Symposium
"Cell-Based Research in Toxicology and Drug Design"

26 January 2023, Zagreb, Croatia

Organised by

Maja Katalinić, PhD
and
Antonio Zandona, PhD

CellToxTargets
HrZZ-UIP-2017-05-7260

Molecular mechanisms underlying the toxicity of antidotes and potential drugs – CellToxTargets

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Programme

8:30-9:00	*Welcome, introduction and registration*
	Session I – CellToxTargets (Chair: Maja Katalinić)
9:00-9:20	**Maja Katalinić** (Croatia) Molecular mechanisms underlying the toxicity of antidotes and potential drugs – HrZZ-UIP-2017-05-7260
9:20-9:35	**Antonio Zandona** (Croatia) Structure – cytotoxicity relationship of cholinesterase-based ligands
9:35-9:50	**Ana-Marija Lulić** (Croatia) Characterization of lysophospholipase PNPLA7 as a potential target for drug development
9:50-10:05	**Ivana Vrhovac Madunić** (Croatia) Interaction of renal and hepatic organic cation transporters (OCT) with oximes
10:05-10:25	***Coffee break***
	Session II – New approaches in drug development (Chair: Antonio Zandona)
10:25-10:55	**Miguel Castanho** (Portugal) NOVIRUSES2BRAIN: Developing broad spectrum brain-targeting drugs against flaviviruses and other envelope viruses
10:55-11:25	**Vera Neves** (Portugal) Peptide shuttles for receptor independent transport across the BBB
11:25-11:45	**Nino Sinčić** (Croatia) Epigenetic biomarkers in liquid biopsies: a new concept in prostate cancer diagnostics
11:45-12:05	**Jelena Krstić** (Austria) Energy metabolism as a target in hepatocellular carcinoma
12:05-12:25	**Pierre-Yves Renard** (France) Photoaffinity labelling: expanding the scope of amenable reactions
12:30-13:40	***Lunch break***
	Session III – Colinesterase ligand-based drugs (Chair: Ivana Vrhovac Madunić)
13:40-13:55	**Tena Čadež** (Croatia) Development of new pseudocatalytic bioscavenger based on BChE and oxime complex as an effective treatment for organophosphorus intoxication
13:55-14:10	**Nikolina Maček Hrvat** (Croatia) Neuroprotection of mice exposed to nerve agent by acetylcholinesterase reactivator RS194B
14:10-14:25	**Anita Bosak** (Croatia) Development of Bioactive Molecules for Treatment of Alzheimer's Diseases
14:25-14:40	**Dajana Gašo Sokač** (Croatia) Synthesis of pyridinium salts novel analogues of vitamin B6 and B3
14:40-15:00	***Coffee break***
	Session IV – Biochemistry in drug related research (Chair: Ivana Vrhovac)
15:00-15:15	**Suzana Žunec** (Croatia) Metabolic stability as a challenge facing drug oriented research
15:15-15:30	**Anja Vidović** (Slovenia) AMPK and glucose modulate the expression of Na^+,K^+-ATPase subunits in cultured myotubes in an isoform-specific manner

15:30-15:45	**Morana Dulić** (Croatia) Aminoacyl-tRNA synthetases: versatile players in the kinetics, thermodynamics, molecular biology, and drug discovery playground
	Section V – Future plans, general discussion and closing remarks
15:45-16:00	**Nevenka Kopjar** (Croatia) Brief overview of the journal Arhiv za higijenu rada i toksikologiju – Archives of Industrial Hygiene and Toxicology
16:00-16:30	**Maja Katalinić** (Croatia) Where we are going from here?

Symposium

"Cell–Based Research in Toxicology and Drug Design"

The Symposium "Cell–Based Research in Toxicology and Drug Design" was held at the hotel Dubrovnik in Zagreb on January 26th, 2023, with the main objective to disseminate results obtained within the project HrZZ-UIP-05-7260 CellToxTargets and to discuss them with colleagues from different field of research. Organization of the Symposium was supported by the Croatian Science Foundation (grant no. HrZZ-UIP-2017-05-7260, CellToxTargets) and by the Institute for Medical Research and Occupational Health. Also, the organization was sponsored by companies Kefo ltd., Jasika ltd. and Medic ltd.

The Symposium brought together 31 participants including experienced scientists, each expert in a specific field (from kinetics to metabolism and cell-based research), along with the PhD students and postdoctoral fellows, as well as the representatives from the industry, who all presented their recent research achievements.

The scientific programme was divided into five sessions spanning topics from new approaches in drug development, cholinesterase ligand-based drugs and biochemistry in drug-related research, giving the participants floor for fruitful discussion and opening new possibilities for collaborations and ideas for future studies.

We were very pleased that we could host eminent scientists Prof Miguel Castanho and Dr Vera Neves from the Institute of Molecular Medicine Lisbon, Portugal, Prof Pierre-Yves Renard from the Normandie Univ, UNIROUEN, Rouen, France, and Dr Jelena Krstić from the Gottfried Schatz Research Center for Cell Signaling, Metabolism & Aging, Medical University of Graz, Austria, delivering four inspiring lectures presenting new and interesting findings in different areas of biochemistry and molecular biology.

The Symposium has been a rewarding scientific and personal experience for us, and we would like to express our sincere thanks to all of the speakers and participants for sharing their results, and contributing to the excellence of our scientific program. We thank Croatian Science Foundation and the Institute for Medical Research and Occupational Health for their support, and the sponsors whose contribution was invaluable to the overall success of the Symposium.

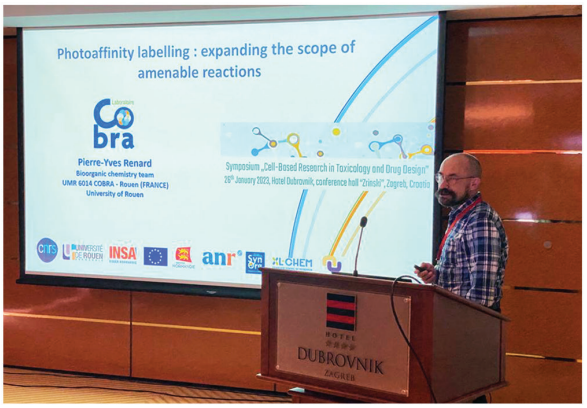
Maja Katalinić and Antonio Zandona
Organisers of the Symposium
HrZZ project 7260 CellToxTargets

Symposium – Cell-Based Research in Toxicology and Drug Design
Arh Hig Rada Toksikol 2023;74:A2-A19

SYMPOSIUM IN PICTURES


Where are we going from here?
HOTEL
DUBROVNIK
ZAGREB


Obrigado!
Thank you!
Hvala vam!
DUBROVNIK


Photoaffinity labelling : expanding the scope of amenable reactions
Pierre-Yves Renard
Bioorganic chemistry team
UMR 6014 COBRA - Rouen (FRANCE)
University of Rouen
Symposium „Cell-Based Research in Toxicology and Drug Design"
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DUBROVNIK


PEPTIDE SHUTTLES FOR RECEPTOR
IDEPENDENT TRANSPORT ACROSS THE BBB
2023-01-26
DUBROVNIK


Medical University of Graz
ENERGY METABOLISM AS A TARGET
IN HEPATOCELLULAR CARCINOMA
Ass.-Prof. Jelena Krstic, PhD
DUBROVNIK


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ABSTRACTS

INVITED SPEAKERS

Symposium – Cell-Based Research in Toxicology and Drug Design
Arh Hig Rada Toksikol 2023;74:A2-A19

Molecular mechanisms underlying the toxicity of antidotes and potential drugs – HRZZ-UIP-2017-05-7260

Maja Katalinić*

Biochemistry and Organic Analytical Chemistry Unit, Institute for Medical Research and Occupational Health, Zagreb, Croatia

This lecture will give a brief overview of the CellToxTargets project which started in 2018 bringing the new innovative approach in the field of antidote research for poisoning with the organophosphorus compounds (OP). The project goals were divided into four major outlines: 1. implementation of an adequate cell-based preclinical screening as a cell-based early safety evaluation in antidote discovery and research; 2. defining the antidote's effects on cell level and the mechanism of cell chemosensitivity to antidotes in OP poisoning; 3. defining the possible structural features/moieties of the tested compounds triggering certain effects; and 4. identifying new possible pharmacological targets on the cell level as well as explore their therapeutic potential. All obtained results proved that we have successfully reached all set goals. When looking at the statistical indicators, during the five project years, we have tested 148 compounds on 10 different cell types, and have implemented 12 new methods in the lab. Until now, overall results were published in 1 book chapter, 13 journal articles and 43 conference abstracts. The project topic has also been addressed in 3 doctoral theses and 4 diploma theses. During the project duration, we had 5 student practices and presented results to general public as well. Furthermore, we have established collaboration with several groups from Croatia as well as from Slovenia, Czech Republic, France and Portugal, helping us reaching our project goals. With the help of our colleagues, we were able to overcome several critical moments triggered by the COVID pandemic, the earthquake damage and the rebuilding of our main Institute. Taken all together, we have obtained a strong background giving us the needed input for further research and continuation of our study focusing on repurposing tested compounds for wider applications in the drug discovery as well.

This research was supported by the Croatian Science Foundation UIP-2017-05-7260.

Structure – cytotoxicity relationship of cholinesterase-based ligands

Antonio Zandona*

Biochemistry and Organic Analytical Chemistry Unit Institute for Medical Research and Occupational Health, Zagreb, Croatia

Oximes and new classes of compounds are being investigated as antidotes in the therapy of poisoning with organophosphorus compounds or as drugs for neurodegenerative diseases. In current research, the leading candidates, chosen solely on the basis of in vitro kinetic studies, are tested immediately on animal models *in vivo* where they often cause side effects or toxicity, which is why they are excluded from further testing. In this way, unnecessary tests on animals are carried out with a large consumption of resources, and valuable data on the effects at the cellular level are lost. A new approach to antidote/drug evaluation, based on cellular assays, would screen compounds with desirable characteristics for further development of new antidote/drug. In this way, the toxic effect can be examined at the cellular level and linked to specific structural elements of the compound, with the aim of defining guidelines for improving the structure of oxime in the early phase of research into their effectiveness as an antidote without conducting in vivo studies. By defining the relationship between the structure and cytotoxicity of the tested compounds and by determining the mechanism of action at the cellular level, new possible pharmacological targets can be determined, as well as the possibility of researching their therapeutic potential.

This research was supported by the Croatian Science Foundation UIP-2017-05-7260 and Slovenian Research Agency (J3-3065 to S.P. and J3-2523, P3-0043 and J7-3153 to S.P. and K.M), Croatian-Slovenian Bilateral grant 2020-2021 (BI-HR/20-21-041). Part of this work was supported through the EMBO short term fellowship STF-8731 to A. Zandona in M. Castanho lab (Lisabon, Portugal) and Foundation of the Croatian Academy of Sciences and Arts (10-102/414-254-2018 and 10-102/384-263-2020).

Characterization of lysophospholipase PNPLA7 as a potential target for drug development

Ana-Marija Lulić[1,*], Katarina Miš[2], Sergej Pirkmajer[2], Morana Dulić[3], Jovica Lončar[4], Maja Katalinić[1]

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[4] Laboratory for Molecular Ecotoxicology, Division for Marine and Environmental Research, Ruđer Bošković Institute, Zagreb, Croatia

Lysophospholipase PNPLA7 is a member of the PNPLA (*patatin-like phospholipase domain containing proteins*) family of nine enzymes sharing a patatin-like catalytic domain. It has been shown that PNPLA7 is highly expressed in the insulin targeted tissues where it can be associated with endoplasmic reticulum and lipid droplets, however, its physiological and molecular roles are not described well. Crystal structure of PNPLA7 is not known, but according to the sequence analysis and homology modelling, it consists of a transmembrane segment near the N-terminal end, three cyclic nucleotide binding sites, and patatin domain where the active site with catalytic dyad Ser-Asp is located. As the first aim of this study, we investigated the physiological role of this enzyme in the cultured human skeletal muscle cells. Our study confirms that human skeletal muscle cells express PNPLA7 mRNA and protein and that it is regulated by metabolic signals, implicating a role for PNPLA7 in skeletal muscle energy metabolism. Gene silencing of PNPLA7 in myoblasts reduced the phosphorylation of S6RP and p70, activators of protein translation, as well as the abundance of α1-subunit of Na^+,K^+-ATPase and acetyl-CoA carboxylase, indirectly suggesting that PNPLA7 is functionally important for these cells. As for the second objective of this study, we have tried to express a truncated PNPLA7 enzyme using *Escherichia coli* as a host. Our results showed that the majority of this enzyme remains in the membrane fractions hence, we are currently working on the optimization of the expression and purification conditions, in order to get the PNPLA7 for possible kinetic characterization.

This research was supported by the Croatian Science Foundation, grant number HrZZ-UIP-2017-05-7260, Slovenian Research Agency (J3-9263 and J3-2523, P3-0043 and J7-8276) and Croatian-Slovenian Bilateral grant 2020-2022 (BI-HR/20-21-041).

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Interaction of renal and hepatic organic cation transporters (OCT) with oximes

Ivana Vrhovac Madunić[1,*], Josip Madunić[2], Marleen Meyer Tönnies[3], Sarah Römer[3], Antonio Zandona[2], Mladen V. Tzvetkov[3], Maja Katalinić[2]

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Organophosphates are highly toxic derivatives of phosphoric acid used in industry and agriculture. As such, they represent an accessible and exceptional danger of poisoning both occupationally exposed workers, and the general population. Acute or chronic exposure to organophosphates can produce varying toxicity levels in humans. Oximes are reactivators of inhibited acetylcholinesterase, clinically used as antidotes in organophosphate poisoning. However, it is unknown how oximes enter and exit cells and whether there is a major accumulation in different human organs. Mostly, oximes either are constantly positively charged quaternary amines, or contain amino groups that could be strongly protonated under physiological conditions. Therefore, organic cation membrane transporters (OCTs) may play an essential role in the pharmacokinetics and organ accumulation of oximes. The aim of this project is to analyze whether oximes, both existing and newly synthesized, are transported via OCTs and MATEs (Multidrug and Toxin Extrusion). These membrane transporters are known to be important for the distribution of drugs in different organs relevant for drug metabolism and elimination. So far, we established LC-MS/MS method for precise quantification of relatively small amounts of known (HI-6 and 2-PAM) and newly synthesized (VII, X and Q5) oximes, which is essential for transport and inhibition experiments. The transporter-mediated uptake of oximes was determined for all five selected oximes at two concentrations in single transfected HEK293 cells overexpressing OCT1. Evaluation of potential role of the known organic cation transporters in the pharmacokinetics and drug-drug-interactions of oximes will open the door for better planning future *in vivo* experiments.

This work was supported by the Croatian Science Foundation (HrZZ-UIP-2017-05-7260) and Croatian-German Scientific Research Project funded by the Ministry of Science and Education of the Republic of Croatia and the German Academic Exchange Service (DAAD).

NOVIRUSES2BRAIN: developing broad spectrum brain-targeting drugs against flaviviruses and other envelope viruses

Miguel Castanho[*] (on behalf of the NOVIRUSES2BRAIN consortium)

Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Portugal

We are developing drugs able to inactivate Zika virus, Dengue virus, and HIV, among others, while having the ability to traverse the blood-brain barrier (BBB). This is deemed important mainly for Zika virus because, so far, there is no effective remedy for infection with this virus due to the limited ability of antiviral drugs to cross blood–placental barrier (BPB) and/or BBB. Chemically, the drugs consist on the conjugation of an antiviral porphyrin to a trans-BBB peptide. Proprietary trans-BBB peptides were obtained from templates based on domains of the capsid protein of Dengue virus.
The activity, toxicology and brain-targeting efficacy of a panel of conjugates were evaluated both *in vitro* and *in vivo*. One of the conjugates, named PP-P1, crossing both BPB and BBB, has shown to be effective against Zika Virus (IC_{50} 1.08 μM) and has high serum stability ($t_{1/2}$ ca. 22 h) without altering cell viability at all tested concentrations.
Project-associated references:

1. Bioconjugate Chem. 2021, 32, 6, 1067–1077; https://doi.org/10.1021/acs.bioconjchem.1c00123
2. Pharmaceutics 2022, 14(4), 738; https://doi.org/10.3390/pharmaceutics14040738

Acknowledgments/Funding: Work supported by the European Union (H2020-FETOPEN-2018-2019-2020-01 grant no 828774).

Peptide shuttles for receptor independent transport across the BBB

Vera Neves*

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The delivery of therapeutic molecules to the central nervous system remain difficult to translate into improved clinical outcomes. This is largely due to the blood–brain barrier (BBB), the most tightly regulated interface in the human body, which can exclude most therapeutics. Therefore, for brain delivery there is need to, either modify the drug to facilitate BBB crossing, or to stimulate BBB modification to allow the passage of the drug. Thus, several strategies have been proposed to achieve this goal that include: i) BBB manipulation or transient opening of tight junctions; ii) drug lipidization to increase passive transcellular diffusion; and iii) physiological approaches, such as carrier-mediated transport (CMT), receptor-mediated transport (RMT) and adsorptive-mediated transport (AMT). The focus of this presentation is the application of AMT for BBB crossing and delivery of drugs. The luminal side of the BBB has a negative charge that can trigger electrostatic interactions whenever a positively charged substance comes into contact with the plasma membrane surface. Thus, AMT can be used either by cationic drugs, or via conjugation of a drug to a positively charged moiety, such as BBB peptide shuttles (BBBpS). BBBpS are capable of BBB crossing without triggering barrier damage, they can be BBB specific and can carry large therapeutic molecules.

This work was supported from the European Union's Horizon 2020 research and innovation program under grant agreement No 952455, from "la Caixa" Foundation (ID 100010434), I.P under the agreement LCF/PR/HP21/52310015, and "Fundação para a Ciência e Tecnologia" (FCT, Portugal) (grant PTDC/BTM-MAT/2472/2021).

Epigenetic biomarkers in liquid biopsies: a new concept in prostate cancer diagnostics

Nino Sinčić[1,2,3,*], Monika Ulamec[1,3,4,5], Tomislav Kuliš[1,3,6], Irena Abramović[1,2,3], Jure Krasić[1,2,3], Lucija Škara[1,2,3], Ivan Pezelj[1,3,7]

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Prostate cancer (RP) is the second most common cancer in men in the global population. It is predicted that the number of men diagnosed with RP will double by 2040. Special challenge in RP diagnostics is the only widely used molecular biomarker, PSA, with its low clinical performaces. Indeed, histopathological analysis of prostate tissue phenotype is the only highly sensitive and specific although highly invasive method used today in clinical practice. Epigenetic changes are a dynamic system of reversible interactions of the environment with the genome without interfering with the DNA sequence. Knowledge of the epigenetics of RP has led to the development of molecular diagnostic tools currently all based on the analysis of prostate tissue. In order to identify new epigenetic biomarkers in liquid biopsies of blood and ejaculate with high sensitivity and specificity in discriminating patients with RP from patients with BHP, we conducted multidisciplinary research within the epiPro project (UIP-HRZZ). This study for the first time identified non-cellular DNA hypermethylation of LGALS3 gene in ejaculate and hyper-expression of miR-375-3p and miR-182-5p in blood in patients with RP compared to patients with BPH. A high degree of sensitivity and specificity in the discrimination of patients with RP compared to patients with BHP was also demonstrated. In conclusion, the listed epigenetic molecular biomarkers of RP show significantly higher sensitivity and specificity than PSA and have a high translational potential for use in diagnostic protocols for RP.

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Energy metabolism as a target in hepatocellular carcinoma

Jelena Krstic[1,*], Zina Riahi[1], Markus Galhuber[1], Helene Michentaler[1], Elisabeth Moyschewitz[1], Meritxell Huch[2], Corina Madreiter-Sokolowski[1], Roland Malli[1,3], and Andreas Prokesch[1,3]

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[2] Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
[3] BioTechMed-Graz, Graz, Austria

Drug resistance is closely intertwined with the metabolic rewiring of cancer cells, calling for novel metabolic combination therapies. We investigated whether simultaneous targeting of the main energy-producing pathways, aerobic glycolysis and oxidative phosphorylation (OxPhos), can be utilized as treatment for hepatocellular carcinoma (HCC). To target aerobic glycolysis, we reduced glucose availability *in vitro* by using glucose-deprived media. To target OxPhos, we used sorafenib, a standard-of-care drug for HCC, and additional OxPhos inhibitors. We show that *in vitro* starvation sensitizes resistant HCC-derived cells, as well as patient-derived organoids to sorafenib. Metabolic characterization shows that sorafenib acts as a potent inhibitor of mitochondrial respiration, causing resistant cells to switch to glycolysis for survival. Synergistic reduction of nutrients prevents this Warburg shift and leads to sorafenib sensitization. Functional experiments show that glucose is the limiting nutrient crucial for curtailing this metabolic flexibility. Furthermore, we show in several HCC cell lines and patient-derived HCC organoids that combining nutrient restriction with other OxPhos inhibitors increases their cytotoxicity, i.e., reduces their IC_{50} values. Together, our data suggest simultaneous targeting of main energy-producing pathways as a potential strategy to overcome resistance and improve the therapeutic efficacy in HCC.

Photoaffinity labelling: expanding the scope of amenable reactions

Pierre-Yves Renard[*], Madeleine Cauwel, Kevin Renault, Cyrille Sabot

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Photoaffinity labelling is one of the main strategies to discover in cellulo the molecular target of a bioactive compound. Yet, only few chemical photoresponsive entities yielding highly reactive moieties have been described and an additional moiety is required to identify and fish-out the photocross-linked products. Discovery of photoresponsive and profluorescent Quinoxalin-2(1H)-ones should allow expanding the scope of photoaffinity labelling amenable reactions.

Development of new pseudocatalytic bioscavenger based on BChE and oxime complex as an effective treatment for organophosphorus intoxication

Tena Čadež*, Zrinka Kovarik

Biochemistry and Organic Analytical Chemistry Unit, Institute for Medical Research and Occupational Health, Zagreb, Croatia

The ability of butyrylcholinesterase (BChE) to bind organophosphorus (OP) compounds lowering their concentration in the body and preventing the inhibition of acetylcholinesterase (AChE) represents a new basis for therapy in OP poisoning. As till now therapy for OP poisoning was based on reactivators of AChE, no effective oxime has yet been found for the reactivation of BChE. Through in silico and *in vitro* approaches, out of 123 compounds, we identified few pyridinium based oximes as the most effective reactivator of phosphorylated BChE, especially in the case of cyclosarin inhibition. Pseudocatalytic pair of this oximes and BChE in *ex vivo* conditions degraded a hundredfold excess of cyclosarin with high recovery of cholinesterase catalytic activity in short period of time. These results were in accordance with impact of tested complexes on neural protection, where pseudocatalytic pair acted by, protecting cells' homeostasis through preserving from 50% to nearly 100% of neural cells. Taken all together our findings offer a platform for further development of oximes and therapy in which effective BChE reactivators would enable pseudocatalytic degradation of OP toxins in the blood.

Acknowledgments: The Croatian Science Foundation (IP-2018-01-7683) supported this work.

Neuroprotection of mice exposed to nerve agent by acetylcholinesterase reactivator RS194

Nikolina Maček Hrvat[1,*], Katarina Ilić[2], Dora Kolić[1], Palmer Taylor[3], Svjetlana Kalanj Bognar[2], Zrinka Kovarik[1]

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Inhibition of the physiological function of acetylcholinesterase (AChE) by organophosphate (OP) molecules that readily cross the blood-brain barrier (BBB) promotes hypercholinergic activity which induces seizures leading to brain damage and neuroinflammation. OP-induced overstimulation of nicotinic and muscarinic membrane receptors in severe cases can lead to hypoxia, vasodepression, and respiratory arrest, followed by death. Currently approved therapy by permanently charged oxime compounds that don't cross BBB readily is inadequate for restoring the activity of synaptic AChE, leaving the brain vulnerable to long-term damage. We anticipate that the treatment with uncharged, but ionizable oximes that cross the BBB and reactivate OP-inhibited synaptic AChE will act protectively on the brain of mice exposed to an organophosphorus nerve agent. For that purpose, a microglial response, detected with IBA-1 protein, glial cells, detected with glial fibrillary acidic protein (GFAP), and neuronal cell viability, detected by the neuronal nuclei antigen NeuN, were all monitored in order to indicate the survival of neurons and neuroprotection by RS194B oxime in the brain of mice exposed to a nerve agent.

This research was supported by the HDTRA-19-1-006-UCSD-113020, HrZZ-IP-2018-01-7683, and HrZZ-IP-2016-06-8636.

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Development of Bioactive Molecules for Treatment of Alzheimer's Diseases

Anita Bosak[1,*], Ana Matošević[1], Ines Primožič[2], Dejan Opsenica[3], Katarina Komatović[3], Antonio Zandona[1], Marija Bartolić[1]

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[2] Faculty of Natural Science, Zagreb, Croatia
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The primary goal of the project is to develop molecules with the potential to alleviate the symptoms and slow down the progression of neurodegenerative diseases that primarily affect the neurons in the human brain that causes problems with movement and/or mental functioning. As the best results in restoration of cognitive functions of patients and alleviating the symptoms of the disease are done using drugs that targets cholinesterases (ChE), the project aims to rationally design dual site binding ChE inhibitors (acting on improving the acetylcholine level in the brain and on Aβ aggregation) and use them as starting points for multitarget-directed ligand (MTDL) design. We focused our study on butyrylcholinesterase (BChE) selective inhibitors due to the role of the BChE in the regulation of brain ACh levels in late AD and the fact that selective inhibition of BChE reduces the occurrence of side effects seen with the acetylcholinesterase (AChE) or nonselective ChE inhibitors currently in use. In design of potential bioactive molecules, we chosen two structural scaffolds, each with different mode of action with ChE's. A carbamate functionality was chosen due to the similarity of mechanism of their interaction with choliesterases with the mechanism of AChE hydrolysis of its physiological substrate ACh. Aminoquinoline, as a structural motive, gained our attention due to their similarity to tacrine, the first centrally acting cholinesterase inhibitor approved for the treatment of Alzheimer's disease.

This work was supported by the Croatian Science Foundation HrZZ-IP-2020-02-9343 and HrZZ-IP-2018-01-7683, and IMI-IP-2017-2.

Synthesis of pyridinium salts novel analogues of vitamin B6 and B3

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In recent years, research has focused on the synthesis of new analogs of vitamins B6 and B3 that can be used in human medicine. Overview of the synthesis of pyridoxal oxime, isonicotinamide and nicotinamide derivatives. Quaternary salts, oximes and dioximes are synthesized by conventional synthesis methods, but also by modern methods that include microwave synthesis, ultrasonic synthesis and synthesis in deep eutectic solvents. Modern methods shorten the reaction time, in most cases they have higher reaction yields. The previous syntheses opened the way for new modifications and functionalization of pyridine derivatives, and future research is focused on the synthesis of quaternary salts of hydrazone derivatives and *N*-acylhydrazone derivatives of vitamin B with potential biological activity.

This research was supported by the Croatian Science Foundation UIP-2017-05-7260 and UIP-2017-05-6593.

Metabolic stability as a challenge facing drug-oriented research

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Drug metabolism studies were previously performed at a late stage of drug development process and very often not until the phase of clinical studies. Therefore, inadequate metabolism and pharmacokinetic parameters frequently presented the major reason of failure for new chemical entities (NCEs). Nowadays, introduction of in vitro approaches into drug metabolism enables the characterization of the metabolic properties of lead candidates at early preclinical studies even in a small-scale academic laboratory. In the framework of the lecture, plans for new perspective testing based on determination of metabolic stability and biotransformation of NCEs with special emphasis on designed cholinesterase-acting compounds will be presented. Using the example of research on interactions between conventional cytostatic and cannabinoids, evidence will be provided to which extent metabolism research is important for better understanding of the biological (pharmacological or therapeutic and/or toxic) effect of a drug.

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AMPK and glucose modulate the expression of Na^+,K^+-ATPase subunits in cultured myotubes in an isoform-specific manner

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Skeletal muscles are not only an important metabolic organ, but also a major site of ion transport. Na^+,K^+-ATPase (NKA), a heterodimeric (α/β) ion pump which plays a key role in maintenance of muscle excitability and contractility, is adversely affected by metabolic dysregulation in diabetes mellitus, but the underlying mechanisms are largely unknown. Our aim was to establish whether expression of NKA subunits (α/β) or phospholemman (FXYD1), a small transmembrane protein that plays a major role in regulation of NKA, depends on AMP-activated protein kinase (AMPK), a cellular energy sensor, or glucose concentrations. Gene-silencing of the catalytic AMPKα1 and AMPKα2 subunits in rat L6 myotubes reduced the protein abundance of NKA α2, while it increased the protein abundance of NKA α1, suggesting AMPK likely regulates NKA α-subunits in an isoform specific manner. In cultured human myotubes, the expression of NKAα1 mRNA was lower after glucose deprivation than in media containing 1 g/L and 4.5 g/L glucose. Conversely, glucose deprivation increased the protein abundance of NKA α1 and NKA α2 subunits in rat L6 myotubes. Pharmacological activation of AMPK with AICAR and A-769662 reduced the levels of Fxyd1 mRNA, while glucose deprivation also suppressed the mRNA expression of Fxyd1, suggesting AMPK acts as a negative regulator of FXYD1. In summary, gene silencing of AMPKα1/α2, AMPK activators and alterations in glucose concentrations modulated the expression of NKA subunits in an isoform-specific manner. Collectively, the results highlight a link between energy metabolism and the expression of NKA subunits in skeletal muscle.

Aminoacyl-tRNA synthetases: versatile players in the kinetics, thermodynamics, molecular biology, and drug discovery playground

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Aminoacyl-tRNA synthetases (aaRS) are enzymes responsible for covalent attachment of amino acid to cognate tRNA, providing substrates for translation. They catalyze reaction in two steps. First, they activate amino acid with ATP to produce aminoacyl-adenylate. In the second step, the aminoacyl moiety is transferred to tRNA. Due to the large number of similar amino acids and tRNAs in the cell, aaRSs have to carefully select only the cognate ones. Despite many specific interactions achieved by the aaRSs, there are chemically similar amino acids among which aaRS cannot discriminate. Since overall accuracy of aaRS is crucial for faithful protein synthesis, they have evolved proofreading mechanisms; pretransfer editing occurring in the synthetic site and posttransfer editing in separate domain. In the Laboratory for Biochemistry we are using various molecular biology and biochemistry techniques to characterize these enzymes, both prokaryotic and eukaryotic ones. Steady state and presteady state kinetics of wild type and mutant enzymes, as well as thermodynamic measurements of affinity of aaRSs for various substrates or inhibitors, offers important insights into mechanisms of different reactions catalyzed by these enzymes, synthetic as well as editing. On the other hand, cell-based assays provide valuable information on the effect of their activities on the behavior of the cell, completing the picture about their function and importance.

Where are we going from here?

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Based on the results obtained within the CellToxTargets project, we have set the direction for continuation of the study addressing biological activity perspectives for designed reversible cholinesterase ligands. Namely, we will screen the wider activity of several defined sets of designed cholinesterases' ligands, that have shown previously in our study to affect cells beyond influencing cholinesterases. With this, we hypothesize identifying compounds with a potential to be developed into drugs for other important therapeutic areas such as diseases affecting multiple cell types (cancer, diabetes, neurodegenerative disorders, muscle diseases etc.). Firstly, we will focus our research on determining to what extent selected compounds alter activity of validated drug targets of choice (like monoamine oxidase, cytochrome enzymes, nicotinic acetylcholine receptor or glucose transporters). Secondly, we will look into the anticancer potential of selected compounds, in example, on prostate and breast cancer cells. Additionally, we will determine the metabolic stability and biotransformation of tested compounds to get the full profile of their potential to be developed into drugs. Within the scope of our future research, we will elucidate whether any of the tested compounds falls into the category of the so-called Pan-Assay Interference Compounds (PAINS), or chemical compounds that often give false positive results in high-throughput screens. Namely, PAINS more likely react nonspecifically with numerous biological targets rather than specifically affecting one desired target, misleading the research and conclusions on the overall biological activity.

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