Workshop "Reactivators and Medical Countermeasures against Nerve Agents and Pesticides"
Zagreb, Croatia, 14-15 May 2018

The workshop "Reactivators and Medical Countermeasures against Nerve Agents and Pesticides" was held at Hotel Palace in Zagreb, Croatia on May 14 and 15 2018. The Workshop was organised by the Institute for Medical Research and Occupational Health and supported by the Croatian Science Foundation (HrZZ project CHOLINESTERASE, IP-2013-11-4307).

The aim was to inform the scientific community on developments and new perspectives in medical countermeasures against organophosphorus compounds, arising from our project CHOLINESTERASE and general advancements in medicinal chemistry directed towards the synthesis of new reactivators of acetylcholinesterase (AChE), an essential enzyme in neurotransmission. Treatment of pesticide and nerve agent poisoning has thus far relied on a combination of agents: antagonists to minimize excessive muscarinic stimulation affecting cardiovascular and respiratory parameters, anticonvulsant agents to avert seizures presumably initiated from central acetylcholine accumulation, and AChE reactivating agents as antidotes to reduce the excessive acetylcholine levels through cholinesterase reactivation. The classic reactivating agents are derivatives of the lead compound 2-PAM shown over 60 years ago to be site-directed to the then presumed active site of AChE. However, this approach has limitations, one of which is the inability of currently applied standard oximes (TMB-4, obidoxime, HI-6 and 2-PAM) to efficiently reactivate AChE inhibited by certain organophosphates. The current therapy is particularly ineffective in cases of tabun and soman. In addition, their capacities are limited to their peripheral activity in being unable to cross the blood-brain barrier in appreciable concentrations to reactivate central AChE.

All of these limitations as well as topics that go beyond have been addressed in our research during the last four years of the project CHOLINESTERASE, and most of our results have been published in highly-rated journals. Therefore, the workshop was an excellent opportunity to discuss new results and exchange ideas and experiences with researchers and experts in antidotal research. Our guests and eminent scientists Horst Thiermann, Florian Nachon, and Fredrik Ekström delivered inspiring lectures devoted to the toxicological aspects of poisoning by nerve agents, chemistry of reactivation, and crystallographic research of AChE. Each lecture was followed by a fruitful discussion that lasted during dinner and a guided Zagreb city tour. The workshop was attended by 25 participants.

We thank *Arhiv* for giving us the opportunity to publish abstracts from the presentation in this issue along with a short biosketch of speakers.

Zrinka Kovarik
Organiser of the Workshop
Principal investigator of the HrZZ project
CHOLINESTERASE

Participants of the workshop: M. Katalinić, H. Thiermann, Z. Kovarik, F. Nachon, F. Ekström (sitting), M. Meštrović, I. Vrhovac Madunić, A. Zandona, A. Bosak, T. Zorbaz, N. Maraković, S. Žunec, A. Matošević, N. Maček Hrvat, and G. Šinko

Introduction to the workshop "Reactivators and Medical Countermeasures against Nerve Agents and Pesticides"

Zrinka Kovarik

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In the last four years, we have been focused on the realization of research planned within the HrZZ project CHOLINESTERASE. Our main goal was to develop more efficient antidotes and improve the treatment of highly toxic organophosphorus (OP) compound poisoning. OP compounds used as pesticides account for over 3,000,000 cases of poisoning per year worldwide. Furthermore, OPs known as nerve agents (soman, sarin, tabun, VX) represent a threat in terrorist attacks and conflicts, as was the case recently in Syria, Malaysia, and the UK. The main targets of OP compounds are acetylcholinesterase (AChE), the essential enzyme in neurotransmission, and butyrylcholinesterase, its back-up enzyme. However, the antidotes currently in use, which act as reactivators of inhibited AChE, had been empirically synthesized before the two enzyme's crystal structures were resolved. Due to structural requirements, their binding affinity and reactivation rate have not been well-balanced. We combined several approaches, including computational and experimental studies of cholinesterase interactions with a wide range of ligands defining favourable characteristic for potential new antidotes, *in silico* design of novel compounds that direct the subsequent synthesis of selected leads, and thorough *in vitro* and *in vivo* experimental evaluation. Moreover, we demonstrated a feasible approach to the development of an oxime-assisted catalytic bioscavenger of soman, tabun, and VX based on AChE mutants in combination with its efficient reactivator. Ultimately, the oxime-assisted catalytic scavenging of the nerve agents in mice improved therapeutic outcomes preventing lethality and resulted in a delayed onset of toxicity symptoms.

KEY WORDS: *cholinesterase; nerve agents; organophosphorus compounds; poisonings, terrorism*

Supported by Croatian Science Foundation project IP-2013-11-4307.

Zrinka Kovarik, PhD permanent scientific adviser, obtained a doctorate from the University of Zagreb, Croatia in 2002. She spent two years at the Department of Pharmacology, University of California at San Diego, La Jolla, USA as a predoctoral and postdoctoral fellow obtaining the Whood-Whelan Fellowship and a one-year fellowship of the Croatian Ministry of Science, Education and Sports. She was the principal investigator of more than 10 international and national research projects, lecturer within postgraduate studies in chemistry, University of Zagreb, and supervisor of four PhD theses. She was involved in the organisation of numerous scientific conferences and meetings. She served for four and six years as president and secretary of the Croatian Society of Biochemistry and Molecular Biology (HDBMB), respectively. Currently, she is the president of the Croatian Society of Natural Sciences and member of the Organisation for the Prohibition of Chemical Weapons (OPCW) Scientific Advisory Board. She is co-author of more than 70 research papers. For her achievements she has been awarded by the Institute and the HDBMB.

ABSTRACTS
Arh Hig Rada Toksikol 2018;69:A32-A43

Reactivators and medical countermeasures against nerve agents and pesticides

Horst Thiermann and Franz Worek

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In organophosphorus compound poisoning, the inhibition of acetylcholinesterase (AChE) results in a cholinergic crisis. While muscarinic signs and symptoms can be treated with atropine, effects at nicotine receptors, *e.g.* respiratory paralysis can only be antagonized by reactivation of inhibited AChE. Although oximes are able to reactivate AChE inhibited by distinct OPs substantially, several circumstances may limit its effectiveness. Effective plasma concentrations are dependent on the respective oxime used and the single OP. In poisonings with several certain OPs, reactivation *per se* is hardly possible, *e.g.* the phosphoramidates tabun and fenamiphos. Others, *e.g.* soman and profenophos, show extremely fast aging thereby preventing relevant reactivation. Finally, high concentrations of persisting OPs or inhibiting metabolites may lead to the re-inhibition of reactivated AChE. The ongoing process of reactivation and re-inhibition may result in enhanced poison elimination as well as prolongation of the aging half time. Hence, a beneficial therapeutic effect might be expected when oximes fulfill several requirements. Generally, the k_r should be higher than 1 min^{-1} and the K_D should be lower than 100 µM in humans. Finally, the respective oxime has to be administered long enough and the effective dose has to be tolerated well in human beings. Obidoxime has shown effectiveness at relevant concentrations against several OPs (*e.g.* diethyl- and dimethyl-OP pesticides, sarin, VX, CVX). HI-6 is able to close several gaps, *e.g.* cyclosarin and VR. Pralidoxime appears less efficient against these agents. In conclusion, a combination of obidoxime and HI 6 could cover the broadest spectrum of OPs in human poisoning.

KEY WORDS: *acetylcholinesterase; antidotes; obidoxime; HI-6; organophosphorus compound poisoning; oximes*

Colonel (MC) Prof Dr Horst Thiermann studied medicine at the University of Regensburg and Technical University, Munich, Germany. After working at the Bundeswehr Hospital Munich, in the departments of anaesthesiology and surgery, he changed to the Bundeswehr Institute of Pharmacology and Toxicology. He specialized in Pharmacology and Toxicology at the Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig Maximilians-University Munich in 1996. In 2002, he completed his advanced studies of Clinical Pharmacology at MDS Pharma Services, Höhenkirchen-Siegertsbrunn, Germany. Since November 2006 he has been the director of the Bundeswehr Institute of Pharmacology and Toxicology. In January 2012, he was appointed Professor at Technical University, Munich. Colonel (MC) Prof. Dr. Thiermann is the president-elect of the European Association of Poisons Centres and Clinical Toxicologists (EAPCCT), vice-chairman of German Federal Institute for Risk Assessment (BfR) Committee for the Assessment of Intoxications, and past president of Clinical and Translational Toxicology Speciality Section (CTTSS) of the Society of Toxicology (SOT).

A decade of French effort in the quest for a broad spectrum and centrally active reactivator

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Organophosphorus nerve agents (NOP) are highly toxic due to their strong inhibition potency against acetylcholinesterase (AChE). Inhibition of AChE leads to the accumulation of acetylcholine and dysfunction of cholinergic receptors. Neurotransmission is seriously impaired, causing seizures in the central nervous system (CNS), muscle fasciculation, and generally death by respiratory arrest. Inhibited AChE can be reactivated using oximes, which remove the phosphyl group from the active site serine. Quaternary oximes are one major component of the immediate treatment in cases of nerve agent intoxication, but they do not readily cross the blood-brain barrier and none of them are able to efficiently reactivate the most common member of the G- and V-agents families. It is only logical then that the development of broad-spectrum reactivators capable of regenerating AChE in the central nervous system has become a hot topic in recent years. A previous study showed that non-quaternary hydroxyl-pyridinaldoxime is a potent reactivator despite its poor affinity toward the inhibited enzyme. We improved the affinity by linking this reactive moiety to a peripheral site ligand of AChE (*e.g.* tacrine, phenylisoquinoline, tryptoline, etc.). The design of these new molecules was heavily supported by docking and X-ray crystallography work. Candidate molecules were tested *in vitro* on inhibited AChE using various methods. These tests led to the identification of molecules with broad spectrum and high activity *in vitro*. The most promising and druggable leads were further evaluated *in vivo* on mice intoxicated with NOP or their surrogates, following the measurement of their pharmacokinetic profile. Surviving test results using the up-and-down method in combination or absence of atropine revealed a promising protective efficacy. This set of novel reactivators constitutes a strong basis for further refinement and development.

KEY WORDS: *acetylcholinesterase; central nervous system; nerve agents; oximes*

Dr Florian Nachon received his PhD degree in Biochemistry and Pharmacochemistry in 1999 from the University of Strasbourg, France. Thereafter, he joined the research center of the French Defense Health Service, and started research on bioscavengers of organophosphorus nerve agents. He took a sabbatical at the University of Nebraska Medical Center in Omaha, USA from 2002 to 2003. He was appointed project manager in 2004, head of the research unit on neurotoxics medical countermeasures at the Biomedical Research Institute of Armed Forces based near Paris in 2009, and became the head of the Departement of Toxicology and chemical risks in 2018. His present main research interests, relying heavily on structural biology, are focused on the development of nerve agent bioscavengers and new generations of reactivators for nerve agent-inhibited cholinesterases with broad spectrum and central nervous system activity.

Design of broad spectrum antidotes

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The design of 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, 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] modelling. The 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 model 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.

KEY WORDS: *acetylcholinesterase; molecular modelling; nerve agents; oximes; QSAR; X-ray crystallography*

Fredrik Ekström earned his PhD in cell and molecular biology at Umeå University, Sweden in 2003. In his thesis, he studied the enzyme phenylalanine hydroxylase using EXAFS and X-ray crystallography. After completing his PhD, Dr Ekström accepted a position at FOI where he has been since. He was appointed project manager in 2004, Deputy Research Director in 2010, team leader for biochemistry and structural biology in 2011, and Research Director in 2018. Dr. Ekström's early work focused on developing a eukaryotic expression system and establishing X-ray crystallography as a platform for mechanistic studies of nerve agents and nerve agent antidotes. Since then, Dr Ekström has further evolved the program, taking a multidisciplinary approach to investigate nerve agents, their antidotes and the molecular recognition of the enzyme acetylcholinesterase. Recent developments include efforts to combine diffusion trap cryo-crystallography with a quantum chemical cluster approach using implicit dispersion-corrected density functional theory (DFT) calculations. Current research interests include structure-based design of nerve agent antidotes, development of selective inhibitors for Malaria and Dengue vector control and the host-pathogen coevolution of alphaviruses.

Design, synthesis, and reactivation efficiency of chiral *N*-substituted 2-hydroxyiminoacetamides

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Using computational methods of molecular modelling, we investigated conformational changes in the active site of acetylcholinesterase (AChE) upon binding various ligands and defined structural characteristics of efficient oxime reactivators of AChE inhibited with warfare nerve agents and defined guidelines for their synthesis. Four new chiral oxime reactivators from the *N*-substituted 2-hydroxiiminoacetamide group were prepared starting from racemic 1-phenyl-allylamine prepared from cinnamyl alcohol. Enantiomers of oximes were separated using high performance liquid chromatography on polysaharidic chiral stationary phases. New oximes were tested for inhibition of AChE and butyrylcholinesterase (BChE) and reactivation of cholinesterases inhibited with tabun, cyclosarin, sarin, and VX. New oximes reversibly inhibit both enzymes with inhibition constant (K_I) in micromolar range. Both enzymes showed greatest affinity toward 2-hydroxyimino-*N*-(3-(4-((2-methylimidazol-1-yl)methyl)-1,2,3-triazol-1-yl)-1-phenylpropyl)acetamide towards which BChE displays significant selectivity and stereoselectivity. All of the new oximes showed reactivation efficiency against cyclosarin-, sarin-, and VX-inhibited BChE, while only oximes with more elaborate structure of structural element binding to peripheral allosteric site showed reactivation efficiency against inhibited AChE. Molecular docking studies concluded that differences in binding of new oximes in AChE and BChE largely result from differences in amino acids at the position of Tyr72, Tyr124, Phe297, and Tyr337 in the AChE active site.

KEY WORDS: *acetylcholinesterase; butyrylcholinesterase; nerve agents; oximes; reactivators*

Supported by Croatian Science Foundation project IP-2013-11-4307.

Nikola Maraković, PhD graduated Chemistry from the Faculty of Science, University of Zagreb, Croatia in 2011 with the thesis title "Approach to synthesis of alkylated benzens with long hydrocarbon chains" written under the supervision of Dr Hrvoj Vančik. From July 2011 he has been employed as a research assistant at the Institute for Medical Research and Occupational Health, where he currently works as postdoctoral researcher. In April 2017, he defended his PhD thesis entitled "Development of new chiral 2-hydroxyiminoacetamide reactivators of phosphylated cholinesterases" done under the supervision of Dr Goran Šinko. His research interests include interactions between cholinesterases and antidotes or inhibitors, reactivation of cholinesterases inhibited with organophosphorus compounds, design and synthesis of antidotes for organophosphorus poisoning, molecular docking and homology modeling. He has published 5 scientific papers in acclaimed international peer-reviewed journals.

RS194B pharmacokinetics and antidotal potential in mice exposed to VX and sarin

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Lipophilic and uncharged organophosphorus compounds (OP) inhibit peripheral and central nervous system (CNS) cholinesterase within minutes of OP exposure due to rapid OP diffusion through biological membranes. Therapeutic treatment consists of a combined administration of an anticholinergic drug (atropine) and oxime-reactivator of acetylcholinesterase (AChE). Therapy by oximes approved for use (2-PAM, obidoxime, HI-6) is somewhat efficient in reactivating inhibited AChE in blood and peripheral tissues but not in CNS because of slow or limited blood-brain barrier penetration for quaternary pyridinium aldoximes with a permanent cation. To develop antidotal therapy capable of efficient reinstatement of CNS AChE activity, we examined the zwitterionic hydroxyimino-acetamido alkylamine oxime known as RS194B amenable to protonation of the non-ionized species that can cross the blood brain barrier. RS194B is as an effective *in vitro* reactivator of human AChE inhibited by VX and sarin, therefore we further examined the pharmacokinetic properties, oral bioavailability, and antidotal efficacy of RS194B against OP exposure in mice. The results show that 2 h sequential administrations to 10 h ensure the steady-state plasma and brain levels of the oxime. Moreover, within the 40 min period brain concentrations of RS194B exceed the plasma concentrations prior to the next administration. Also, RS194B substantially protected mice when administered by gastric lavage prior to OP exposure, whereas 2-PAM exhibited no protection when similarly administered. Furthermore, the observed recovery of the mice brain activity after administering RS194B to mice exposed to sarin and even VX is consistent with its rapid tissue disposition and BBB penetration. Those results, along with low toxicity of RS194B in mice, make this oxime a lead candidate for further efficacy, tissue disposition, and pharmacokinetics analysis in other animal species.

KEY WORDS: *acetylcholinesterase; blood-brain barrier; central nervous system; oximes; plasma*

Partially supported by Croatian Science Foundation project IP-2013-11-4307.

Nikolina Maček Hrvat, PhD is a postdoctoral researcher at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. She defended her PhD in Chemistry – Biochemistry in 2015 with a thesis researching cholinesterases and oximes as pseudo-catalytic bioscavengers of nerve warfare agents. Her field of interest include enzyme kinetics of cholinesterase interactions with various organophosphates and newly developed oximes as potential reactivators of organophosphate-inhibited cholinesterase activity, as well as the bioscavenging potential of acetylcholinesterase mutants and butyrylcholinesterase in the event of organophosphate poisoning.

Potent lipophilic 3-hydroxy-2-pyridinium aldoxime reactivators of phosphorylated cholinesterases

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Organophosphate compounds (OPs) poisoning can occur due to exposure to OP pesticides (*e.g.*, parathion) or nerve agents (*e.g.*, sarin, cyclosarin, VX, tabun). OPs irreversibly inhibit acetylcholinesterase (AChE) causing the accumulation of acetylcholine in synapses and cholinergic crisis, which can lead to respiratory arrest and death or result in long-term neurological impairments in survivors. Reactivation of OP-inhibited AChE is one of the possible therapy approaches, but standard oxime reactivators (2-PAM, HI-6, obidoxime) are not potent enough for every OP and achieve low brain concentrations due to a permanent charge (quaternary nitrogen). New class of oximes, 3-hydroxy-2-pyridine aldoximes, without permanent charge have been synthesized and tested in detail. They were proven to be potent reactivators of AChE inhibited by VX, sarin, cyclosarin, tabun, and paraoxon with the affinity of AChE in the micromolar range. Moreover, molecular docking studies predicted optimal orientation of the oximes in the active site of OP-inhibited AChE. Furthermore, these oximes were proven to be lipophilic and by analysing various physicochemical properties that describe their size, charge, lipophilicity, and polarity we predicted their penetration through the blood-brain barrier (BBB). The permeability of these oximes across the membrane was confirmed *in vitro* using an artificial membrane that mimics the BBB. In addition, most of the oximes were shown to be metabolically stable when incubated with human microsomes and were not cytotoxic for neuroblastoma and astrocytoma cell lines. Finally, the lead oxime crossed the BBB *in vivo* in mice after intramuscular application proving its potential to be used as a centrally active antidote.

KEY WORDS: *AChE; CNS; molecular docking; nerve agents; oximes; synthesis*

Supported by Croatian Science Foundation projects (IP-2013-11-4307 and UIP-2017-05-7260) and the COGITO project (2015-2016; 33068WE).

Tamara Zorbaz graduated at the Faculty of Pharmacy and Biochemistry at the University of Zagreb, Croatia (Master of Medical Biochemistry) in 2014. Since 2015, she has been a research assistant at the Institute for Medical Research and Occupational Health within the Croatian Science Foundation Project (no. 4307, CHOLINESTERASE; PI: Zrinka Kovarik, PhD), and a PhD student at the Faculty of Science at the University of Zagreb working on the doctoral thesis entitled "A new approach to the analysis of oximes designed to protect central nervous system in organophosphorus poisoning". In 2016, she received the French Government Scholarship for professional development at the University Paris Descartes, Paris, France, under the supervision of Eric Krejci, PhD (Cognition and Action Group, CNRS). She has been awarded with two travel grants to conferences, and she received three poster awards at international conferences in 2016 (Marseille), 2017 (Munich), and 2018 (Hradec Kralove). She is the co-author of five research papers.

Molecular mechanisms underlying the toxicity of antidotes and potential drugs

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In organophosphorus poisoning (OP) antidote research, the lead candidates are selected mostly based on *in vitro* kinetic studies and are further tested *in vivo* without any of cell toxicity studies performed previously. This way, adverse effects of oximes as xenobiotics can be masked by a stronger system response to the applied *in vivo* treatment. This becomes visible in the next steps of drug development process and due to negative effects on the cell level, previously designated lead oximes cannot be further considered for use as antidotes. Therefore, to overcome this shortcoming, the here presented work focuses on the implementation of an adequate cell-based preclinical screening or cell-based early safety evaluation in antidote discovery prior to any *in vivo* testing. We try to define the exact mechanism of cell chemosensitivity to potential antidotes and define the possible structural features/moieties of the tested compounds triggering certain effects. This will ensure more efficient feedback to researchers designing these drugs and aid the challenging development of a more effective treatment for OP poisoning.

KEY WORDS: *apoptosis; cell-based preclinical screening; cell chemosensitivity; oxidative stress*

This work was supported by Croatian Science Foundation project UIP-2017-05-7260.

Maja Katalinić, PhD works as a senior research associate at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. In 2004, she obtained her biochemical engineering diploma at the Faculty of Food Technology and Biotechnology. In 2011, she defended her doctoral thesis in Biochemistry and Medicinal Chemistry at the Faculty of Science of the University of Zagreb. Her primary research focus is in the assessment of new cholinesterase reactivators as potential antidotes in organophosphorus compound poisoning and the implementation of the cell-based preclinical screening. She has received many awards among which also the "B.P. Doctor Young Investigator Award", awarded to the successful young scientists in the field of cholinesterase research. She is a member of the Executive Board and Secretary of the Croatian Society of Biochemistry and Molecular Biology.

Novel insight into the principles of oxime bioactivity

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Pyridinium oximes are an essential part of antidotal therapy used in organophosphorous (OP) compounds poisoning. Through reactivation of phosphorylated acetylcholinesterase (AChE), oximes ameliorate symptoms arising from acute OP poisoning. However, survivors of OP exposures often have delayed neurologic symptoms of memory loss and cognitive dysfunction. Recent studies have shown that oxidative stress mediates secondary OP injury so long-term neurological consequences may be amenable to antioxidant therapy. Pyridiunium oximes have incorporated iminium and oxime moieties that are electron-affinic which enables them various physiological properties and might contribute to their overall therapeutic efficacy. Currently none of the four conventional oximes (2-PAM, TMB-4, HI-6 and obidoxime) promote the recovery of OP intoxicated patients to a satisfactory extent. Another issue is their low blood-brain barrier permeability and relatively high toxicity in mammals. Our research group has recently adopted a simultaneous approach for studying the biochemical properties of oximes together with the evaluation of their cyto-/genotoxicity on human white blood cells *in vitro* as well as in somatic cells of rats *in vivo*. Our results have shown the weak antioxidant potential of conventional oxime HI-6 and experimental oxime K048. Taking into account that genotoxicity studies on oxime compounds have thus far been rare, our results provide insight into the toxicity of HI-6 and K048 toward human non-target cells. Since this compounds should provide pharmacological protection by minimizing the adverse effects, it is important to elucidate their safety profile. Our studies have shown that HI-6 and K048 applied at concentrations/doses relevant for therapeutic interventions did not possess DNA-damaging potential.

KEY WORDS: *antidotal therapy; antioxidative potential; genotoxicity; oximes*

Suzana Žunec, PhD is a research associate employed at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. She defended her PhD in biochemistry and medicinal chemistry in 2012 with a thesis researching new effective antidotes against organophosphorous compounds poisoning. She expanded the research involving anticholinesterase poisoning to the evaluation of additional non-cholinergic mechanisms (*e.g.* oxidative stress) that could contribute to organophosphate toxicity and also carried out research on the cyto/genotoxicity and antioxidative activity of potential antidotes. Currently, her research interests are the oxidative potential of novel and conventional pesticides and their connection with biomarkers of neurologic risks and carcinogenesis, and examinations of the properties of second-generation chemotherapeutics and their effects on the cholinergic system.

Carbamates of bronchodilators and Cinchona-based alkaloids as selective BChE inhibitors

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The inhibition of BChE in human tissues is important for the detoxification and scavenging of xenobiotics such as organophosphates. Increased BChE activity in advanced Alzheimer's disease (AD) has led to the hypothesis that BChE needs to be inhibited to restore the brain levels of ACh. With an aim to evaluate the preferable characteristic of novel potential therapeutics based on BChE inhibition with respect to AChE, we studied the inhibition potency and selectivity of carbamates bearing the structural motif of the bronchodilator bambuterol, a very selective BChE inhibitor, bronchodilators with a resorcinol-, catechol- and saligenin-containing structure, and cinchonies and cinchonidines, quinine-based alkaloids. All of the synthesised carbamates are potent and selective BChE inhibitors with carbamylation rates similar to that of bambuterol and an inhibition potency dictated by the disposition of carbamate groups on the benzene ring, where the *meta*-position is preferred over the *ortho*-position. Both AChE and BChE were stereoselective with an about five time higher preference for (*R*)- over (*S*)-carbamates. The studied bronchodilators reversibly inhibited AChE and BChE with an inhibition potency dependent of the size of the hydroxyaminoethyl chain on the benzene ring. Generally, the studied bronchodilators had a higher preference to BChE without displaying any stereoselectivity. Furthermore, a series of 20 synthesised synthetic quaternary derivatives of cinchonidines and cinchonines reversibly inhibited BChE and AChE with inhibition constants in nanomolar to micromolar range and with a higher preference for BChE and an up to 510 times higher inhibition selectivity toward BChE over AChE. The most selective and potent BChE inhibitor was cinchonidine with bromidium in the *para*-position on the benzene ring.

KEY WORDS: *acetylcholinesterase; butyrylcholinesterase; inhibition potency; steroselectivity*

Supported by Croatian Science Foundation project IP-2013-11-4307.

Anita Bosak, PhD, a scientific associate, has been employed at the Biochemistry and Organic Analytical Chemistry Unit of the Institute for Medical Research and Occupational Health, Zagreb, Croatia since 1999. She has actively participated in the implementation of several national and international projects. Her research activity is focused on cholinesterases and paraoxonase, primarily in the study of enzyme kinetics of these enzymes in interaction with certain esterifying inhibitors and many reversible inhibitors, some of which are used as drugs. In her scientific work, Anita Bosak has published a total of 26 scientific papers. She is a member of the Croatian Society of Biochemistry and Molecular Biology, the Croatian Society of Toxicology, and the Croatian Society of Natural Sciences (treasurer and member of the Executive Council from 2016). For her work, Anita Bosak has received four annual IMROH awards and the annual award of the Croatian Society for Biochemistry and Molecular Biology.

Kinetic model as a tool for prediction of multiple ligand binding in ChEs

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Since Michaelis and Menten proposed a kinetic model of enzyme activity in 1913, it has been demonstrated that not all enzymes follow this simple kinetics characterised by the formation of an enzyme-substrate reversible complex (Michaelis-Menten complex). This is the case for cholinesterases, serine hydrolases, which have a high catalytic turnover. Acetylcholinesterase (AChE, EC 3.1.1.7) is one of fastest enzymes known that hydrolyses acetylcholine (ACh), a neurotransmitter in the synaptic cleft thus enabling a high rate of impulse propagation. AChE shows substrate inhibition at substrate concentrations higher than 2 mmol dm^{-3}. The related enzyme butyrylcholinesterase (BChE, EC 3.1.1.8) can also hydrolase acetylcholine but it can also react with larger choline esters or xenobiotics. BChE shows substrate inhibition by ACh at concentrations higher than 75 mmol dm^{-3}. Advanced kinetic models have been developed to describe highly efficient ATC hydrolysis by ChEs and inhibition by various different ligands. It has been shown that in wide substrate concentration range ($2x10^{-6}$ to $2x10^{-1}$ M), a 7-parameter model of substrate hydrolysis can be effectively used to describe interaction of ChEs with inhibitors after enlargement of the kinetic model by introducing corresponding enzyme-inhibitor or enzyme-substrate-inhibitor species. Previous kinetic measurements predicted the formation of multiple inhibitor (ligand) species with an enzyme. There are several crystal structures of AChE or BChE complexes in the Protein Data Bank that confirm multiple ligand binding into the active site of AChE or BChE.

KEY WORDS: *acetylcholinesterase; butyrylcholinesterase; hydrolysis; Michaelis-Menten kinetics*

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