, pre-symptomatic therapy in guinea-pigs poisoned by VX via the dermal route. Data will be presented on the efficacy of bioscavenger before, on and after the appearance of signs of poisoning and the influence of introducing a delay between initial treatment and the administration of bioscavenger. Treatment regimens including bioscavenger offered near-complete protection against the VX challenge, in the absence of continuing supportive therapy.

The potential for bioscavenger use within the treatment chain could range from pre-exposure to hospital use. Inclusion of bioscavenger has potential to reduce the level of medical care, monitoring and therapeutic intervention for casualties that have been poisoned percutaneously. The results will be discussed in the context of the UK military medical management chain and the considerations and constraints of the operational environment.

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Keywords: Bioscavengers; Medical Management; VX; guinea-pigs

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

BORDERLINE BETWEEN CATALYTIC AND NON-CATALYTIC BIO SCAVENGERS: THE EXAMPLE OF ALBUMIN AND REVERSIBLE B-ESTERASES

Eugenio Vilanova, Jorge Estévez, Miguel Ángel Sogorb, Iris Mangas and Antonio Monroy

University Miguel Hernandez of Elche (Alicante) Spain

Protective mechanism against organophosphorus compounds (OPs) toxicity are mainly based in molecular processes frequently divided conceptually in (A) catalytic and (B) non-catalytic bio-scavengers. Modified natural proteins and small molecules have been developed for applying in therapy and protection. The catalytic ones are mainly associate to the classical concept of A-esterases (phosphotriesterases, PTEs, i.e. paraoxonase); they hydrolyze carboxylesters and OPs by a divalent cation dependent mechanism. The non-catalytic scavengers are mainly associated to covalent binding to proteins, especially B-esterases with a serine or tyrosine residue, which hydrolyzes carboxylesters. However, if an OP is bound (organophosphorylation), its represents an enzymatic inhibition in some cases considered "the target" of toxicity or initial molecular event (IME) in their mode of action developing toxicity (adverse output pathway, AOP). The binding to proteins also represents a sequestration avoiding the OP interaction to other protein. However, there are protein binding OPs (non-catalytic bioscavengers) which can be slowly dephosphorylated, having a role as catalytic scavenger. A proportion of B-esterase activity in serum and brain shows reversible inhibition and their protective role just in situ in the target tissue of toxicity need to be investigated. Serum albumin is other example of B-esterase mainly thorough a tyrosine residue; its role in detoxication have been demonstrated and adducts applied as biomarker of exposure. Moreover, for a specific phosphoramidate family hydrolysis capacity may be enhanced by copper, probably by a mechanism not related with its B-esterase activity. Therefore, we have examples in the borderline between non-catalytic and catalytic scavengers.

Keywords: A esterase; B esterase; scavengers; albumin; phosphorylation

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

CATALYTIC SCAVENGERS PROVIDE BROAD-SPECTRUM PROTECTION AGAINST ORGANOPHOSPHORUS NERVE AGENTS

Shane A. Kasten, Sandra J. DeBus, Thuy L. Dao, Michael V. Boeri*, Zachary A. Canter*, Sean M. Hodgins*, Robyn. B. Lee, Douglas M. Cerasoli, and Tamara C. Otto
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Efforts to develop a single enzyme capable of catalyzing the hydrolysis of a broad spectrum of organophosphorus (OP) compounds into non-toxic products have produced multiple candidate enzymes on different structural scaffolds. While protection against multiple OPs from a single enzyme has been obtained, no single enzyme has been identified that can provide protection against all G- and V-type OP nerve agents. The most promising candidate enzyme platform is the bacterially produced recombinant variant of organophosphorus hydrolase (OPH) from *B. diminuta*. *In vivo* protective efficacy of candidate OPH scavengers as prophylactics was tested in guinea pigs by administering the enzyme via a carotid catheter, followed 20 minutes later by a subcutaneous injection of increasing doses of the OP nerve agents GA, GB, GD, GF, VX, VR, or VM. A stage-wise, adaptive dosing experimental design was used to determine the median lethal dose (LD_{50}) of each OP in the context of enzyme prophylaxis. We report that a combination of two different OPH variants is capable of providing protection against at least 2 x LD_{50}s of all of the OPs tested. The results indicate that broad spectrum prophylactic protection against OP intoxication can be provided with a cocktail of two different catalytic scavengers with appropriate catalytic activity. Formulation of the enzymes to promote circulatory stability will be discussed.

Keywords: catalytic scavenger; prophylaxis; organophosphorus hydrolase

The views expressed in this abstract are those of the author(s) and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. This work was supported by the NIH CounterACT Center of Excellence grant U54 NS058183 (to D.M.C.) and by the Defense Threat Reduction Agency-Joint Science and Technology Office, Medical S&T Division.

*This research was supported in part by an appointment to the Postgraduate Research Participation Program at the U.S. Army Medical Research Institute of Chemical Defense administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and USAMRMC.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

PARAOXONASE 1 VARIANT I-F11 GENE THERAPY USING ADENO-ASSOCIATED VIRUS8 (AAV8) OFFERS LONG-TERM PROTECTION AGAINST G-TYPE CHEMICAL WARFARE NERVE AGENTS

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The paraoxonase 1 variant I-F11 affords asymptomatic protection against the lethal effects of G-type chemical warfare nerve agents (CWNA). Here, we tested whether adeno-associated virus8 (AAV8) is able to deliver I-F11 for extended periods of time and at levels affording asymptomatic protection against 2-5LD50 doses of G-type CWNA in mice. I-F11 gene expression levels in mouse blood were assessed under the influence of three different promoters and found to be significantly higher with TBG compared to CMV and CASI. A single tail vein or intramuscular injection of AAV8-TBG-I-F11 resulted in robust production of the enzyme, which reached concentrations of up to 1 to 2 mg/ml in mouse blood for up to 6 months. Mice containing 0.75 mg/ml or higher concentrations of I-F11 in their blood were afforded asymptomatic protection against multiple 5LD50 exposures of GD, GF, GA, and GB, a total of 9 exposures over a seven-week period. We also conducted studies showing that I-F11 is most efficacious in offering protection against GD followed by GF, GB and GA. Analysis of the mouse blood for serum chemistry and hematology parameters, and tissues by H&E staining, indicated no appreciable changes between control mice, mice overexpressing 1-F11 for 6 months, and mice surviving repeated G-agent exposures. These data suggest that AAV8-mediated catalytic bioscavenger gene therapy using 1-F11 is a safe, efficacious, and long-lasting pre-treatment strategy against G-agents.

Keywords: Gene Therapy; AAV8; Chemical Warfare Nerve Agents; Paraoxonase 1 variant I-F11; Safety and Efficacy

Disclaimers/Acknowledgments

The views expressed in this abstract are those of the author(s) and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. The experimental protocol was approved by the Animal Care and Use Committee of the US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD and all procedures were conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. Funding for this work was provided by Joint Science and Technology Office (JSTO), Defense Threat Reduction Agency (DTRA), Department of the Army.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

ORGANOPHOSPHATE HYDROLASE (OPH) DESIGNED AS A TETHERED MONOMER

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Organophosphate hydrolase (OPH) mutants have shown potential use as a medical countermeasure against organophosphorus compounds (OPs). OPH is typically expressed in bacteria as a homodimer. Two separate subunits (35 kDa each) self-assemble through non-covalent bonding at the enzyme face close to the putative active site. OPH homodimers do not secrete expediently from mammalian cells. This causes potential problems when trying to express the protein from a heterologous plasmid or viral delivery system. To enhance secretion of OPH from mammalian cells, we sought to increase protein solubility without catastrophic detriment to activity and without addition of fusion proteins. To this end, we designed OPH to be expressed as a tethered monomer by joining two OPH subunits with a poly-glycine linker. We created the single polypeptide OPH with a tether 10 or 35 amino acids in length between the two halves, and named them T10 and T35 respectively. Western blot analysis and paraoxon hydrolysis assays revealed that T10 was being produced and retained some activity against paraoxon. This was a surprise as we expected T10 to have no enzymatic activity. T35 monomer (75 kDa) was also being produced and retained 71% of specific activity against paraoxon compared to untethered OPH. T10 and T35 showed no significant decrement in activity against the nerve agent sarin. Both constructs showed high molecular weight aggregates greater than 250 kDa in dynamic light scattering and native polyacrylamide gels. These tethered constructs are the first attempts known for producing OPH as a single polypeptide.

Keywords: Organophosphate hydrolase; tether; monomer; sarin

The views expressed in this abstract are those of the author(s) and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. This research was supported by the Defense Threat Reduction Agency – Joint Science and Technology Office, Medical S&T Division. This research was supported in part by an appointment to the Postgraduate Research Participation Program at the U.S. Army Medical Research Institute of Chemical Defense administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and USAMRMC.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

A NEW ANIMAL MODEL TO INVESTIGATE ORGANOPHOSPHORUS POISONING AND ENZYMATIC DECONTAMINATION

Laetitia Poirier[1], Pauline Jacquet[2], Laure Plener[2], Cédric Torre[3], Eric Ghigo[3], David Daudé[2], Eric Chabrière[1]
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Freshwater planarians from Platyhelminthes, harboring a mammal-like cholinergic nervous system, have emerged as a promising in vivo model for investigating neurotoxicity. Moreover a large proportion of stem cells provide planarian an unconventional capacity of regeneration allowing for developmental disruption studies. *Schmidtea mediterranea (Smed)* was used as model for organophosphorus (OP) poisoning and for evaluating the efficacy of detoxifying enzymes.

Acetylcholinesterase and butyrylcholinesterase from planarian (*Smed*-AChE and *Smed*-BChE) share 35% identity with their human counterpart (*Hs*-AChE and *Hs*-BChE). Structural predictions revealed strong similarities between planarian and human enzymes. Cholinesterase activities were detected in crude planarian homogenates after grinding and were inhibited after organophosphorus exposition. *In situ* Hybridization was further used to localize cholinesterases in planarians and showed two different patterns, *Smed*-AChE being mainly detected in cephalic ganglion and ventral nerve cords while *Smed*-BChE distribution was diffuse.

Survival, behavior and regeneration were analyzed in whole planarian exposed to four OP [1]. The toxicity of OP degradation products generated by enzymatic hydrolysis with the robust phosphotriesterase enzyme *Sso*Pox, from the archea *Sulfolobus solfataricus* [2], was further evaluated. OP were found to be highly toxic to planarians causing severe mortality and behavior disruption at sublethal concentrations as well as growth disruption during regeneration after cutting. Enzymatic decontamination drastically reduced toxicity and enhanced both mobility and development. These results underline that degradation products have a lower impact than initial organophosphorus substrates. A biotechnological application based on a filtration column incorporating detoxifying enzymes was developed to decontaminate wastewater with planarian as biosensor.

Keywords: Organophosphorus poisoning; Planarian; Cholinesterase; Pesticides; Bioremediation

Acknowledgement

This work is supported by Direction Générale de l'Armement (DGA)

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Military Medical Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

PARAOXONASE-2 DEPENDENT REDOX CONTROL OF PLATELET PHYSIOLOGY

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Background and Objective: Platelets are not only central players in hemostasis and thrombosis but also important modulators of immune responses, inflammation and cancer. Activated platelets generate reactive oxygen species (ROS) that modulate platelet function through redox signaling and oxidative stress. The anti-oxidative enzyme paraoxonase-2 (PON2) is known to counteract inflammation and atherosclerosis. Recently, we showed that PON2-deficient mice exhibit tissue factor-dependent hypercoagulability[1]. Here, we investigated the role of PON2 in ROS production, phenotype and activation of platelets from PON2-deficient mice.

Methods: Platelet count and mean platelet volume (MPV) were determined by a cell counter. Flow cytometry was used to quantify platelet surface receptors, intracellular ROS and platelet function in diluted citrate-anticoagulated platelet-rich plasma. Platelet aggregation was analyzed by light transmission aggregometry in platelet-rich plasma.

Results: Platelets from PON2-deficient mice displayed increased basal and agonist-induced ROS levels accompanied by decreased platelet count but increased MPV compared to wildtype platelets. PON2-deficient platelets showed increased surface expression of the von Willebrand receptor (vWF) GPIbα, vWF-binding, P-selectin surface expression, but no αIIbβ3 integrin/fibrinogen receptor activation *ex vivo*. Botrocetin induced enhanced binding of vWF to PON2-deficient platelets in vitro. However, agonist-induced αIIbβ3 integrin activation, P-selectin surface expression and platelet aggregation were impaired compared to wildtype platelets. Interestingly, addition of 0.5 mM Ca^{2+} to platelet-rich plasma normalized platelet hyporeactivity.

Conclusion: Our data demonstrate that PON2 plays a crucial role in platelet ROS production, phenotype and function. Reactivity of platelets from PON2-deficient mice depends on extracellular Ca^{2+}-concentration.

Keywords: Paraoxonase-2; Reactive oxygen species; Platelets; Hemostasis; Ca^{2+} homeostasis

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

COPPER WITH CHICKEN SERUM ALBUMIN SHOW STEREOSELECTIVE HYDROLYSIS OF CHIRAL PHOSPHORAMIDATES

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Chiral analogous compound of methamidophos insecticide are only poorly hydrolyzed by Ca^{2+}-dependent phosphotriesterases in mammals tissues including the human serum. We reported the hydrolysis of O-hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) in chicken serum. The hydrolysis of the R-(+)-HDCP isomer is strongly increased in vitro in the presence of 30-250 µM copper. It is the opposite estereoselectivity of that showed by liver Ca^{2+}-dependent activity. We name it as "antagonistic stereoselectivity". Diluted chicken serum (10 µL in 1 mL solution of 400 µM HDCP) or the equivalent amount of commercial chicken serum albumin (CSA 216 µg/mL) with 100 µM Cu^{2+}, showed about 50% and 75% of R-(+)-HDCP hydrolysis after 60 and 120 min. In the same conditions other commercial serum metalloproteins with high affinity to Cu^{2+} (cuproproteins) as human serum ceruloplasmin or horse kidney metallothionein did not showed significant Cu^{2+}-dependent hydrolysis. Moreover, other divalent cations (Zn^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+} and Mg^{2+}) did not showed this activation. The results confirm that the CSA is the protein responsible of "antagonistic stereoselectivity" that had been observed in the chicken serum. The effect of copper on the hydrolysis of HDCP by other animal albumins is shown in this work.

Keywords: Albumin; copper, hydrolysis; stereospecificity; phosphotriesterases; organophosphorus

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

INSIGHTS INTO THE YIN AND THE YANG OF ACETYLCHOLINESTERASE INHIBITION BY MECHANISTIC X-RAY CRYSTALLOGRAPHY

M. Bartolini [1], M.L. Bolognesi [1], J. Korábečný [2,3], K. Kuca [4], D. Lamba [5], A. Pesaresi [5], X. Zha [6]

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Drug discovery and development is a complex and expensive process. Thanks to the exponential growth of molecular data and advancement in technologies, efforts have been tremendously amplified. Among new approaches multipotent compounds are emerging as the next paradigm in drug discovery [1] and includes: (i) single drug acting on multiple targets of a unique disease pathway, or (ii) single drug acting on multiple targets pertaining to multiple disease pathways. These compounds are thought to have best beneficial effects in the treatment of complex diseases, like Alzheimer's Disease, in which the simultaneous regulation of various pathological aspects may more efficiently interfere with the disease progression. Systematic integration of the data derived from different disciplines including computational modeling, X-ray crystallography, synthetic chemistry, *in vitro / in vivo* pharmacological tests, is mandatory for the selection of best-in-class compounds. In this context, we report on the key contribution of X-ray crystallography in highlighting peculiar mode of interaction of promising multi-target directed ligands, designed by combining the tacrine fragment to distinct pharmacophores i.e. juglone [2], benzofuran [3] and L-tryptophan with a linker of a suitable length.

Overall, the structural analysis highlights the molecular determinants responsible for the optimal binding of the multi-target ligands to AChE and pinpoints the utility of hybridization strategies in structure-based drug design programs. It also unveils the validity of X-ray crystallographic structures determination at certain milestones along the development of interacting inhibitory drugs based on molecular modeling studies.

Keywords: Acetylcholinesterase Inhibition; Structure-Based Drug Discovery; X-ray Crystallography; Alzheimer's Disease; Multi-target directed ligands

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Science Letters
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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

PHOTO-INDUCED RELEASE OF AN ACETYLCHOLINESTERASE INHIBITOR

Eugenio de la Mora [1,2*], Johannes Broichhagen [3,4,5], Peter Mayer [3], Elisabet Artursson [6], Fredrik Ekström [6], Joel Sussman [7], Israel Silman [8], Dirk Trauner [2,3,9], Giorgio Schirò [1] and Martin Weik [1]
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Light–induced isomerization of enzyme ligands allows controlling specific biological processes in time and space. Photoisomerisable azobenzene-based inhibitors allow photo-control of acetylcholine (ACh) signalling by regulating acetylcholinesterase (AChE), the enzyme that catalyses ACh hydrolysis in the central and peripheral nervous system. By regulating AChE, this family of inhibitors would allow spatial and temporal regulation of ACh levels in the synaptic cleft. Adequate regulation of ACh levels is an essential part of Alzheimer's disease (AD) treatment and other common pathologies. Win this work we present the crystal structures of AChE in complex with three different azobenzene derived inhibitors, we confirmed AzoTHA-1 as the only photoactive compound and we determined its structure in its *cis*- and *trans*- isomeric forms bound to AChE. Three-dimensional structures, supported by online UV-Vis spectroscopy and kinetic data, explain why only AzoTHA-1 is an effective photoactive AChE inhibitor and suggest possible ways to improve photoactive drugs. We utilised S/WAXS to follow photo-isomerisation induced-changes in the wide-angle scattering region to demonstrate that photoisomerisation of the inhibitor induces its release from AChE's active site.

Keywords: Photopharmacology; Alzheimer disease; acetylcholinesterase dynamics

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

STRUCTURAL STUDIES OF *Anopheles gambiae* ACETYLCHOLINESTERASE PROVIDE INSIGHT TOWARDS IMPROVED INSECTICIDES FOR MALARIA VECTOR CONTROL

Jonah Cheung [1], Arshad Mahmood [1], Ravi Kalathur [1], Lixuan Liu [2], Max Totrov [3], Paul Carlier [2]
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Malaria is transmitted by the *Anopheles gambiae* mosquito in sub-Saharan Africa and tropical regions where the disease is prevalent. Indoor spraying with anticholinesterase insecticides is a proven method to control populations of the mosquito and to reduce spread of the disease; however, widespread use of insecticides has led to the rise of an insecticide-resistant G119S mutant acetylcholinesterase in the mosquito which threatens ongoing disease-control efforts. We have solved high resolution X-ray structures of the G119S mutant acetylcholinesterase of *An. gambiae* (G119S AgAChE), in the ligand-free state and in complex with a potent difluoromethyl ketone inhibitor, revealing the structural basis of insecticide resistance[2]. Although resistance-breaking inhibitors of G119S AgAChE exist, they also inhibit human acetylcholinesterase and thus lack the necessary species selectivity to be safely used as insecticides. In our structures, we see specific features within the active site gorge, including an open "back door", that are distinct from human acetylcholinesterase. These differences provide a means for improving species-selectivity in the rational design of improved insecticides for malaria vector control.

Keywords: acetylcholinesterase; structure; malaria; insecticide

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

ROOM-TEMPERATURE CRYSTALLOGRAPHY AND NEUTRON SCATTERING STUDIES OF HUMAN ACETYLCHOLINESTERASE TO INFORM THE DESIGN OF OXIME REACTIVATORS

Oksana Gerlits [1], Mikolai Fajer [2], Xiaolin Cheng [2], Donald Blumenthal [3], Palmer Taylor [4], Zoran Radić [4], Andrey Kovalevsky [5]
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Human acetylcholinesterase (hAChE) is responsible for degrading neurotransmitter acetylcholine at synapses of the nervous system. Organophosphate (OP) nerve agents and pesticides inactivate hAChE through chemical modifications of the catalytic serine. The current generation of oxime antidotes is not highly efficient. Insights into the molecular structures of AChEs from various species reveal possible limitations in enhancing reactivation rates, but provide only limited information, because the structures have been obtained at cryo-temperatures. Moreover, X-ray crystallography usually cannot resolve positions of hydrogen atoms involved in proton transfer processes during reactivation. Thus, we use room-temperature X-ray and neutron crystallography to obtain structures at physiological conditions and to visualize hydrogen atoms.

Several X-ray structures of native and VX and POX-conjugated hAChE in complex with oxime reactivators, RS2-170B and RS-194B have been obtained. hAChE crystallized in a unit cell (a=124.3, c=129.1 Å; $P3_1$) amenable to neutron crystallography. For the first time we show how RS2-170B binds in the non-modified and OP-conjugated active site gorge at room temperature. RS-194B is observed with its oxime group pointing away from the catalytic Ser203 and the reactivator is pushed out to bind at the peripheral site in the VX-modified structure. Dynamics of hAChE was probed by neutron vibrational spectroscopy to look at harmonic vibrations. POX binding induces significant changes in the acyl pocket loop conformation expelling the weakly binding RS-194B from the active site gorge completely, and the loop becomes more dynamic. We hypothesize that increased dynamics of the acyl pocket loop contributes to the POX-conjugated hAChE resistance to reactivation.

Keywords: room-temperature crystallography; neutron vibrational spectroscopy; oxime reactivator; protonation state; hydrogen bonding; protein dynamics

Acknowledgement

Supported by Grant 1U01NS083451 by the CounterACT program from NINDS (NIH).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

CRYSTAL STRUCTURES OF HUMAN CHOLINESTERASES IN COMPLEX WITH SUPRAMOLECULAR LIGANDS

José Dias [1], Xavier Brazzolotto [1], Xiao-Yu Cao [2], Artur Stefankiewicz [3], Jean-Marie Lehn [4], Florian Nachon [1]
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Human acetylcholinesterase (hAChE) and butyrylcholinesterase (hBChE) are related enzymes. hAChE plays a key role in neurotransmission and is the target of organophosphorus nerve agents. hBChE is good a natural stoichiometric scavenger of nerve agents, preventing their diffusion to the central and peripheral nervous system where they inhibit hAChE.

hAChE and hBChE display different specificities for substrates and ligands due to differences in the number of aromatic residues lining the active site gorge. These aromatic residues are essential for the binding of quaternary and aromatic ligands.

Some molecules containing quaternary and/or aromatic moieties form supramolecular structures by chelating Zinc. The nature of these molecules suggested that they could have affinity for the aromatic residues in the active site gorge of human cholinesterases. It was confirmed by determining their inhibition properties. A key question was whether these supramolecular ligands bind to human cholinesterases as their Zn-complex or monomeric form? The X-ray structures of two supramolecular complexes binding to the gorge of the hAChE and the hBChE reported herein showed that either cases are possible. These structural data on two new types of ligand can be used to design original cholinesterases inhibitors or reactivators.

Keywords: Acetylcholinesterase; butyrylcholinesterase; inhibitors; metallosupramolecular complexes

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

MODIFICATIONS OF CHOLINESTERASE STRUCTURE AND FUNCTION IN COVALENT ORGANOPHOSPHATE CONJUGATES VISUALIZED IN 2D, 3D AND VR

Zihan Zheng, Wanlu Yu, Jacqueline Rohrer, Alexandria Tran and Zoran Radić
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Backbone conformations in hundreds of PDB deposited cholinesterase (ChE) X-ray structures show surprising similarity with typical variability of ~1Å or less among native and liganded acetylcholinesterases (AChEs; 3.1.1.7) and as low as ~2 Å between AChEs and butyrylcholinesterases (BChEs; 3.1.1.8). The largest backbone deviations are observed in their covalent conjugates with organophosphate (OP) inhibitors. Those deviations are likely to influence approach, binding and reaction efficacy of nucleophilic oxime reactivators of ChEs the only true antidotes of OP intoxicated individuals and therefore need to be considered in structure based design of improved oxime antidotes.

We developed a novel, reference point based principle for overlay-independent pairwise comparison of liganded and non-liganded Cα conformations from respective PDB structures and encoded it in JAVA based computer algorithm for quick analysis. Comparisons are based on differences in distances between each Cα pair based on differences in the angle between center of mass, reference point and each of Cα in the comparison, revealing a subset of Cα in two structures that maintains their relative positions in the 3D space best and that can be used as tethering points for overlay of compared structures.

Using NanoPro (Nanome Inc.) VR software, we visualized results of pairwise structure analyses creating .pdb format 3D graphs to identify interaction matrices between amino acids revealed upon ligand binding.

Structure comparisons will be paralleled to OP inhibition and oxime reactivation parameters for some of analyzed ChE-OP-oxime systems to emphasize the importance for complete molecular target template characterization in the structure based antidotes design.

Keywords: organophosphates; oxime reactivators; backbone conformation; 3D structure; VR

Acknowledgement

This work was supported by the CounterACT Program, NIH, Office of the Director, Grant R21 NS098998 from NINDS.

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Military Medical Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

CHOLINERGIC MECHANISMS AT THE CORE OF SKELETAL AND RETINAL HISTOGENESIS

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Recently we could establish major cholinergic impact on vertebrate *in vivo* and *in vitro* skeletogenesis (1,2). Cholinergic mechanisms are also at the core of formation of the vertebrate retina. Retinal histogenesis of a so-called inner plexiform layer (IPL) was disturbed in an AChE KO mouse (3). Characterized best by their ChAT expression, the only cholinergic cells in all vertebrate retinae are so-called *starburst amacrine cells* (SACs), which send processes into synaptic IPL sublaminae. We documented that SACs are derived from a larger pool of postmitotic $AChE^+$ cells. A developmental comparison of $ChAT^+$ and $AChE^+$ cells revealed a close spatial localization of both proteins first within individual cells (nuclear ChAT, vs. extranuclear AChE), and later between adjacent cells, e.g., ACh-secreting and -degrading cells have the same cell lineage origin, and later remain in close apposition (4). Using our 3D stem cell organoid approach (retinal spheroids), we could show that $ChAT^+$ cells were first to initiate IPL formation by establishing two synaptic sublaminae. Unexpectedly, the earliest $ChAT^+$ cells co-expressed markers of Müller glial precursors (MCPs), indicating that a direct SAC precursor i) gives rise to neurons and glial cells, and ii) that these premature cholinergic cells drive earliest processes of network formation in vertebrate retinae, e.g. could function as *IPL founder cells* (5, cf. also 6,7). These findings could have profound relevance for a basic understanding of neuronal network formation.

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Military Medical
Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

BUTYRYLCHOLINESTERASE AS A GHRELIN MODULATOR IMPACTING ANXIETY, STRESS, OBESITY, AND DRUG CRAVINGS

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Our recent studies on butyrylcholinesterase (BChE) have led us to conclude that this enzyme has a major physiological role in regulating levels and impact of ghrelin, the "hunger hormone." A key step toward this realization was finding that, over time, group-housed mice given AAV8-BChE expression vector showed a sharp drop in fighting. Eventually we linked this reaction to a large decrease in plasma ghrelin, which is involved in food-seeking and stress. At first, we assumed that lowered ghrelin was reducing stimulation of growth hormone secretagogue receptors in brain. Instead, treated mice showed *larger* pulses of circulating growth hormone after i.v. ghrelin injection. In other words, high plasma BChE enhanced sensitivity of ghrelin's target, the growth hormone secretagogue receptor, involved in emotional behaviors. That also fits BChE's impact on feeding. BChE knockout mice have high ghrelin levels that drive overeating and obesity. BChE-enhanced mice have low plasma ghrelin, they resist obesity on high-fat diet and show less rebound weight gain after a forced low-calorie diet. These findings suggest that BChE gene transfer could have substantial therapeutic impact on obesity and other conditions that involve ghrelin.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

ASSEMBLY OF PRIMA-LINKED FORM OF ACETYLCHOLINESTERASE IN NEURONS: THE ROLE OF ENZYME INHIBITOR ACTING AS CHEMICAL CHAPERON

Karl W. K. Tsim [1,2], Etta Y. L. Liu [1,2], Miranda L. Xu [1,2], Xiang P. Kong [1], Qiyun Wu [1,2], Ran Duan [1,2], Tina T. X. Dong [1,2]
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Acetylcholinesterase (AChE) is anchored onto cell membranes by a transmembrane protein PRiMA (Proline-Rich Membrane Anchor) as a tetrameric globular form that is prominently expressed in vertebrate brain. Several lines of evidence suggest that the dimer formation probably represents an intermediate in the assembly of the tetramer. In addition, the assembly of AChE tetramers with PRiMA requires the presence of a C-terminal "t-peptide" in the AChE catalytic subunit ($AChE_T$). This protein assembly could be affected by chaperons. AChE inhibitors (AChEIs) are the most established treatment strategy for Alzheimer's disease (AD). Many AChEIs are membrane permeable, and thus which could act as chemical chaperons in affecting the protein assembly of PRiMA-linked AChE in the endoplasmic reticulum (ER). In cultured neuroblastoma or cortical neuron, application of AChEIs, including tacrine (Cognex), rivastigmine (Exelon), but not donepezil (Aricept) and galantamine (Razadyne), caused an accumulation of the unfolded AChE being retained in ER fraction: the AChEI-bound enzyme was not able to transport to Golgi/plasma membrane fraction. As a result, the transcripts encoding AChE and PRiMA were decreased by 50% in the AChEI-treated cultures. In parallel, an increase of ubiquitin-associated enzyme degradation was revealed. The treatment of AChEI in the cultures induced the expression of apoptotic markers, e.g. cleaved caspase 3. In parallel, the apoptotic cell number and mitochondrial membrane potential (MMP) were increased in a dose-dependent manner. The AChEI-bound enzyme retained intracellularly could induce a result of ER stress, as indicated by increased expressions of BiP and CHOP in the treated cultures. The AChEI-induced ER stress resulted with an activation of cAMP signaling, which could regulate the expressions of miR132 and miR212. These findings provide guidance for the drug design and discovery in AD based on inhibition of AChE.

Acknowledgement

This work was supported by Shenzhen Science and Technology Committee Research Grant (JCYJ20160229205726699, JCYJ20160229205812004, JCYJ20160229210027564, CKFW2016 082916015476, JCYJ20170413173747440, ZDSYS201707281432317 and 20170326).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

EVOLUTION OF THE FIRST DISULFIDE BOND IN THE CHOLINESTERASE-CARBOXYLESTERASE (COESTERASE) FAMILY: POSSIBLE CONSEQUENCES FOR CHOLINESTERASE EXPRESSION IN PROKARYOTES

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Within the alpha/beta hydrolase fold superfamily of proteins, the COesterase group (carboxylesterase type B, block C, cholinesterases…) diverged from the other groups through addition of an N-terminal disulfide bond and simultaneous increase in the mean size of the protein (1). This disulfide bond creates a large loop, which is essential for the high catalytic activity of cholinesterases through formation of the upper part of the active center gorge. In some non-catalytic members of the family, the loop may be necessary for heterologous partner recognition. The shuffling of this portion of protein occurred at the time of emergence of the fungi/metazoan lineage. Homologous proteins with this N-terminal disulfide bond are absent in plants but they are found in a limited number of bacterial genomes. In prokaryotes, the genes coding for such homologous enzymes may have been acquired by horizontal transfer. However the cysteines of the first disulfide bond are often lost in bacteria. Natural expression in bacteria of CO-esterases comprising this disulfide bond may have required compensatory mutations or expression of new chaperones. This disulfide bond may also challenge expression of the eukaryote-specific cholinesterases in *E. coli*. Recently , catalytically active human acetylcholinesterase and butyrylcholinesterase were successfully expressed in *E. coli*. The key was the use of a peptidic sequence optimized through the Protein Repair One Stop Shop process, an automated structure- and sequence-based algorithm toward expression of properly folded, soluble eukaryotic proteins with an enhanced stability (2,3). Surprisingly however, the crystal structure of the optimized butyrylcholinesterase variant expressed from bacteria revealed co-existing 'close' and 'open' states of the first disulfide bond. Whether the 'open bond' involves two cysteines (i.e., the bond never formed) or two half-cystines (i.e., the bond properly formed, then broke during the production/analysis process) cannot be inferred from the structural data. Yet, this observation suggests that this first bond is difficult to maintain in *E. coli*-expressed cholinesterases.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

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9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

ACETYLCHOLINESTERASE IN NEUROMUSCULAR SYNAPTIC CLEFTS OF VERTEBRATES

Edna Blotnick-Rubin and Lili Anglister
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Precise positioning and density of acetylcholinesterase (AChE) in the synaptic cleft is required to correctly control the duration of transmitter action in cholinergic synapses according to the particular functional demands of the synapse. We had previously evaluated the densities of AChE at neuromuscular junctions (NMJs) by EM-autoradiography, using radiolabeled probes. The current study addressed fundamental issues concerning the precise location and distribution of the enzyme in the cleft, i.e., whether it is associated with pre- or postsynaptic membranes, or with synaptic basal lamina (BL), and whether it is present only in the primary cleft (PC) or also in postjunctional folds. Quantitative EM-analysis using nanogold labeled anti-AChE probes demonstrated that AChE sites are almost exclusively located on the BL rather than on pre- or postsynaptic membranes and are distributed in the PC and down the postjunctional folds, with a defined pattern. This localization pattern of AChE is suggested to ensure full hydrolysis of acetylcholine bouncing off receptors, thus eliminating its harmful re-binding. The methodology developed for normal NMJs provides a benchmark for studying other peripheral and central nervous system synapses under physiological or pathological conditions.

Keywords: nanogold; acetylcholinesterase; basal lamina; synaptic cleft; postjunctional folds

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

RESPIRATION DURING ORGANOPHOSPHATE AND CARBAMATE INTOXICATION WHEN ACETYLCHOLINESTERASE IS NOT ANCHORED AT CHOLINERGIC SYNAPSES

Eric Krejci [1], Aurélie Nervo [1,2], Imene Kellout [1], Anne Sophie Hanak [1,2], Guilhem Calas [1,2], Florian Nachon [2]
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Intoxications with organophosphate or carbamate shut down control of breathing in minutes. These central apneas are reversed by atropine the well-known antidote of acetylcholinesterase (AChE) inhibitors. But how the excess of ACh triggers the crisis remains unclear. If the buildup of ACh on the post-synaptic receptors at cholinergic synapses is critical, we expected that mice in which the synaptic transmission is adapted to the deficit of AChE should resist to intoxication with carbamates. AChE is specifically anchored in the synapses by ColQ at the neuromuscular junction (NMJ) and by PRiMA in central nervous system (CNS). We have thus intoxicated mice with paraoxon, physostigmine or pyridostigmine and recorded in great details the modifications of breathing in double chamber plethysmography. Physostigmine triggers very long end inspiration pauses (EIP) in WT whereas pyridostigmine provokes only short EIP. The duration of EIP was changed with physostigmine or pyridostigmine in PRiMA KO mice when the brain was adapted to a huge excess of ACh. Surprisingly, when AChE is absent at the NMJ, EIP were much shorter with physostigmine. If AChE in the respiratory center is a key target, we expected long EIP when AChE is normal in the brain and reduced in muscles. Altogether these observations do not support that the change of the synaptic transmission explains the central shutdown control of breathing when cholinesterases are inhibited. In addition, we observed that methacholine provokes similar alteration of breathing when injected subcutaneously to mice. I will discuss a novel model to reconciliate these observations.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

SINGLE NUCLEOTIDE POLYMORPHISMS IN THE GENES ENCODING AChE AND ITS miR-608 REGULATOR CO-MODULATE ANXIETY AND BLOOD PRESSURE

Alon Simchovitz [1,4], Nimrod Madrer [1,4], Rotem Haviv [1], Geula Hanin [1], Shani Shenhar-Tsarfaty [2], Einor Ben Assayag [2], Shlomo Berliner [2], Zehava Solomon [3], Hermona Soreq [1]

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[4] contributed equally to this work

Cholinergic-regulated phenotypes including anxiety, cardiac and immune-related properties show inter-individual variability which might be affected by genomic Single Nucleotide Polymorphisms (SNPs) in the corresponding protein coding genes and their targeting microRNAs (miRs), but the combined impact of such SNP pairs is unknown. We have recently shown that the rs17228616 SNP in the Acetylcholinesterase (AChE) gene reduces the affinity of AChE mRNA to the primate-specific miR-608 and elevates both AChE levels in brain and blood as well as trait anxiety and blood pressure (1) while affecting PTSD-related neural circuits and downregulating numerous brain miR-608 targets (2). Others reported that the rs4919510 SNP in the miR-608 gene reduces miR-608 levels *in vitro* and limits the risk of sepsis following head injury *in vivo* (3). To explore the combined effect of these two SNPs, we tested 444 healthy 30 years old US donors and 101 Israeli ex-prisoners of the 1973 war (EWP), 76 of whom returned with post-traumatic stress disorder(PTSD). Genotyping combined with R-statistics of the corresponding biomedical evidence demonstrated that the rare allele of the AChE SNP was more abundant among non-PTSD EWP donors compared to PTSD patients in this cohort (33 vs 19%, Chi-square 0.03). Moreover, we found in both of these cohorts interaction between the effect of the two SNPs on blood pressure, inflammation and anxiety-related parameters, with the miR-608 SNP stratifying the corresponding impact of the rare allele of the AChE SNP on these parameters. Our findings indicate an interaction between the SNPs in the AChE and miR-608 genes, possibly reflecting modified impact of this primate-specific miR on its numerous downstream targets.

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Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DIOXIN SUPPRESSES AChE EXPRESSION IN NEURON AND MUSCLE

Heidi Qunhui Xie [1,2], Yingjie Xia [1,2], Tuan Xu [1,2], Yangsheng Chen [1,2], Yali Luo [1,2], Rui Sha [1,2], Yiyun Liu [1,2], Li Xu [1,2], Bin Zhao [1,2]

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Acetylcholinesterase (AChE, EC3.1.1.7) plays an important role in the cholinergic neurotransmission in central and peripheral nervous systems, which has been widely recognized as a biomarker for monitoring pollution of organophosphate and carbamate pesticides. Recently, a broad spectrum of environmental toxic substances has been found to decrease AChE activity in various species. Dioxin is one of the emerging environmental AChE disruptors, which is a typical persistent organic pollutant with multiple toxic effects on the nervous system. We have reported that dioxin suppresses the expression of neuronal AChE via aryl hydrocarbon receptor (AhR), in which both transcriptional and posttranscriptional regulations could be involved. Moreover,, muscular AChE expression was also disturbed by dioxin exposure. During myogenic differentiation of C2C12 cells, the mRNA expression of AChE T subunit and the enzymatic activity of AChE were significantly suppressed by dioxin exposure in parallel with the disturbances on the myotube formation. However, the addition of AhR antagonist was not able to reverse the suppressive effect of dioxin, suggesting a distinct role of AhR during the myogenic differentiation process. These results further support the notion that dioxin is a novel environmental AChE disruptor which acts on the biosynthesis processes via multiple molecular mechanisms.

Acknowledgement

This work was supported by Natural Science Foundation of China (Nos. 21177150, 21377160, 21525730), and the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDB14030400).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

Wnt3a INDUCES THE TRANSCRIPTION OF ACETYL-CHOLINESTERASE: AN ENZYME PLAYING A ROLE IN OSTEOBLASTIC DIFFERENTIATION

Miranda L. Xu [1,2], Etta Y. L. Liu [1,2], Qiyun Wu [1,2], Duan Ran [1,2], Tina T. X. Dong [1,2], Karl W. K. Tsim [1,2]
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Acetylcholinesterase (AChE) plays hydrolytic role to terminate cholinergic transmission in vertebrate. AChE is intensively reported to exist in different tissues, and may participate in differentiation process. Here, AChE was demonstrated to participate in osteoblastic differentiation. In rat-derived bone tissues and primary cultured osteoblasts, the expression of AChE was increased in parallel with bone development, as well as osteoblastic differentiation. Transcriptional expression and protein of AChE in differentiating osteoblast could be enhanced by application of Wnt3a. Runx2, a downstream transcription factor in Wnt/β-catenin signaling pathway, played crucial role in Wnt3a-induced AChE expression in osteoblasts. This was confirmed by identification of Runx2-binding site in the *ACHE* gene promoter, over-expression of Runx2 and deletion of the Runx2-binding site in the *ACHE* promoter. Bone defect was observed in *ACHE*-/- mice. The non-enzymatic role of AChE in osteoblast was determined by over-expression system and application of AChE inhibitors. By transcriptomics, AChE was found to influence gene expressions of Wnt/β-catenin signaling components, and may participate in osteoblastic function, e.g. affecting osteoclastogenesis and cell adhesion of osteoblast. A notion of non-cholinergic role of AChE in osteoblast, as well as an insight for elucidating other possible mechanisms in regulation of bone formation was provided.

Acknowledgement

This work was supported by Shenzhen Science and Technology Committee Research Grant (JCYJ20160229205726699, JCYJ20160229205812004, JCYJ20160229210027564, CKFW2016 082916015476, JCYJ20170413173747440, ZDSYS201707281432317 and 20170326).

MMSL Military Medical Science Letters Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

RESTORING MITOCHONDRIA (DYS)FUNCTION AND ACETYLCHOLINE LEVELS AS A PROSPECTIVE THERAPEUTIC STRATEGY FOR ALZHEIMER'S DISEASE

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Alzheimer´s disease (AD) is a progressive and degenerative neurological disorder resulting in memory loss and cognitive decline. The severity of AD dementia was found to correlate with the extent of the cholinergic loss and acetylcholine (ACh) depletion.

In brain synapses ACh can be hydrolyzed by two cholinesterases (ChEs), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which were found in neurons and glial cells as well as in AD neuritic plaques and tangles. AChE is the prevalent enzyme in the healthy brain, while BChE is considered to play a minor role in the regulation of synaptic ACh levels. However, in AD advanced stages, AChE activity is decreased while BChE activity is unchanged or even increased, making both ChEs stimulating targets for the treatment of AD. Current AD therapy is based on AChE inhibitors, although they have very modest clinical effects in treating the symptoms of the disease and are unable to halt disease progression.

Oxidative stress (OS) and mitochondrial dysfunction are also considered critical factors in AD pathogenesis. As a result, targeting mitochondrial oxidative stress (OS) in the prodromal phase of AD to slow or prevent the neurodegenerative process and restore neuronal function is thus viewed as a valid therapeutic approach.

As part of our drug discovery program focused in oxidative stress-related diseases, and following a multi-target strategy, new mitochondriotropic antioxidants based on natural scaffolds acting as dual and bifunctional cholinesterase inhibitors have been developed. The results will be reported in this communication.

Keywords: Alzheimer disease; mitochondriotropic antioxidant; cholinesterase inhibitor

Acknowledgement

This work was funded by FEDER funds through the Operational Programme Competitiveness Factors-COMPETE and national funds by FCT – Foundation for Science and Technology under research grants (QUI/UI0081/2013, NORTE-01-0145-FEDER-000028 and PTDC/DTP-FTO/2433/2014

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

FROM DUAL BINDING SITE AChE INHIBITORS TO CHAMELEON MOLECULES: DISCOVERY OF POTENT BuChE INHIBITORS

Carlos Roca [1], Talita P.C. Chierrito [1], Concepción Perez [2], Loreto Martinez [1], Nuria Campillo [1], Ana Martinez [1,*]

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Current pharmacotherapy for Alzheimer's disease (AD) involves compounds aimed at increasing the levels of acetylcholine in the brain through inhibition of AChE. These drugs, known as acetylcholinesterase inhibitors, have been shown to improve cognition and global functions but have little impact on improving the eventual progression of the disease. However, there are evidences that other cholinesterases such as butyrylcholinesterase (BuChE) can play an important role in cholinergic function in the brain, and the long-suspected non-cholinergic actions of acetylcholinesterase, mainly the interference with the beta-amyloid protein cascade, have recently driven a profound revolution in cholinesterase drug research [1-2].

We will present our journey from dual binding site AChE inhibitors as potent beta-amyloid modulators to the more recent serie of indolylpiperidines hybrids with an unexpected and very potent *h*BuChE inhibition. Experimental and computational studies have revealed the chameleon behavior of these molecules able to change their bioactive conformation depending on the cholinesterase binding site. Based on the potent activity of these compounds targeting BuChE, the low cellular toxicity and the *in vivo* target engagement, we can propose these indolylpiperidine derivatives as valuable tools for the study of the role of BuChE in AD and probably as potential drugs candidates for its future pharmacotherapy.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

DISCOVERY AND DEVELOPMENT OF NEUROPROTECTIVE AND DISEASE-MODIFYING ANTI-AD DRUG LEADS FROM THE CHINESE MEDICINE

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Alzheimer's disease (AD) represents a chronic and progressive brain disorder, and has now become the most common neurodegenerative disorders among the older population. Although the disease is now seen as major public health problems, the currently available therapeutics only offer temporary symptomatic relieves. Therefore, research and development of more effective and disease-modifying agents for the prevention and/or treatment of AD will have tremendous value from both scientific and economic standpoints.

Over the past few years, our series of studies have identified some highly promising anti-AD drug leads, including those derived from the Chinese medicines, with disease-modifying potential. In this presentation, the multi-neuroprotective effects and the underlying mechanisms of those promising candidates will be comprehensively illustrated and discussed.

Keywords: Neuroprotective effect; Dimers; Alzheimer's disease; Disease modifying; Multiple functions

Acknowledgement

This work was supported by the research grants from PolyU (G-YBGQ, G-YZ95), the Research Grant Council of Hong Kong (15101014), ITSP-Guangdong–Hong Kong Technology Cooperation Funding Scheme (GHP/012/16GD), and Shenzhen Basic Research Program (JCYJ20160331141459373).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

FIFTY SHADES OF CHOLINESTERASE IMMOBILIZATION AND THEIR APPLICATION TO DRUG DISCOVERY

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New screening methodologies capable of identifying new enzyme inhibitors in a faster, more reproducible and automated way may help early drug discovery. Indeed high throughput screening methodologies for the identification of new cholinesterase inhibitors can reduce screening time and screening costs. In this frame, "immobilized enzymes" [1] can serve as handy and efficient alternatives to conventional in-solution methods. On the other hand, other than massive screening, highly informative approaches may provide decisive information in the selection of best-in-class compounds. Hence, combination of several parameters spanning from inhibition, binding mechanisms and kinetic parameters is important to be considered. In particular, estimation of residence time has recently emerged as critical feature [2]. Therefore, accessing kinetic information on drug binding events at initial stages of the drug discovery process is gaining increasing interest among medicinal chemists.

In the light of these considerations, the talk will present different approaches involving immobilized human cholinesterases (ChEs). Micro-immobilized enzyme reactors (IMERs) can be used in combination with HPLC systems while SPR biosensing technology can be exploited for binding and kinetic investigation. ChE-based IMERs and single or multiple sensing surface(s) can be used in combination as valuable screening tools, which allow to quickly retrieve a set of highly useful information which can assist scientists in the selection of new chemical entities to be further developed.

Keywords: human cholinesterases; automation; bioreactors; sensing surfaces; binding events

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

SERUM CHOLINESTERASE ACTIVITY AND ALZHEIMER DISEASE COMORBIDITIES - CAN BARIATRIC SURGERY CHANGE YOUR SYMPATHETIC PRONE STATE?

Shani Shenhar-Tsarfaty, Shiri Sherf-Dagan, Galia Berman, Shira Zelber-Sagi, Oren Shibolet, Itzhak Shapira, David Zeltser, Shlomo Berliner and Ori Rogowski
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Alzheimer disease comorbidities, such as hypertension, obesity, metabolic syndrome, diabetes mellitus and inflammation are all associated with impaired sympathetic/parasympathetic response.

Inherited and/or acquired sympathetic prone state, expressed by elevated serum Acetylcholinesterase (AChE) can lead to excessive inflammatory load and cognitive decline.

To evaluate the sympathetic/parasympathetic balance we measured serum cholinesterase activities in stroke, myocardial infarction, diabetes mellitus, morbid obese patients and apparently healthy control. Our findings identify the potential value cholinesterases as possible biomarkers in diseases associated with cerebro-cardiovascular outcome.

Recently we found that serum AChE activity increased with BMI in a dose-dependent manner until it reached a peak level at BMI of 30-35 kg/m², followed by a plateau ($p<0.001$, $n=1,450$). Similarly, AChE activity increased with waist circumference categories ($p<0.001$ for men and $P=0.013$ for women).

The Obesity-related AChE resistance phenotype may be reversed following laparoscopic sleeve gastrectomy (LSG) surgery and correlates with metabolic outcomes (% excess weight loss, %fat, and delta Homeostasis Model Assessment (HOMA)).

Further long-term studies will be needed to validate and evaluate the beneficial effect of AChE reduction post bariatric surgery and its possible relation to cognitive decline.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

INDAZOLYLKETONES: HIT TO LEAD OPTIMIZATION OF A MULTITARGET DRUGS

Pedro González-Naranjo [1], Natalia Pérez [1], Concepción Pérez [1], Carlos Roca [2], Rocio Girón [3], Eva Sánchez-Robles [3], Ángeles Martín Requero [2], Maria L. de Ceballos [4], Nuria E. Campillo [2], Juan Antonio Páez [1]

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A new family of indazolylketones with a multitarget profile as modulators of cholinergic and BACE-1 enzymes and cannabinoids receptors [1] was designed based on our previous results [2]. We present the synthesis, computational studies and biological evaluation and of a new family of heterocyclic compounds.

Pharmacological evaluation include *in vitro* inhibitory assays in AChE/BuChE enzymes and BACE-1. In addition, functional activity for cannabinoid receptors has been carried out. The results of the pharmacological tests have revealed that some of these derivatives behave as CB2 cannabinoid agonists and simultaneously show BuChE and/or BACE-1 inhibition. Furthermore, studies in human neuroblastoma SH-SY5Y cells and in the lymphoblasts of patients with Alzheimer's disease have shown neuroprotective effects of this family of compounds, as well as their capacity to blunt the abnormal enhanced proliferative activity of AD lymphoblasts. Based on the *in vitro* and functional studies we performed *in vivo* studies of those best compounds employing transgenic mouse (TgAPP) model. The results of the *in vivo* study revealed that some of these compounds could be very promising candidates for the treatment of Alzheimer's disease.

Keywords: Alzheimer's disease; BACE-1 inhibitor; BuChE inhibitor; CB2R agonist; indazolylketone; multitarget drug

Acknowledgement

Financial support of this research has been provided by Ministerio de Economía y Competitividad (CTQ2015-66313-R).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

BUTYRYLCHOLINESTERASE GENETIC POLYMORPHISM AND NEUROIMAGING BIOMARKERS IN ALZHEIMER'S DISEASE

DeBay, Drew R.[1]; Maxwell, Selena[1]; Luke, David[1]; Fisk John D.[2]; Burrell, Steve[3]; Bowen Chris V.[3]; Song, Xiaowei; Black, Sandra E[5].; Darvesh, Sultan[1,6]

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Objective: The influence of butyrylcholinesterase (BChE) genetic polymorphism in Alzheimer's (AD) remains controversial. *BCHE*-K and *BCHE*-A genetic variants cause reduction of BChE, an enzyme implicated in AD. Some studies have reported a protective effect of *BCHE*-K, others suggest increased AD risk, particularly when associated with *APOE4*. We utilized a candidate gene-driven analyses to determine the effects of *BCHE*-K and *BCHE*-A on AD biomarkers using ADNI data (http://adni.loni.ucla.edu/).

Methods: Participants were genotyped for *BCHE*-K (615) and *BCHE*-A (785), each stratified into control (C), MCI or AD groups. MRI, ^{18}F-FDG and amyloid-PET were assessed. ANCOVA compared main effects of i)diagnosis, ii)^{BCHE}-K, iii)*BCHE*-A and iv)*APOE4* status on each biomarker with age, education and sex as covariates.

Results: The allelic frequency was 20.8%, 4.6% and 26.5% for *BCHE*-K, *BCHE*-A and *APOE4*. For MRI, main effects for diagnosis were significant ($p<0.0001$), with reduction in whole-brain and selected regional volumes (7-27%, $p\leq6x10^{-6}$) in AD vs. C. For 18FDG-PET, the main effect for diagnosis was significant (p=5x10-9), with 14% decrease in metabolism in AD vs. C ($p=7x10^{-10}$). For amyloid-PET, the main effects for diagnosis and *APOE4* status were significant (p=0.034; $p=3x10^{-6}$), with 12% increase in retention in AD vs. C (p=0.023) and 16% increase among carriers of at least one *APOE4* allele vs. non-carriers ($p=8x10^{-6}$). No significant effects of these biomarkers were observed due to *BCHE*-K or *BCHE*-A status ($p\geq0.209$).

Conclusions: These data suggest *BCHE*-K or *BCHE*-A may not significantly effect structural, metabolic or molecular AD biomarkers. Further ROI/voxel-wise analyses are warranted to uncover potential regional changes among AD *BCHE* variants.

Keywords: Alzheimer's disease; butyrylcholinesterase genetic variants; neuroimaging; amyloid-PET; FDG-PET

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

CASE STUDIES FOR SUCCESSFUL COMBINATION OF ChE INHIBITORS AND GPCR LIGANDS (CANNABINOID 2 AND HISTAMINE 3 RECEPTORS)

Dominik Dolles [1], Fouad H. Darras [1], Antonios Drakopoulos [1], Andrea Strasser [2], Hans-Joachim Wittmann [2], Christoph A. Sotriffer [1], Steffen Pockes [2], Bassem Sadek [3], Tangui Maurice [4], Michael Decker [1]
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The combination of cholinesterase inhibitors with GPCR ligands in hybrid molecules seems highly promising for Alzheimer's disease (AD) therapy, since two very different molecular targets can be addressed at the same time. Nevertheless, significant challenges come with this rationale: a) hybrids might possess too high molecular weights to be orally bioavailable and/or pass the blood-brain-barrier, b) the compounds might act in different concentration ranges, c) and selectivity and affinity has to be optimized for several very distinct targets.

We have designed – applying computational methods - and synthesized dual-acting ChE-inhibitors that act with high potency and selectivity also at the histamine 3 receptor (hH$_3$R) [1], and the same could be achieved for cannabinoid 2 receptors (hCB$_2$R) [2, 3], both GPCRs represent important AD targets. Regarding dual-acting ChE inhibitors and hCB$_2$R agonists both covalently connected hybrids using the unselective ChE inhibitor tacrine as well as merged small molecules with high butyrylcholinesterase (BChE) selectivity have been obtained and pharmacologically characterized *in vitro*. Representative examples from all sets of compounds have been investigated *in vivo* in different AD mice models [3].

The case studies demonstrate that it is possible to obtain dual-acting compounds that a) act highly selectively and with high affinity at the respective targets, b) work in the same concentration range ("balanced affinity"), c) exhibit pronounced *in vivo* activity.

Keywords: GPCR; cannabinoid; histamine; merged ligands; hybrid molecules

Acknowledgement: German Science Foundation (Deutsche Forschungsgemeinschaft under DFG DE1546/6-3); Elite Network of Bavaria (Elitenetzwerk Bayern under the International Doctoral Program "Receptor Dynamics"); German Academic Exchange Service (DAAD) with funds of the Federal Ministry of Education and Research (BmBF)

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

FROM ACETYLCHOLINESTERASE INHIBITORS TO MULTI-TARGET-DIRECTED LIGANDS (MTDLs): A STEP FORWARD IN ALZHEIMER'S DISEASE DRUG DISCOVERY

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Notwithstanding clinical effectiveness evidences continue to suggest benefit from the acetylcholinesterase inhibitors (AChEIs) in alleviating Alzheimer's disease (AD) symptoms, these drugs do not appear to delay or prevent the underlying neurodegeneration. In this context, novel prospects are offered by the strategy of developing single chemical entities able to modulate multiple targets, i.e. the multi-target-directed ligands (MTDLs). On this basis, several multifunctional AChEIs have been rationally designed with the deliberate aim of enlarging their biological profiles, beyond the ability to inhibit cholinesterases. This is because it has been recognized that a balanced simultaneous modulation of multiple targets critically intertwined in AD pathological cascade can provide a superior therapeutic and toxicological profile compared to the action of a selective AChEI.[1]

Building on this founding principle, we and others have developed several series of anti-AD MTDL compounds that combine cholinesterase inhibition with anti-aggregating, anti-oxidant, and anti-neuroinflammatory properties.[2] As a further step, to explore the possibility to discover new MTDLs based on inexpensive resources, we have developed a series of MTDLs obtained by properly modifying constituents from the cashew nut shell liquid (CNSL), a waste from cashew nut processing factories.[3] Such hybrid compounds, obtained from renewable and inexpensive material, might be promising bio-based, sustainable MTDLs for AD drug discovery.

Working in the field for almost 20 years, we should draw lessons from the past and try our best to chart innovative directions and hopefully address the scientific and societal challenges of neurodegenerative diseases.

Keywords: Alzheimer's disease; amyloid; acetylcholinesterase; multitarget compounds; neuroinflammation

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

FROM SELECTIVE BUTYRYLCHOLINESTERASE INHIBITORS TO MULTI-TARGET-DIRECTED LIGANDS AS LEAD COMPOUNDS FOR ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is characterized by severe basal forebrain cholinergic deficit, which results in progressive and chronic deterioration of memory and cognitive functions. Similar to acetylcholinesterase, butyrylcholinesterase (BChE) contributes to the termination of cholinergic neurotransmission. Its enzymatic activity increases with the disease progression, thus classifying BChE as a viable therapeutic target in advanced AD. Potent, selective and reversible human BChE inhibitors were developed. First, a hierarchical virtual screening was performed followed by biochemical evaluation of highest scoring hit compounds. Three compounds showed significant inhibitory activities against BChE and the best inhibitor was selected for further SAR studies. More than 100 different analogues were synthesized and among them, two compounds were found to be promising lead compounds as they were not cytotoxic, they crossed the blood-brain barrier and improved memory, cognitive functions and learning abilities of mice in a model of the cholinergic deficit that characterizes AD, without producing acute cholinergic adverse effects. The solved crystal structures of human BChE in complex with the most potent inhibitors revealed their binding modes and provided the structural basis for their further development into multi-target-directed ligands, which in addition to good inhibition of BChE possess good antioxidant, metal chelating, neuroprotective and other properties beneficial for AD.

Keywords: Alzheimer's disease; butyrylcholinesterase; multi-target-directed ligands

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DISCOVERY AND CHARACTERIZATION OF TACRINE/HUPRINE-TRYPTOPHAN HETERODIMERS AS NOVEL MULTIPOTENT COMPOUNDS AGAINST ALZHEIMER'S DISEASE

Jan Korabecny [1,2], Katarina Spilovska [1,3], Manuela Bartolini [4], Barbara Monti [4], Doriano Lamba [5], Rosanna Caliandro [5], Alessandro Pesaresi [5], Vendula Hepnarova [2,6], Daniel Jun [2,6], Martina Hrabinova [2,6], Rafael Dolezal [2], Jana Zdarova Karasova [2,6], Ondrej Soukup [1,2], Eva Mezeiova [1,2], Eugenie Nepovimova [3], Maria Laura Bolognesi [4] and Kamil Kuca [2,3]

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Combination of tacrine/huprine, connected through a different linker tether length, with tryptophan led to the generation of a novel, highly-potent family of multi-target directed ligands targeting key molecular mechanisms of Alzheimer's disease. Based on *in vitro* biological profile, the 6-chloro-tacrine-$(CH_2)_6$-L-tryptophan heterodimer S- K1035 was found to be the most potent inhibitor of human acetylcholinesterase (*h*AChE) and human butyrylcholinesterase (*h*BChE) within the series, with nanomolar IC_{50} values (6.31 and 9.07 nM, respectively). Moreover, S K1035 showed good ability to inhibit $A\beta_{42}$ self-aggregation and *h*AChE-induced $A\beta_{40}$ aggregation. The X-ray crystallographic analysis of *Tc*AChE in complex with S-K1035 highlighted the utility of the hybridization approach used in the structure based drug design. S K1035 also exerted moderate inhibition against neuronal nitric oxide synthase (nNOS). *In vivo* studies displayed low toxicity profile compared to parent tacrine. S-K1035 also significantly ameliorated performances of scopolamine-treated animals.

Acknowledgement

Supported by Ministry of Health of the Czech Republic, grant nr. 15-30954A.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

NOVEL CONJUGATES BASED ON γ-CARBOLINES, CARBAZOLES, PHENOTHIAZINES, AND AMINOADAMANTANES AS MULTIFUNCTIONAL AGENTS FOR ALZHEIMER'S DISEASE TREATMENT

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Neurodegenerative diseases are multifactorial. Therefore, their treatment requires drugs that can act simultaneously on multiple pathogenic targets. We synthesized several series of hybrid structures combining certain pharmacophores essential for neurodegenerative disease treatment: γ-carbolines, carbazoles, phenothiazines, and aminoadamantanes [1-3]. Inhibitory activity of these conjugates against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CaE) was studied along with their ability to competitively displace propidium iodide from the peripheral anionic site of electric eel AChE to assess their potential effect on AChE-induced aggregation of β-amyloid. Antioxidant properties were examined computationally with density functional theory and measured experimentally using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and oxygen radical absorbance capacity (ORAC-FL) assays. Binding modes of conjugates to AChE and BChE were studied using quantum mechanical-assisted molecular docking. Results revealed structures that were selective inhibitors of BChE [1,2] or that combined high potency and selectivity toward BChE with high radical-scavenging activity, e.g., conjugates of γ-carbolines and tetrahydrocarbazoles [3]. Conjugates of γ-carbolines and cycloalcaneindoles with the phenothiazine derivative Methylene Blue demonstrated high potency against AChE and BChE combined with effective displacement of propidium from the peripheral anionic site of AChE. Additionally, the conjugates were extremely active in both antioxidant tests. All conjugates were poor CaE inhibitors and therefore expected to lack drug-drug interactions by this pathway. Good agreement was found between experimental and computational results. Lead compounds were identified for future optimization and development of new multi-target drugs against neurodegenerative diseases that combined cognition enhancement with neuroprotective potential.

Keywords: Alzheimer's disease; multifunctional agents; γ-carboline; phenothiazine; aminoadamantane

Acknowledgement

This work was supported by RSF grant #14-23-00160P and partly by RFBR grant #17-03-00984

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

PLEIOTROPIC PRODRUGS: A NOVEL POLYPHARMACOLOGY APPROACH TO TREAT NEURODEGENERATIVE DISEASES

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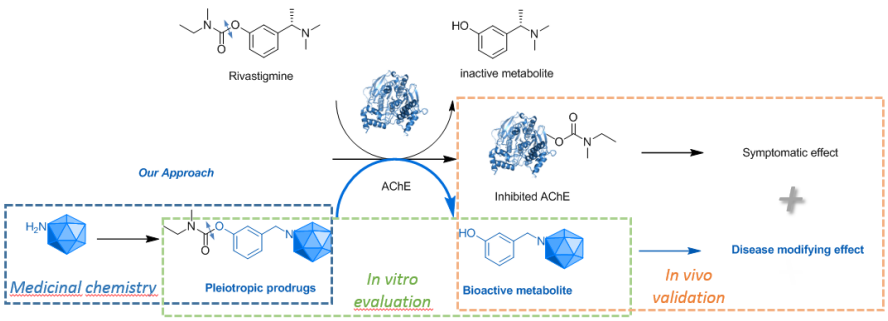
Today, treatment of Alzheimer's Disease (AD) mainly involves acetylcholinesterase inhibitors (AChEIs). AChEIs display solely a symptomatic benefit, alleviating the cognitive disorders associated to AD through a temporary restoration of the cholinergic neurotransmission impaired by the neurodegeneration. The gradual loss of efficiency for AChEIs led to associate them to drugs exhibiting potential disease-modifying properties.

The "Multi-Target-Directed Ligands" (MTDLs) was used in the recent years with a great potential benefit towards multiple targets implicated in the complex AD,[1] as well as other neurodegenerative syndromes, which involve multiple pathogenic factors.

Our contribution to the field led recently to the discovery of Donecopride, the first 5-HT_4R partial agonist, which possesses important acetylcholinesterase (AChE) inhibition properties currently under preclinical development.[2,3] Based on this experience, we have recently developed a novel pleiotropic prodrugs approach to generate promising in vivo active compounds. Based on the structure of rivastigmine, novel MTDLs were designed, acting as prodrugs, able to temporarily covalently bind and inhibit AChE (for a symptomatic effect). and to secondarily release a drug able to selectively reach another AD target (for a potential disease-modifying effect)



This concept was applied to several secondary targets, including different 5-HT receptors of interest[4] for the treatment of AD. The concept, the synthetic development, *in vitro* and *in vivo* evaluation of these candidates and our undisclosed results will be presented for the first time in this communication.

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Military Medical Science Letters
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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

TOWARD AN INNOVATIVE TREATMENT OF ALZHEIMER'S DISEASE: DESIGN OF MULTI-TARGET DIRECTED LIGANDS (MTDLs) TARGETING ACETYLCHOLINESTERASE (AChE) AND alpha-7 NICOTINIC RECEPTORS (alpha-7 nAChRs)

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Alzheimer's disease (AD) is a complex and progressive neurodegenerative disorder. The available therapy is limited to the symptomatic treatment and its efficacy remains unsatisfactory [1]. In view of the prevalence and expected increase in the incidence of AD, the development of an effective therapy is crucial for public health. Due to the multifactorial etiology of this disease, the multi-target-directed ligand (MTDL) approach is a promising method in search for new drugs for AD. Aiming at developing new MTDLs, this project consists on the development of new multifunctional agents, which will act simultaneously on the different players in AD pathology. The project aims at developing MTDLs by combining an AChE inhibitory activity with an alpha-7 nAChR activation [2].

Keywords: Alzheimer's disease; MTDLs; AChE; nAChR

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

MOLECULAR MODELING IN SEARCH OF NEW, MULTI-TARGET LIGANDS AGAINST ALZHEIMER'S DISEASE. EXPLORING THE BIOCHEMICAL MULTIVERSE.

Jakub Jończyk [1], Dawid Panek [1], Anna Więckowska [1], Justyna Godyń [1], Marek Bajda [1], Tomasz Wichur [1], Anna Pasieka [1], Damijan Knez [2], Anja Pišlar [2], Jan Korabecny [3], Ondrej Soukup [3], Vendula Sepsova [3], Raimon Sabaté [4], Janko Kos [2], Stanislav Gobec [2], Barbara Malawska [1]

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In response to the complex and still not fully understood pathomechanism of Alzheimer's disease, many researchers have turned towards the promising paradigm of designing ligands with a multi-target nature[1]. One of the possible benefits of this approach in Alzheimer's disease is an opportunity to merge activity against cholinesterases, which are used in the current symptomatic therapies, with disease-modifying targets associated with β-amyloid and tau protein pathways[2]. Optimization of ligand with respect to several biological targets while maintaining good physicochemical parameters is not an easy task. Computer modeling can be a huge help in this task. Computer modeling in the design of biologically active substances can be used to effectively search through the huge, available chemical space, or provide support for drawing conclusions of results obtained during the study[3]. In the work presented here, we would like to describe how the molecular modeling methods were used to design and obtain new series of 1-benzylamino-2-hydroxyalkyl derivatives that are effective against both acetyl- and butyrylcholinesterase as valid, symptomatic targets with an anti-aggregating properties against Tau protein, β-amyloid and inhibition properties against β-secretase (BACE-1) as disease-modified targets[4].

Acknowledgement

This work was supported by the National Science Center of Poland (Grants UMO-2016/21/B/NZ7/01744 and UMO-2016/21/N/NZ7/03288), the European Cooperation in Science and Technology COST Action CA15135, and the Slovenian Research Agency (research program P1-0208 and research project L1-8157).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

DESIGN OF A BUTYRYLCHOLINESTERASE MUTANT FOR DETOXIFYING COCAINE AND ITS TOXIC METABOLITES IN CONCURRENT USE OF COCAINE AND ALCOHOL

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Cocaine abuse is a major medical and health problem. There is no FDA-approved medication for treatment of cocaine overdose and addiction. Statistical data show that 92% of cocaine users also consume alcohol. The risk of immediate death is 18 - 25 times greater for cocaine co-ingested with alcohol than for cocaine alone. Alcohol can react with cocaine to get a series of toxious compounds in body including cocaine, cocaethylene, norcocaine, norcocaethylene and benzoylecgonine.

In combination of our "virtual screening of transition states" computational protocol and artificial intelligence, a novel approach was used to design BChE mutants as multiple functional cocaine hydrolases (mfCocHs) for treatment of toxicity caused by concurrent use of cocaine and alcohol. Comparing the kinetic parameters of *native human BChE* and mf*CocH* against cocaine as well as its four toxic/harmful metabolites (i.e. norcocaine, cocaethylene, norcocaethylene and benzoylecgonine) determined by us, the most effective mfCocH has at least a ~1000-fold improved catalytic efficiency against three of the substrates (cocaine, norcocaine, and cocaethylene), ~100-fold and ~10-fold improved catalytic efficiency against norcocaethylene and benzoylecgonine, respectively.

In vivo studies have revealed that the mfCocH can effectively hydrolyze cocaine and its four metabolites in rats produced from the concurrent abuse of cocaine and alcohol in both addiction and overdose models. The mfCocHs was powerful antidote to treat cocaine (w/ or w/o alcohol) induced toxicity, even from the lethal toxicity after co-administrated 1 g/kg alcohol (IP) and 180 mg/kg cocaine (IP), at any time point as long as the subject is alive before treatment.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

7-METHOXYDERIVATIVE OF TACRINE IS A 'FOOT-IN-THE-DOOR' BLOCKER OF GluN1/GluN2 AND GluN1/GluN3 NMDA RECEPTORS

Martina Kaniakova [1,2], Lenka Kleteckova [1,3], Katarina Lichnerova [1,2], Kristina Holubova [1,3], Kristyna Skrenkova [2], Miloslav Korinek [1], Jan Krusek [1], Tereza Smejkalova [1], Jan Korabecny [4], Karel Vales [1,3], Ondrej Soukup [4], Martin Horak [1,2]
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N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels that mediate excitatory neurotransmission in the mammalian central nervous system (CNS), but their dysregulation results in the aetiology of many human CNS disorders. Several NMDAR modulators including memantine have been used successfully in clinical trials. Indeed, 1,2,3,4-tetrahydro-9-aminoacridine (tacrine; THA) was the first approved drug for Alzheimer's disease (AD) treatment. 7-methoxyderivative of THA (7-MEOTA) is less toxic and showed promising results in patients with tardive dyskinesia. Here, we employed electrophysiological recordings in HEK293 cells and rat neurones to examine the mechanism of action of THA and 7-MEOTA at the NMDAR. We showed that both THA and 7-MEOTA are "foot-in-the-door" open-channel blockers of GluN1/GluN2 and GluN1/GluN3 NMDARs and that 7-MEOTA is a more potent but slower blocker than THA. Furthermore, the inhibitory potency of 7-MEOTA at synaptic and extrasynaptic hippocampal NMDARs was similar, and 7-MEOTA exhibited better neuroprotective activity in rats exposed NMDA-induced lesions in hippocampus when compared with THA and memantine. Finally, intraperitoneal administration of 7-MEOTA attenuated MK-801-induced hyperlocomotion in rats. We conclude that 7-MEOTA is a promising candidate for the treatment of diseases associated with the dysfunction of NMDARs.

Keywords: glutamate receptor; patch-clamp technique; inhibitor; excitotoxicity

Acknowledgement

This work was supported by a project from the Czech Science Foundation (no. 16-08554S) and from European Regional Development Fund: Project "PharmaBrain" (no. CZ.CZ.02.1.01/0.0/0.0/16_025/0007444).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

THE *Caenorhabditis elegans* PHARYNX AS A MODEL SYSTEM TO INVESTIGATE AND MITIGATE AGAINST THE EFFECTS OF ANTI-CHOLINESTERASE DRUGS

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C. elegans is a free-living worm widely used as model to study neurotoxicology. Despite its simplicity, *C. elegans* has a high level of genetic and molecular conservation with vertebrates. Similar to mammals, intoxication with anti-cholinestereses triggers the accumulation of synaptic acetylcholine causing continuous stimulation of both nicotinic and muscarinic receptors, hypercontracting the muscles of the worm[1]. The pharynx, the nematode feeding organ, depends on cholinergic function. Pharyngeal movements, readily observed in whole organism, are disrupted by impairments in cholinergic transmission. Therefore, quantitative analysis of pharyngeal structure and function has excellent potential to probe anti-cholinesterase mode of action that may translate to human toxicology.

We establish the IC_{50} values for the carbamate aldicarb and the organophosphates paraoxon-ethyl, paraoxon-methyl and DFP, highlighting a distinct dose-time dependence inhibition of pharyngeal activity. In recovery experiments, aldicarb and paraoxon-ethyl but not paraoxon-methyl or DFP intoxicated worms recover the pharyngeal function onto empty and oxime plates. A cycle of aldicarb intoxication-recovery-intoxication revealed aldicarb-induced plasticity as a reduced sensitivity of pre-conditioned worms to a subsequent drug exposure. We investigated molecular determinants of this plasticity by using uncoordinated locomotion and reduced pharyngeal movement mutant worms due to impairments in cholinergic transmission. Interestingly, preconditioned mutant worms exhibits a switch in the aldicarb-induced plasticity observed in wild type, becoming more sensitive to post-exposure of aldicarb. Defining the molecular identity of this mutant will reveal pathways that mediate cholinesterase induced structural reorganization at the pharyngeal NMJ. Thus, the drug and genetic tractability of *C. elegans* offers a new route to anti-cholinesterase poisoning antidotes.

Keywords: anti-cholinesterase intoxication; cholinergic plasticity; C. elegans

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Military Medical Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

PHARMACOKINETICS OF BIS-PYRIDINIUM MONO-ALDOXIMES

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Bis-pyridinium mono-aldoxime (BPMA) compounds are potential antidotes against organophosphorus inhibitors of either acetylcholinesterase or these of butyrylcholinesterase. From the points of drug distribution and pharmacokinetics essential characteristics were determined (concentration versus time curves).

Experimental results of pharmacokinetics of BPMA will be detailed with special focus on drug distribution and HPLC analysis of oxime K117.

The concentration of BPMAs decreases fast in the body of rats, and thus they fulfil the basic requirement for antidotes: elimination should be as fast as possible. Their elimination curve should be characterized by the term „tenth-life" rather than half-life.

BPMA compounds penetrate into the brain in considerable amounts of their concentration in the serum. As blood-brain penetration can have vital importance, time of the maximum extent of blood-brain barrier should also be conceived as a novel pharmacokinetic parameter.

This research has been supported by Kalász Teaching and Research Co.(Budapest, Hungary), by the NN126968 grant of Hungarian National Research,Development and Innovation Office (Budapest, Hungary) and Ministry of Education, Youth and Sports of the Czech Republic (no. BF17004).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

3D STRUCTURE OF NATURAL TETRAMERIC FORM OF HUMAN BUTYRYLCHOLINESTERASE OBTAINED BY CRYO-ELECTRON MICROSCOPY

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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.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

BUTYRYLCHOLINESTERASE-PROTEINS INTERACTIONS IN HUMAN BLOOD SERUM

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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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DIMERIZATION INTERFACE OF CHOLINESTERASES: ANALYSIS OF CRYSTAL STRUCTURES, FREE ENERGY MOLECULAR DYNAMICS CALCULATIONS,AND *IN SILICO* ALANINE SCREENING

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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)

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

4-AMINOQUINIOLINES AS REVERSIBLE INHIBITORS OF HUMAN CHOLINESTERASE ACTIVITY

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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 (K_i) 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

Acknowledgement

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)

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

SYNTHESIS OF NERVE AGENTS' SURROGATES FROM DIALKYL ALKYLPHOSPHONATES FOR ANTIDOTE SCREENING AND TOXICOLOGICAL STUDIES

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Abstract: 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

Acknowledgement

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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

BIOLOGICAL EVALUATION OF CYSTEINE TARGETED INSECTICIDES

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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.

Keywords: malaria; acetylcholinesterase; insecticide; cholinesterase inhibitor; cysteine

Acknowledgement

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).

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

AN ALTERNATIVE SUBSTRATE FOR HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY DETECTION

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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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Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

ACETYLCHOLINESTERASE REACTIVATORS BASED ON OXIME-FUNCTIONALIZED BIODEGRADABLE IONIC LIQUIDS

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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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Military Medical
Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

IN SILICO SCREENING OF NOVEL BChE-REACTIVATORS

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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, pKa 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

Acknowledgement

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".

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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] and Zrinka Kovarik [1]
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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.

MMSL Military Medical Science Letters Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

IN SILICO AND *IN VITRO* EVALUATION OF TWO NOVEL OXIMES K456 AND K733 AGAINST PARAOXON INHIBITED HUMAN ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE

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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. R50 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

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Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

FACILE SYNTHESIS OF CYSTEINE-ACETYLCHOLINESTERASE TARGETED INSECTICIDES

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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.

Acknowledgement

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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

INHIBITION OF HUMAN ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE BY METHYLENE VIOLET 3RAX

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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 Ki 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; $\alpha=\infty$. 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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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 Musilek [2], Daniel Jun [5] and Tanos C. C. França [1,3]
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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

Acknowledgement : 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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

CHLORINATED PYRIDINIUM OXIMES ARE POTENT REACTIVATORS OF ACETYLCHOLINESTERASE INHIBITED BY NERVE AGENTS

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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

Acknowledgement

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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

BISTABLE DYNAMIC BEHAVIOR OF ENDOGENOUS BUTYRYLCHOLINESTERASE EXPRESSED IN Expi293 CELLS

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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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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

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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}. kr 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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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]

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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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

INHIBITION OF CHOLINESTERASES FOLLOWING PERCUTANEOUS INTOXICATION WITH V AGENTS IN RATS

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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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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

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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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DESIGN, SYNTHESIS AND IN VITRO EVALUATION OF A PROMISING NEW CLASS OF BIFUNCTIONAL UNCHARGED HYBRID REACTIVATORS FOR NERVE AGENT-INHIBITED HUMAN ACETYLCHOLINESTERASE

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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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

THE EARLY TISSUE ALTERATION INDUCED BY DIFFERENT OXIMES IN RATS

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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 LD_{50} 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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

CYTOTOXICITY STODY OF OXIME@CB7 COMPLEXES FOR CENTRAL NERVOUS SYSTEM PENETRATION OF QUATERNARY ACETYLCHOLINESTERASE REACTIVATORS

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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.

Acknowledgement

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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

BRAIN EXPOSURE OF BIS-PYRIDINIUM OXIME KR-26256

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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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

ANALYSIS OF BIS-PYRIDINIUM MONO-ALDOXIMES IN SERUM AND ORGANS USING A HIGH-THROUGHPUT HIGH PERFORMANCE LIQUID CHROMATOGRAPHY APPROACH

Gellert Karvaly, Kornélia Tekes, Huba Kalász

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.

Acknowledgement

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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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

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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

Acknowledgement

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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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]

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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.

Acknowledgement

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.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DECONTAMINATION OF WARFARE AGENT

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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.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

IN VITRO CHARACTERIZATION OF THE STANDARD ACETYLCHOLINESTERASE REACTIVATORS

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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 (M1 subtype), nicotinic (α7 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

Acknowledgement

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.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

UNCHARGED REACTIVATORS OF CHOLINESTERASES INHIBITED BY ORGANOPHOSPHORUS NERVE AGENTS

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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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

IN VITRO DETERMINATION OF OXIDATIVE STRESS INDUCED BY OXIME REACTIVATORS USING CHROMATOGRAPHIC METHODS

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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.

Acknowledgement

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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

IN VITRO EVALUATION OF QUINUCLIDINIUM OXIMES AS REACTIVATORS OF HUMAN CHOLINESTERASES INHIBITED BY ORGANOPHOSPHORUS COMPOUNDS

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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

Acknowledgement

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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

13th INTERNATIONAL MEETING ON CHOLINESTERASES, 7th INTERNATIONAL CONFERENCE ON PARAOXONASES
9. - 14.9.2018, University of Hradec Kralove, Hradec Kralove, Czech Republic, www.13thche.cz

MEETING ABSTRACTS

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. and Babakov V.N.
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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 ($R^2 \geq 0.98$). 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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

AN *IN-VITRO* INDUCTION OF PARAOXONASE 3 ACTIVITY IN HEPATOCYTES BY RESVERATROL

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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

Acknowledgement

ICMR, New Delhi

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

SMART & INNOVATIVE TOOLS FOR CHOLINESTERASES RELATED APPLICATIONS

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Presenting author: Emilie David

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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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

IN VITRO EVALUATION OF STANDARD ACETYLCHOLINESTERASE REACTIVATORS AS REACTIVATORS OF HUMAN PLASMA BUTYRYLCHOLINESTERASE

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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”.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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]

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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.

Acknowledgement

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

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Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

BUTYRYLCHOLINESTERASE AND ITS VARIANTS (rs3495 & rs1803274) ASSOCIATION WITH MAJOR DEPRESSIVE DISORDER

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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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

ALKALOIDS DERIVED FROM TRADITIONAL CHINESE MEDICINE ARE INHIBITORS FOR INFLAMMATION AND ACETYLCHOLINESTERASE

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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.

Acknowledgement

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).

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Science Letters
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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

ACETYLCHOLINESTERASE REGULATES INFLAMMATORY RESPONSES IN CULTURED MACROPHAGES: A PLAYER IN CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

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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.

Acknowledgements

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.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

GENISTEIN, A PHYTOESTROGEN IN SOYBEAN, INDUCES THE EXPRESSION OF ACETYLCHOLINESTERASE VIA G PROTEIN-COUPLED RECEPTOR 30 IN PC12 CELLS

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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.

Acknowledgements

This work was supported by Shenzhen Science and Technology Committee Research Grant (JCYJ20160229205726699, JCYJ20160229205812004, JCYJ20160229210027564, CKFW2016 082916015476, JCYJ20170413173747440, JCYJ20151030164022389, 20170326 and ZDSYS201707281432317). Etta Y. L. Liu hold a Lee's Pharmaceutical-Kanya Lee Postgraduate Scholarship.

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Military Medical
Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

STATUS OF CHOLINESTERASES IN HEROIN, HASHISH (CANNABIS) AND POLYDRUG ADDICTS

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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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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

INDIRECT EFFECTS OF DIOXIN ON NEURONAL AChE EXPRESSION VIA ASTROCYTES

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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

Acknowledgement

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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Science Letters
Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

NON-NEURONAL CHOLINERGIC SYSTEM IN RAT AORTA

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We have showed before that both cholinesterases are present in rat aorta, while inhibition of butyryl-cholinesterase 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.

Acknowledgement

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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

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]
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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.

Acknowledgement

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.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DUAL BINDING SITE INHIBITORS OF ACETYLCHOLINESTERASE AS THERAPEUTIC TREATMENTS FOR ALZHEIMER'S DISEASE: ANY NEED FOR AN UPDATE?

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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.

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

DETECTION OF ALZHEIMER'S DRUG CANDIDATE BY SURFACE-ENHANCED RAMAN SPECTROSCOPY

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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 ≒m 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.

Acknowledgement

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

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Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

BUTYRYLCHOLINESTERASE INHIBITORS GRAFTED WITH ANTIOXIDANT AND NEUROPROTECTIVE ACTIVITIES: NOVEL MULTIFUNCTIONAL LIGANDS FOR ALZHEIMER'S DISEASE

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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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MMSL Military Medical Science Letters Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2018, 87(supplementum 1)
ISSN 2571-113X (Online)

MEETING ABSTRACTS

7-MEOTA-DONEPEZIL HYBRIDS: POTENTIAL CHOLINESTERASE INHIBITORS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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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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