CHEPON 2024

15th International Meeting on Cholinesterases
9th International Conference on Paraoxonases

Brdo pri Kranju, Slovenia
15 — 18 September, 2024

Organised by

UNIVERSITY OF LJUBLJANA | MF Faculty of Medicine

UNIVERSITY OF LJUBLJANA | FFA Faculty of Pharmacy

Programme and Book of Abstracts

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Edited by:
Anže Meden, Damijan Knez,
Aljoša Bavec, Marko Goličnik,
and Samo Mahnič-Kalamiza

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Scientific Committee

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Ljubljana, 2024

Foreword

It is a great pleasure to welcome you to the 15^{th} International Meeting on Cholinesterases and the 9^{th} International Conference on Paraoxonases at the Brdo Congress Centre in Slovenia, organized by the University of Ljubljana, Faculty of Medicine, and Faculty of Pharmacy. We are honored to host this meeting in Slovenia again after more than four decades, following the 2^{nd} International Meeting on Cholinesterases held nearby in Bled in 1983.

The program offers an excellent opportunity to present your scientific achievements on the most current topics in the field of both enzymes, strengthen collaborations with existing partners, and establish new connections with scientists from the region and around the world. Established researchers, those at the beginning of their scientific careers, as well as graduate and undergraduate students, will have the chance to present their research findings through oral presentations and poster sessions.

In addition to a dynamic scientific program, we encourage you to enjoy our social events. On the first evening, we will host a welcome party. On Tuesday afternoon, you are invited to a tour of Bled and Bled Castle, followed by a gala dinner at the Brdo Estate in the evening.

We are confident that the Brdo Congress Centre will meet your expectations, and you will enjoy its beautiful green surroundings. We wish you a productive and enjoyable meeting and a pleasant stay in Slovenia.

Sincerely yours,
The Organizing Committee

Information for Participants

Registration and Information Desk

Registration will take place at the registration desk in the lobby of the Elegans Hotel on Sunday, 15 September 2024, starting from 14:00 till 16:30, and in the lobby of the Brdo Congress Centre from 16:30 till 19:00. The certificate of attendance will be provided at the registration desk.

The information desk will be located in the lobby of the Brdo Congress Centre on the following days/hours:

Monday, 16 September 2024, 8:15 – 11:00
Tuesday, 17 September 2024, 8:15 – 11:00

Name Badges

All participants are kindly requested to wear badges at all times during the conference. The badges are necessary for the admission to all conference events (lectures, posters, welcome party, gala dinner, lunches and coffee breaks etc).

Oral presentations

Lectures will be held in the Grandis Hall of the Brdo Congress Centre. Speakers are kindly requested to deliver their presentations on an USB device to the computer technician in the Grandis Hall at least half an hour before the start of the session. Slides should be prepared in either MS PowerPoint or PDF format, and should come either together with any supporting materials (e.g. videos) in the same folder (alert the technical staff to copy those to the presenters' computer as well!) or should be embedded into the PowerPoint presentation so they will be at your disposal at the time of your presentation.

Technical assistance will be provided on-site to help you with the download/upload to provided laptop PCs with Windows OS, Office and Acrobate Reader software. Apple Mac computer users are asked to export their presentations to a PC-compatible format (recommended PDF files) to avoid compatibility issues. The use of presenters' own computers will not be allowed.

The projected screen will be in a wide, 16:9 (width:height) format. Please prepare your slides accordingly! All presenters are asked to respect the time limits when giving their talks.

Wi-Fi and computers with wire internet access will be available at the conference hall.

Poster presentations

There will be two poster sessions:

The Poster session I will take place on Monday, 16 September 2024 from 18:15 till 19:30.
The Poster session II will take place on Tuesday, 17 September 2024 from 11:00 till 12:15.
Complete list of posters with poster board numbers can be found further down in the Programme section of this Book.

Poster presenters are asked to mount their posters before 8:30 in the morning and remove them in the evening on the same day of the session. All presenters should look-up the numbers assigned to posters in the Poster Sessions and pin up the posters on the display boards with the corresponding number. The authors of posters are kindly asked to stand by posters for the duration of the session.

Poster format and dimensions

The recommended size/format for poster is the A0 print format, i.e. 841 x 1189 mm (width x height). Please consider using the recommended format, although you can also choose other size but up to a maximum size of 900 x 1300 mm. In any case, your poster must be oriented vertically.

Social Events

Social programme includes Welcome party in the lobby of Brdo Congress Centre, 15 September 2024, and Gala dinner on the Duck Island in the Brdo Park, 17 September 2024.

Bled half-day trip will be organized for the participants who show the interest on the registration form.

Committees

Scientific Committee
Aljoša Bavec, Ljubljana (Slovenia)
Marko Goličnik, Ljubljana (Slovenia)
Stanislav Gobec, Ljubljana (Slovenia)
Jure Stojan, Ljubljana (Slovenia)

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Jure Stojan, Ljubljana (Slovenia)

PROGRAMME

Plenary and other Distinguished Lectures

Oral Presentations

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Alexandre Champault, Julie Knoertzer, Armelle Rancillac, Grégory Dal Bo, Karine Thibault

15:40 OR-14 **Blood-brain barrier controlled-opening with ultrasound to improve neuroprotection after nerve agent exposure** 40
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16:45 OR-15 **Development of the post-exposure RS194B oxime against lethal sarin vapour and organophosphate insecticides in macaques** 42
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17:45 OR-18 **Attenuating organophosphate-induced neuroinflammation in mice by oxime therapy** 43
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17:55 OR-19 **Design and Evaluation of Acetylcholinesterase Reactivators: A study of reactive oxime- and peripheral site-moieties** 44
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Chair: Eugenio Vilanova

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Session: **Session VI - Translational research of inhibitors of cholinesterases and paraoxonases** **49**
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Poster Presentations

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Session: **Poster Session** **76**

PLENARY LECTURES' ABSTRACTS

Plenary and other Distinguished Lectures

Sunday - Opening Session
Sep 15, 18:00 - 19:30

PL-1

A single nucleotide polymorphism in the human AChE gene alters miR-608 levels, anxiety and inflammatory status

Hermona Soreq, Nimrod Madrer, Yonat Tzur, Adi Bar, Estelle Bennett, Alon Simchovitz, David Greenberg

The Hebrew University of Jerusalem, Israel

Introduction: The human AChE gene harbors a single nucleotide polymorphism (SNP), rs17228616, the impact of which remained incompletely understood. This SNP is located in an AChEmRNA region that is targeted by microRNA (miR)-608, which also targets transcripts coding for the pro-inflammatory interleukin-6 (IL6) and the CDC42 kinase. Therefore, impaired miR-608-AChE interactions could elevate AChE levels and enable increased targeting of CDC42 and IL6 by miR-608, altering the CDC42 and IL6-regulated physiological processes, including inflammation and stress.

Methods: We studied human carriers of rs17228616 and established transgenic mice carrying miR-608 flanked by its human intronic 250 nucleotides-long regions, which expressed human miR-608 in several tissues.

Results: Among 368 healthy people, carriers of the minor rs17228616 SNP allele presented elevated blood pressure and intensified anxiety, and chronically stressed soldier carriers of the SNP presented elevated amygdala activity. Nevertheless, among 98 aged ex-prisoners of war (ePW), SNP carriers consisted of a greater fraction than expected in the 26 non-PTSD ePW group. Moreover, studying the transcriptional regulation of miR-608 identified an active promoter located in the 150 nucleotides 5' to the pre-miR-608, that elevated miR-608 levels by 100-fold. Surprisingly, pulldown and mass spectrometry assays revealed that this 150 nucleotides-sequence interacted with the ribosomal protein L24 (RPL24), an interaction that inhibited miR-608 expression. Inversely, RPL24 depletion bidirectionally altered the levels of multiple miRs and transfer RNA fragments (tRFs) in both human and plant systems, demonstrating an active contribution by RPL24 to the regulation of diverse small non-coding RNAs.

Discussion: Our findings extend reports that Arabidopsis thaliana RPL24 binds short sequences 5' to pre-miRs and regulates their expression. Moreover, we show that RPL24's supervision of the biogenesis of small regulatory RNAs is pan-evolutionary, and shed new light on the traumatic impact of impaired cholinergic networks.

PL-2

PON1, as a common denominator of neurodegenerative and cardiovascular diseases: proactive player or static biomarker?

Carlo Cervellati, Raffealla Riccetti, Gianmarco Mola, Valentina Rosta, Alessandro Trentini

University of Ferrara, Italy

Paraoxonase 1 (PON1) is a multifunctional protein with pleiotropic proprieties. This calcium-dependent enzyme exhibiting lactonase and ester hydrolase activity that exert multiple defensive roles in the human body. In particular, PON1 is able to protect from damage due to lipid peroxidation damage, exacerbated inflammation and several types of endogenous and exogenous toxicants. This beneficial action can be exerted at systemic level since most of PON1 is carried by circulating high density lipoprotein (HDL). It is now widely recognized that PON1 is one of the main contributors of the anti-atherogenic proprieties of these particles.

It has also become increasingly apparent that atherosclerosis is one of the main risk factors for Alzheimer's disease (AD). Indeed, vascular pathology plays a relevant role in the etiology of AD by decreasing cerebral blood

and impairing brain amyloid beta (Aβ, the putative initial trigger of neurodegeneration) homeostasis. This potential pathogenic link accounts, at least in part, for the growing body of evidence showing that patients affected by AD have consistently lower blood (serum/plasma) PON1 activity compared to cognitively healthy controls. This 'peripheral scenario' may also reverberate in the brain. Indeed, recent studies on AD animal models suggest that PON1 may be transferred through HDL to central nervous system, where it seems to participate in AD pathogenesis. In conclusion, despite the lack of conclusive mechanistic evidence, PON1 may not be merely a static biomarker of vascular and neurological diseases, but a proactive player involved in their onset and progression.

Plenary and other Distinguished Lectures

Wednesday - Memorial Lecture
Sep 18, 16:00 - 16:30

PL-3

Memorial Lecture

Eugenio Vilanova

Plenary and other Distinguished Lectures

Wednesday - Closing Lecture
Sep 18, 16:30 - 17:15

PL-4

Structural Characterization of Orphan and Taxonomically Restricted Proteins

Israel Silman

Department of Brain Sciences, Weizmann Institute of Science, Israel

Until 20 years ago, it was believed that all proteins are derived from homologous proteins in organisms further down the evolutionary ladder. In a dramatic paradigm change, it is now accepted that all organisms contain 'orphan' proteins which are devoid of identifiable ancestors. In other cases, similar proteins occur in several related species, again without detectable ancestors. These are known as a taxonomically restricted. The de novo proteins are generated by expression of novel Open Reading Frames in non-coding DNA sequences. Since most research on Orphan and taxonomically restricted proteins has been performed by geneticists, only sparse structural data are available for them. Thus, only three crystal structures of orphans are deposited in the Protein Data Bank. We could identify seven additional proteins, with well-defined functions, which had been expressed and purified. They had undergone partial physicochemical characterization, but structural data were lacking. The development of AlphaFold revolutionized protein structure prediction. We utilized AlphaFold2, and two other algorithms, to predict the structures of the three proteins with available crystal structures, as well as the other seven. All three confirmed the crystal structures, two of which, interestingly, displayed novel folds. Two of the other proteins, which were predicted to be disordered based on their sequences, were predicted to be disordered by all three algorithms. The remaining five were predicted to be compact, with two exceptions for AlphaFold2. All three algorithms made similar and high-quality predictions for a large nematode protein. We conjecture that this is due to many homologs in its taxonomically restricted family, and to the fact that several non-related proteins have similar folds. Overall, orphan and taxonomically restricted proteins are often predicted to have compact 3D structures, sometimes with a novel fold that is a consequence of their novel sequences, which are associated with appearance of new biological functions.

ORAL PRESENTATIONS' ABSTRACTS

Session I - Structure and dynamics of cholinesterases, paraoxonases and phosphotriesterases

Monday - Morning Lectures
Sep 16, 8:30 - 10:20

KN-01

An Unusual α/β Hydrolase Fold Enzyme from an Antarctic Bacterium

Joel L. Sussman

Department of Chemical and Structural Biology, Weizmann Institute of Science, Israel

Keynote Lecture

We here report the discovery of a new type of bacterial carboxylesterase. GLase is produced by the psychrophilic bacterium, Glaciecola pallidula, which inhabits Antarctic sea-ice habitats. Catalytic bio-scavengers, e.g. bacterial phosphotriesterases (PTE) and mammalian paraoxonase1 (PON1), have been developed for the detoxification of organophosphate (OP) pesticides and chemical warfare nerve agents. All native OP hydrolases (OPH) are active toward the less toxic Rp (+) stereo-isomer of asymmetric OPs. Consequently, novel OPH variants with reversed stereo-selectivity and improved hydrolytic potency were developed using enhanced evolution and protein engineering approaches. GLase, is inhibited by OPs such as paraoxon and VX. Notably, following inhibition by (-)-VX or by (-)-EMP-MeCyC, the more toxic stereoisomers of these OPs, and by paraoxon, the enzyme recovers spontaneously within 60-90 minutes. Conversely, GLase inhibited by either (+)-VX or (+)-EMP-MeCyC does not recover after inhibition. Thus, it is the first known carboxylesterase that reactivates spontaneously after inhibition by the toxic isomers of anti-acetylcholinesterase phosphonates. GLase may serve as a structural template for developing new catalytic bio-scavengers of toxic OPs by directed evolution combined with protein engineering.

The uniqueness of the primary structures of GLase and its close homologs is that they include 6 highly conserved Cys residues out of a total of 9 Cys residues in GLase but with no S-S bonds. GLase was successfully cloned, expressed, and purified from E. coli. It was crystallized, and its X-ray structure was solved at 2.95 Å resolution, revealing a canonical α/β hydrolase fold.

OR-01

Phosphotriesterase catalyzed synthesis of chiral precursors to antiviral prodrugs

Frank Raushel

Texas A&M University, United States

Nucleoside analogs are among the most common medications given for the treatment of viral infections and cancers. The therapeutic effectiveness of nucleoside analogs can be dramatically improved by phosphorylation. The ProTide approach was developed at Cardiff University using a phosphorylated nucleoside that is masked by esterification with an amino acid and phenol forming a chiral phosphorus center. The FDA has approved Sofosbuvir, Tenofovir Alafenamide, and Remdesivir for use as a treatment for hepatitis C, HIV, and COVID-19, respectively. The biological activity of the ProTides depends, in part, on the phosphorus stereochemistry and thus it is imperative that efficient methods be developed for the chemical synthesis and isolation of diastereomerically pure ProTides. Chiral ProTides are often synthesized by direct displacement of a labile phenol (p-nitrophenol or pentafluorophenol) from a chiral phosphoramidate precursor with the appropriate nucleoside analog. The ability to produce these chiral products is thus dictated by the synthesis of the chiral phosphoramidate precursors. The enzyme phosphotriesterase (PTE) from Pseudomonas diminuta is well known for its high stereoselectivity and broad substrate profile. Screening PTE variants from enzyme evolution libraries enabled the

identification of variants of PTE that can stereoselectively hydrolyze chiral phosphoramidate precursors. The variant G60A-PTE exhibits a 165-fold preference for hydrolysis of the RP-isomer, while the variant In1W-PTE has a 1400-fold preference for hydrolysis of the SP-isomer. Using these variants of PTE, the SP- and RP-isomers of the ProTide precursors were isolated on a preparative scale with no detectable contamination of the opposite isomer. This approach enabled the chemo-enzymatic synthesis of the pure (RP)-diastereomer of Remdesivir. This work was supported in part by the National Institutes of Health.

OR-02

Disentangling the mechanism underlying the covalent MSF-AChE adduct formation and evolvement: mechanistic insights into an aged-like inactive complex susceptible to reactivation by a combination of nucleophiles

Jure Stojan[1], Alessandro Pesaresi[2], Anže Meden[3], *Doriano Lamba*[2]

[1]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

[2]Institute of Crystallography, National Research Council, Trieste Outstation, Trieste, Italy

[3]Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia

[4]Institute of Crystallography, National Research Council, Trieste Outstation, Trieste; Interuniversity Consortium "Biostructures and Biosystems National Institute", Roma, Italy

Chemical warfare nerve agents and pesticides, known as organophosphorus compounds inactivate cholinesterases (ChEs) by phosphorylating the serine hydroxyl group located at the active site of ChEs. Over the course of time, phosphorylation is followed by loss of an organophosphate-leaving group and the bond with ChEs becomes irreversible, a process known as aging. Differently, structurally related irreversible catalytic poisons bearing sulfur instead of phosphorus convert ChEs in its aged form only by covalently binding to the key catalytic serine.

Kinetic and crystallographic studies of the interaction between Torpedo californica acetylcholinesterase and a small organosulfonate, methanesulfonyl fluoride (MSF), indeed revealed irreversibly methylsulfonylated serine 200, to be isosteric with the bound aged sarin/soman analogues. The potent bulky reversible inhibitor 7-bis-tacrine (BTA) adopts, in the active site of the crystal structure of the MSF-enzyme adduct, a location and an orientation that closely resemble the one being found in the crystal structure of the BTA-enzyme complex. Remarkably, the presence of BTA accelerates the rate of methanesulfonylation by a factor of two. This unexpected result can be explained on the basis of two facts: i) the steric hindrance exerted by BTA to MSF in accessing the active site and ii) the acceleration of the MSF-enzyme adduct formation as a consequence of the lowering of the rotational and translational degrees of freedom in the proximity of the catalytic serine.

It is well known that pralidoxime alone or in the presence of the substrate acetylcholine cannot reactivate the active site serine of the TcAChE-MSF adduct. We show that the simultaneous presence of pralidoxime and the additional neutral oxime, 2-[(hydroxyimino)methyl]-1-methylimidazol, triggers the reactivation process of TcAChE within the hour timescale.

Overall, our results pave the way toward the likely use of a cocktail of distinctive oximes as a promising recipe for an effective and fast reactivation of aged cholinesterases.

OR-03

Acetylcholinesterase dynamics determine the potency and selectivity of inhibitors targeting disease-transmitting mosquitoes

Anna Linusson Jonsson[1], Rashmi Kumari[1], Cecilia Lindgren[1], Rajendra Kumari[1], Nina Forsgren[2], David Andersson[1], Fredrik Ekström[2]

[1]Umeå University, Sweden

[2]CBRN Defence and Security, Swedish Defence Re-

search Agency, Sweden

Vector control of mosquitoes with insecticides is an important tool for preventing the spread of mosquito-borne diseases including malaria, dengue, chikungunya, and Zika. New active ingredients for insecticides are urgently needed because existing active ingredients exhibit off-target toxicity and are subject to increasing resistance. We therefore aim to develop non-covalent inhibitors of the validated insecticidal target acetylcholinesterase 1 (AChE1) from mosquitoes.

In this study, we use molecular dynamics simulations to identify structural properties essential for the potency of reversible inhibitors targeting AChE1 from Anopheles gambiae (AgAChE1), the malaria-transmitting mosquito, and for selectivity relative to the vertebrate mus musculus AChE (mAChE). We show that the main collective motions of apo AgAChE1 and mAChE differ, with AgAChE1 exhibiting less dynamic movements. Opening and closing of the gorge, which regulates access to the catalytic triad, is enabled by different mechanisms in the two species, which could be linked to their differing amino acid sequences. Inhibitor binding reduced the overall magnitude of dynamics of AChE. In particular, more potent inhibitors reduced the flexibility of the Ω loop at the entrance of the gorge. The selectivity of inhibitors for AgAChE1 over mAChE derives from the positioning of the α-helix lining the binding gorge.

Our findings emphasize the need to consider dynamics when developing inhibitors targeting this enzyme and highlight factors needed to create potent and selective AgAChE1 inhibitors that could serve as active ingredients to combat disease-transmitting mosquitoes.

OR-04

Structure and dynamics of hAChE and oxime interactions in structure-based design of novel uncharged bis-oxime reactivators

Andrey Kovalevsky[1], Oksana Gerlits[2], Thibault Alle[3], Carlo Ballatore[3], Zoran Radic[3]

[1]Neutron Scattering Division, Oak Ridge National Laboratory, Oak Ridge, United States

[2]Department of Natural Sciences, Tennessee Wesleyan University, Athens, United States

[3]University of California San Diego, United States

We have recently solved nearly a dozen X-ray structures of human acetylcholinesterase (hAChE; EC 3.1.1.7) in complex with novel heterocyclic bis-oxime reactivators, including complexes with organophosphate (OP)-inhibited, inactive hAChE. Structures provide unique insights into dynamics of both protein and small molecule oxime interactions informative for structure-based design and optimization of uncharged nucleophilic bis-oximes. Our previous inelastic neutron scattering (INS) studies of native and OP-conjugated hAChE revealed the acyl pocket loop as a primary hAChE domain whose vibrational dynamics is affected by POX phosphorylation. We have now investigated effects of both uncharged heterocyclic oximes as a new class of CNS-active reactivators as well as pyridinium oxime MMB4 in terms of enzyme dynamics as inferred from a diversity of static conformations of the acyl-pocket loop in those structures. In addition, various conformations of new uncharged heterocyclic bis-oximes reveal that single attachment point to hAChE via central heterocycle allows for enhanced conformational flexibility of nucleophile-bearing arms critical in accessing the targeted phosphorus atom in the conjugated OP. We have shown in corresponding structures how non-productive oxime-bearing arm orientation could be straightened and converted into productive binding conformation in 1,4-bisoximes without shifting the central heterocyclic anchoring ring. Additionally, conformational flexibility of the nucleophile-bearing arm

of the single bis-oxime could be captured in both non-productive and reactivation-productive orientation in corresponding X-ray structures. Our new X-ray structures illustrate specific structural advantages of uncharged heterocyclic bis-oximes compared to pyridinium-based oximes while allowing us to learn about the protein dynamics of hAChE structural domains.

This research was supported by the Counter-ACT Program, NIH Office of the Director (OD), and the NINDS, [Grant Number 1R21NS120839-01A2].

Session II - Interactions of cholinesterases (AChE and BChE) with substrates, inhibitors and reactivators

Monday - Late morning Lectures
Sep 16, 10:45 - 12:35

KN-02

Evaluating cholinesterase's interactions with inhibitors and potent reactivators towards efficient treatment in organophosphate poisoning

Zrinka Kovarik, Dora Kolić, Tena Cadez, Goran Šinko, Nikolina Maček Hrvat

Institute for Medical Research and Occupational Health, Croatia

Keynote Lecture

The main action mechanism of organophosphorus compounds (OP) is the inhibition of acetylcholinesterase (AChE) that causes the accumulation of the neurotransmitter acetylcholine leading to the paralysis of cholinergic synaptic transmission. Although BChE is generally considered as having no natural physiological function, the most likely function for BChE is as backup for AChE and protection of synaptic AChE from man-made and naturally occurring poisons. Both enzymes should be reactivated by strong nucleophiles such as oximes to avoid severe health effects after exposure to OP. However, both inhibition and reactivation of both enzymes are fine-tuning chemical processes that depend on the structure of all reactants. Recently, we evaluated the inhibition of cholinesterase activity with OP herbicides. It is worth pointing out that herbicides - anilofos, bensulide and piperophos inhibit both cholinesterases through a network of non-covalent and covalent interactions, while butamifos inhibits only with covalent binding to the catalytic serine. These findings give insight into the potential toxic effects of herbicides in use. Our focus was also the reactivation of OP nerve agents-inhibited AChE with newly synthesized oximes that were proven to be more efficient reactivators than standard oximes. New OPs such as Novichoks urgently ask for an efficient medical countermeasure based on reactivators of both AChE and BChE.

Supported by the European Regional Development Fund (KK.01.1.1.02.0007), Next Generation EU (BioMolTox project), and Croatian Science Foundation (IP-2022-10-6685).

OR-05

Towards the fourth generation of effective uncharged bis-oxime reactivators of organophosphate-inhibited human AChE.

Andrey Kovalevsky[1], Thibault Alle[2], Oksana Gerlits[3], Nikolina Maček Hrvat[4], Carlo Ballatore[2], Zrinka Kovarik[4], ***Zoran Radic***[2]

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Guided by the X-ray structures of uncharged zwitterionic monoxime RS-194B in complex with native and OP-conjugated human acetylcholinesterase (hAChE; EC 3.1.1.7) and by in silico computational evaluation we developed the first generation of uncharged,

heterocyclic bis-oximes with superior in vitro reactivation, very good in vivo therapeutic efficacy in organophosphate (OP)-exposed mice, and with favorable pharmacokinetic (PK) properties. The X-ray structures of representative first-generation bis-oximes in complex with hAChE revealed opportunities for their structural improvement towards better steric fit into geometry of the active center gorge of hAChE that led to synthesis of the second generation heterocyclic bis-oximes. The in vitro reactivation potency of the second generation was, however, inferior to the bis-oximes of the first generation for reasons rationalized by their corresponding X-ray structures with hAChE. That led us to design the third generation of bis-oximes with specifically modified central heterocyclic core resulting in improved in vitro kinetics of reactivation of hAChE inhibited by OPs paraoxon, VX, sarin, cyclosarin and fenamiphos. PK properties of representative bis-oximes of the third generation appeared reasonably good and improved over the second generation. In expectation of more detailed in vivo therapeutic characterization of the third bis-oxime generation we are now developing a prototype fourth generation in silico for further improved nucleophilic attack geometry and bioavailability, towards achieving a goal of highly efficient centrally active reactivating antidotes against OP intoxication.

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OR-06

Development and therapeutic potential of uncharged cholinesterase reactivators

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Acetylcholinesterase (AChE), a critical enzyme in the Central Nervous System (CNS), breaks down a vital neurotransmitter, acetylcholine. Organophosphorus nerve agents (OPNAs) and pesticides irreversibly inhibit AChE, disrupting nerve communication and potentially leading to death if left untreated. Butyrylcholinesterase (BChE), another closely related enzyme, is crucial but has broader functions beyond nerve signaling. Unlike AChE, BChE is found mainly in blood plasma and is also inhibited by OPNAs. The French military's current treatment for OPNA exposure utilizes an auto-injector containing a methanesulfonate salt of 2-PAM to reactivate AChE, alongside atropine, an anticholinergic drug, and avizafone, a prodrug of diazepam designed to mitigate convulsions. However, this treatment struggles to reach the brain effectively (limited CNS bioavailability), only

works against a limited range of nerve agents (narrow spectrum of action), and doesn't always achieve optimal results. Uncharged reactivators for inhibited cholinesterases offer a promising alternative. Our research group has been developing and testing new generations of these molecules for over a decade. These reactivators aim to surpass current treatments by better accessing the brain, having a broader impact on various nerve agents, reactivating both AChE and BChE and potentially influencing nicotinic receptor activity. Our upcoming presentation will explore these advancements in uncharged reactivators and discuss the future of this field.

OR-07

Direct Determination of the hydrolysis of a poor substrate by human plasma

Jure Stojan

Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia

Plasma cholinesterase is non-specific for its substrates. Like butyryl(thio)choline, it can successfully hydrolyse also different other non-physiological esters which enter the blood stream as xenobiotics. Usually, their degradation is significantly slower then that of butyryl(thio)choline. One of such substances is the depolarizing skeletal muscle relaxant succinyldicholine, used mostly during emergency surgrery. When injected intravenously it is normally hydrolysed by plasma butyrylcholinesterse within a few minutes. However, under different pathologic states, like liver failiure, but also in the case of atypical enzyme the paralysis may last dangerously long. Since direct determination of kinetic parameters for the hydrolysis of succinyldicholine is very tricky, we suggest a simple method, based on its inhibition of butyrilthiocholine hydrolysis of plasma samples. In the first experiment the inhibition parameters are detrmined and in the second one, the remaining concentration of succinyldicholine is assesed from the degree of inhibition after different incubation times of plasma with an appropriate starting succinyldicholine concentration. In a subsequent third step, the final determination of the catalytic and Michaelis constant is obtained by the simultaneous fit of first two data sets together with the curve of time course of residual succinyldicholine concentration.

OR-08

Inhibition of human butyrylcholinesterase by A-series nerve agents, functional and structural characterization

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Institut de Recherche Biomédicale des Armées, France

Butyrylcholinesterase, a circulating homolog of acetylcholinesterase, is a crucial bioscavenger against nerve agents, particularly evident in prophylaxis but also for treatment against percutaneous exposure to persistent nerve agents like VX. In response to the emerging threat posed by a new class of nerve agents, known as Novichoks or A-series agents, the international authorities have revised the OPCW Schedule 1 table to strengthen proliferation control. These agents, characterized by their stability and potent inhibition of cholinesterases, have focused significant attention in the recent literature.

Our investigations focused on the inhibitory effects of three A-series agents, namely A-230, A-232, and A-234, on recombinant human butyrylcholinesterase (hBChE), determining their respective inhibition constants of the same order as VX, demonstrating the high bio-scavenging potential of hBChE for these agents. Both A-232 and A-234 possess alkoxy substituents similar to other nerve agents or pesticides. Once the agent is covalently bound to the active site serine of hBChE, the alkoxy substituent may undergo dealkylation over time, known as aging, resulting in a phosphylate anionic adduct that is refractory to the reactivation by oxime-based coun-

termeasures. Employing LC/MS and LC/MS2 techniques, we probed for potential aging in hBChE following inhibition by A-232 and A-234 and did not observe discernible dealkylation even after 11 days.

To elucidate the absence of aging, we determined the crystallographic structures of hBChE inhibited by the A-agents. The respective phosphyl-adducts on the catalytic serine residue exhibited similarities. The amidine substituent orients toward Trp82 within the choline-binding pocket. The phosphyl oxygen fits in the oxyanion hole while the respective alkoxy substituents, or methyl group in the case of A-230, are directed toward Trp231 within the acyl-binding pocket. Such a position of the alkoxy substituents away from the catalytic histidine, which plays a crucial role in the aging mechanisms, explains the absence of aging for A-232 and A234 in hBChE.

OR-09

Inhibition kinetics of cholinesterases from various species by the organophosphate CBDP in vitro

Gabriele Horn, Franz Worek

Bundeswehr Institute of Pharmacology and Toxicology, Germany

Introduction: An increasingly reported illness during air travel referred to as aerotoxic syndrome is accompanied by neurological symptoms and has been associated to the exposure to tri-ortho-cresyl phosphate (TOCP), which is a toxic component included in fumes escaping from the engine into the bleed air of the cabin (i.e. "fume event"). TOCP is converted in vivo to the metabolite 2-(2-cresyl)-4H-1,3,2-benzodioxaphosphorin-2-oxide (CBDP) by cytochrome P450. CBDP is a known inhibitor of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). In this in vitro study, species differences of the inhibitor CBDP were assessed, which are important for the design of animal model studies.

Methodology: The inhibition kinetics of human, Cynomolgus monkey, pig, mini pig, guinea pig, mouse, and rat AChE as well as BChE by CBDP were determined in the presence of substrate to obtain the bimolecular rate constants for the inhibition (ki).

Results: Human BChE was found to be most sensitive towards CBDP, indicating a ki value of 3.24 ×108 M- 1min- 1. The determination of the inhibition kinetics of human AChE by CBDP resulted in a ki value of 2.84 × 105 M- 1min- 1. In addition, markedly more pronounced inhibition rates of BChE from the species guinea pig, mini pig, pig, rat, Cynomolgus monkey, and mouse by CBDP were found as compared to those of AChE from the respective sources, indicating 2.0- to 89.6-fold higher ki values.

Conclusions: The inhibition kinetics of cholinesterases from various species by CBDP showed apparent species differences and a wide variation of the inhibition rate constants ki. From the results of the present study a Cynomolgus monkey model seems most appropriate with respect to the similarities in the CBDP inhibition kinetics and the plasma composition in comparison with humans.

Session III - Post exposure organo phosphates strategies and toxicology

Monday - Afternoon Lectures
Sep 16, 14:00 - 15:50

KN-03

Novichok class of organophosphorus compounds and the efficacy of current countermeasures against A-234

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[2]University of Defence, Czech Republic

Keynote Lecture

A-series agent belongs to a generation of nerve agents that were developed during the 1970s but got to wide public knowledge

just recently. The poisoning of a former Russian spy Sergei Skripal and his daughter in 2018 by A-agent representative A-234 led to the inclusion A-series agents into the Chemical Weapons Convention. Even though five years have already passed, there is still very little information on its chemical properties, biological activities, and treatment options with established antidotes. We experimentally assessed A-234 stability, determined its inhibitory and reactivation potential towards human acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). We also assessed its toxicity in rats and therapeutic effects of different antidotal approaches. The results of spontaneous A-234 hydrolysis confirmed its stability during the first 72 h. A-234 was found to be a potent inhibitor of both human ChEs (AChE IC50 = 0.101 ± 0.003 M and BChE IC50 = 0.036 ± 0.002 M), whereas the five marketed oximes have negligible reactivation ability toward A-234-inhibited HssAChE and HssBChE. The acute toxicity of A-234 is comparable to that of VX and in the context of therapy, atropine and diazepam effectively mitigate A-234 lethality. Even though oxime administration may induce minor improvements, selected oximes (HI-6 and methoxime) do not reactivate ChEs in vivo.

OR-10

How to increase bioavailability and follow effect of charged oximes in the central nervous system?

Kamil Musilek[1], David Malinak[1], Eliska Prchalova[1], Rudolf Andrys[1], Adam Skarka[1], Zbynek Heger[2], Zenon Starcuk[3]

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The cholinesterase reactivators (so called "oximes") are used as causal antidotes in case of organophosphorus intoxications. The clinically used oximes have mono- or double-charged molecules that are only poorly penetrating the blood-brain barrier (BBB), and thus they have limited impact on reactivation of brain acetylcholinesterase. For this reason, various strategies are being developed to overcome this drawback, i.e., uncharged reactivators, conjugation with molecules that have transporters in the BBB, bypassing the BBB through intranasal delivery, inhibition of BBB efflux transporters or nanoparticle drug delivery systems.

The nanoparticle drug delivery seems to be very promising approach that can be used even for double-charged oximes with low BBB penetrability. For this reason, double-charged oxime K027 was conjugated to BODIPY fragment and it was encapsulated into the human recombinant ferritin nanovehicles. The oxime or encapsulated oxime were found to be bioaccumulated primarily in liver and kidneys of mice, but some amount was distributed also to the brain, where it was detectable even after 24 h indicating better CNS bioaccumulation and tissue retention. In addition, oxime K027 conjugated to DOTA fragment was encapsulated into the human recombinant ferritin nanovehicles. This conjugate seems to allow real-time observation of oxime biodistribution in vivo via magnetic resonance imaging (MRI), i.e., the BBB penetration and bioaccumulation in mice. Most recently, monocharged pyridinium reactivators with excellent reactivation of OP-inhibited AChE were prepared and proved to enhanced brain reactivation of mice poisoned by VX agent. These approaches seem to be of benefit for crucial reactivation of acetylcholinesterase in CNS. This work was supported by Czech Science Foundation (no. GA22-14568S) and University of Hradec Kralove (Faculty of Science).

OR-11

Novel Strategies for Optimizing the Treatment of Organophosphate Poisoning in a Mouse Model

Marilène Trancart, Anne-Sophie Hanak, Karine Thibault, Grégory Dal Bo, Alexandre Champault, Méliati Madi, Gwladys Meesemaecker, ***André-Guilhem Calas***

Institut de Recherche Biomédicale des armées, France

Organophosphate compounds (OP), such as VX, used as chemical warfare nerve agents (CWNA) pose significant threats due to their impact on the vital physiological systems of the poisoned organism. A primary concern is the risk of respiratory failure resulting from cholinergic dysregulation following systemic cholinesterase (ChE) enzyme inhibition. Therefore, it is essential to have effective treatment. To treat OP poisoning promptly, several countries have developed self-injectable devices. These devices typically contain a competitive muscarinic receptor antagonist, atropine, a benzodiazepine to prevent convulsions, and an oxime to reactivate inhibited ChE. However, the efficacy of oximes varies depending on the OP, and their limited ability to cross the blood-brain barrier presents significant limitations.

To address the weaknesses of the current therapeutic system, we focused on three areas of research:

1) Assessment of the relevance of the No-Observed-Adverse-Effect-Level (NOAEL) dose as an optimized dose of oxime in the treatment of CWNA poisoning.

2) Combination of two oximes as a treatment for CWNA poisoning. A suitable combination could broaden the spectrum of therapeutic efficacy and protect both peripheral and central ChE.

3) Identification of the mechanisms causing respiratory failure during CWNA poisoning. We aimed to characterize respiratory pathophysiology in mice following subcutaneous sub-lethal VX exposure. Candidate compounds targeting cholinergic signaling pathways associated with the CWNA poisoning toxidrome or implicated in respiratory disorders with similar symptoms (such as asthma, opioid overdose, etc.) were evaluated. Their efficacy in preventing ventilatory abnormalities induced by VX exposure was assessed by dual-chamber plethysmography. Finally, 24-hour survival tests were conducted to confirm their therapeutic effectiveness against VX.

OR-12

Choline-O-acetyltransferase as a potential therapeutic target for nerve agent poisoning: unveiling an ion sensitive regulatory mechanism

Fredrik Ekström, Nina Forsgren, Frida Jonsson, Cecilia Engdahl, Tomas Bergström

Swedish Defence Research Agency, Sweden

This research focuses on developing antidotes to counteract the devastating effects of nerve agent intoxication, a pressing concern in warfare, terrorism, and targeted assassinations. We center our investigation on the enzyme Choline-O-acetyltransferase (ChAT), a critical component in neurotransmitter production and a promising drug target. Our study explores arylvinylpyridinium-based compounds, traditionally viewed as inhibitors, revealing them to be substrates in a coenzyme A-dependent hydrothiolation reaction that forms active inhibitors in-situ. This novel mechanism results in an adduct embedded within ChAT's active site tunnel, establishing significant interactions in a hydrophobic pocket near the choline binding site. These findings pave the way for the development of more potent and bioactive ChAT inhibitors, potentially useful for symptomatic treatment of nerve agent intoxications. Additionally, we unveil the discovery of a molecular switch responsive to ionic strength and regulatory phosphorylations that modulate ChAT's structure and dynamics. This discovery holds broad implications for understanding and manipulating the functions of the choline/carnitine enzyme

family across various diseases. Moreover, we highlight the critical role of X-ray synchrotron radiation in fragment-based screening. This advanced technique provides unique opportunities for discovering compounds targeting ChAT and other challenging drug targets, significantly enhancing drug discovery efforts.

OR-13

Investigation of neuroprotection conferred by a new therapeutic strategy after organophosphorus exposure

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Introduction:

Organophosphate (OP) nerve agent (NA) like VX are irreversible acetylcholinesterase (AChE) inhibitors. Acute NA exposure leads to a cholinergic syndrome, due to acetylcholine accumulation in synapses, manifested by fasciculation, tremors, muscle paralysis and a respiratory distress leading to death. Antidote treatment includes a muscarinic cholinergic receptor antagonist (atropine) and an oxime able to reactivate OP-inhibited AChE. In central nervous system, oximes action is limited due to their weak ability to cross the blood brain barrier (BBB). The goal of this study was to test therapeutic potential of a new oxime (RM048) designed to cross the BBB, alone or in association with another oxime, HI-6 unable to cross the BBB.

Methodology:

To evaluate the potential antidote effect of RM048, nine-week-old male Swiss mice exposed to a lethal dose of VX were used and received therapy containing atropine with either HI-6, RM048 or both oximes (Mix).

Results:

Using EEG recordings, we showed that RM048 alone and Mix treatments prevented the onset and recurrence of epileptic seizures. We demonstrated the beneficial effects of Mix treatment on the long-term behaviour of mice and its ability to attenuate neuroinflammation. These results showed that RM048 combined to HI-6, provided a better neuroprotection after VX exposure. Finally, patch-clamp recording on mouse brain slices showed that RM048 modulates neuronal activity by increasing the amplitude and the duration of hyperpolarization of pyramidal neurons.

Conclusion:

HI-6/RM048 association improved mice central and peripheral recovery after VX exposure at short and long term. Our study suggests that the use of RM048 is very promising for improving the medical care of NA exposures.

OR-14

Blood-brain barrier controlled-opening with ultrasound to improve neuroprotection after nerve agent exposure

Lucie Lépinard[1], Sarah Leterrier[2], Laurène Jourdain[2], Karine Thibault[1], Anthony Novell[2], *Grégory Dal Bo*[1]

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Introduction:

Organophosphate compounds (OP) found in the most toxic chemical warfare agents (such as VX), are also widely used as pesticides in several parts of the world. These highly lipophilic chemical compounds cross easily the blood brain barrier (BBB) and irreversibly inhibit cholinesterase (ChE), resulting in an increase in acetylcholine (ACh) concentration at cerebral and systemic levels. Acute exposure to OP causes an acute cholinergic toxidrome that can lead to death if not treated. The current antidote therapy combines a muscarinic receptor antagonist (atropine sulfate) to limit ACh signal, and oximes (pralidoxime or HI-6) to reactivate the inhibited ChE. However, these oximes are unable to cross the BBB and there-

fore cannot reactivate brain ChE, which can result in long-term neurological dysfunctions.

Methodology:

The objective of this study was to assess the potential of focalized ultrasound (FUS)-induced BBB opening to enhance the passage of oximes (pralidoxime or HI-6) into the brain and optimize neuroprotection in VX-exposed mice.

Results:

Our results showed a significant reactivation of cerebral ChE in mice exposed to a sublethal dose of VX when the therapy associated FUS and HI-6 compared to the animals that received HI-6 without FUS. Conversely, pralidoxime demonstrated no capacity to reactivate, regardless of the presence or absence of FUS. The beneficial effects of FUS + HI-6 were then evaluated in animals exposed to a lethal dose of VX. The results demonstrated that the animal recovery was significantly improved by the FUS/HI-6 association, with an AChE reactivation in the main cerebral structures and a significant reduction of neuroinflammation at different time points after the intoxication. Finally, we report improved 24h survival with FUS + HI-6 after lethal VX-exposure.

Conclusion:

The combination of FUS and oxime has the potential to significantly enhance the medical care of individuals exposed to OP.

Session IV - Cholinesterase pharmacology, inhibitors and antidotes

Monday - Late afternoon Lectures

Sep 16, 16:15 - 18:05

KN-04

Balancing central and peripheral nervous system antidotal actions of oxime reactivators to acetylcholinesterase inhbition

Palmer Taylor, Kwok-Yiu Ho, Zoran Radic

University of California San Diego, United States

Keynote Lecture

Achieving antidotal actions of oximes in the treatment of toxicity from irreversible cholinesterase inhibitors, such as the organophosphates (OPs), may well require separate dosing schedules depending on whether toxicity is due to acute or chronic exposures. These encounters may arise from longer term exposures to pesticides, an acute exposure from a larger dose of the toxic pesticide and from distinct modes of exposure. To investigate these differences, we have carried out systematic studies beginning with exposure to the particular organophosphate under consideration and subsequent treatment with the reactivating oxime. We have also distinguished the time intervals between exposure and oxime reactivator treatment. We began these studies with isolated and purified human cholinesterases, expressed in tissue culture cell after transfection of the human acetylcholinesterase gene sequence into mammalian cells. Such initial studies also require measurements of the extent and rate of inhibition. They were followed by studies in mice after parenteral injection or oral exposure to the organophosphate. Mice carry the advantages of purpose of the experiments to get a range of dosages of both the organophosphate and antidotal oxime, and estimates of duration of action related to re-synthesis active enzyme, as well as the initial pharmacokinetic parameters of the oxime. Also, mice carry the advantages of doing statistics on animal number and costs as well as the availability of genetic knock-out stains. However, as a small rodent species, they fall short of allowing studies to proceed to humans.

Our session is devoted dealing with the complexities of an antidotal reactivating agent, where both toxicity of the offending toxic organophosphate and the antidote should be considered in terms of pharmacokinetics and pharmacodynamics. Studies have been supported by the NIH, CounterACT program.

OR-15

Development of the post-exposure RS194B oxime against lethal sarin vapour and organophosphate insecticides in macaques

Yvonne Rosenberg[1], Dennis Sullivan[2], Zoran Radic[3], Palmer Taylor[3]

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Introduction: Deliberate nerve agent releases as well as occupational and self-inflicted insecticide exposures resulting in fatalities, emphasize the need for organophosphate (OP) countermeasures for both military and civilian populations. Therapeutic countermeasures against OP neurotoxins involve several strategies: (i) preventing OP poisoning through administering pre-exposure bioscavenger treatments that scavenge OPs before they inhibit their physiological AChE targets in the brain and in the periphery (ii) post-exposure oxime plus atropine that can rapidly reactivate OP-inhibited AChE and reduce severe symptoms or (iii) a combination of both.

Methodology: Macaques were exposed to lethal OP doses of sarin vapor (49.6ug/kg) and paraoxon (100ug/kg) by inhalation or orally to the phosphorothioate insecticides chlorpyrifos and parathion (50mg/kg). When clinical symptoms were severe, macaques were injected IM with 50-80mg/kg of a new low MW zwitterionic oxime RS194B (developed at UCSD) plus low-dose atropine and monitored clinically and for AChE and BChE activity.

Results: The simplified structure and neutral nature of post-exposure RS194B enabled rapid blood-brain barrier (BBB) passage and resulted in rapid reactivation of OP-inhibited RBC-AChE and circulating BChE with dramatic reversal both early and advanced clinical OP symptoms.

Conclusions: Centrally acting RS194B is currently the most efficacious post OP exposure treatment developed ; requiring only a single IM injection for rapid recovery and AChE and BChE reactivation in severely intoxicated macaques. RS194B can potentially be delivered to military and civilian personnel using an autoinjector favoured by the US military or more appropriately delivered orally as a pill in rural clinics and hospitals in the case of agricultural workers following insecticides due to the slower onset of symptoms.

OR-16

Detection of selective inhibitors of BChE in structurally and functionally different groups of molecules

Anita Bosak[1], Ana Matošević[1], Marija Bartolić[1], Ines Primožič[2], Alma Ramić[2], Xavier Brazzolotto[3], Florian Nachon[3], Nikola Maraković[1]

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Selective inhibition of butyrylcholinesterase (BChE) has proven to be a very promising direction in the development of drugs against Alzheimer's disease (AD) based on the hypothesis of cholinergic dysfunction. Although AD is one of the most common neurodegenerative disorders, accounting for about 60-80% of all dementia cases, its pathogenesis is complex and not fully understood. In recent years, therapies for AD primarily focused on beta-amyloid and tau have received more attention. However, various beta-amyloid- and tau-targeting agents have failed in clinical trials, leaving improvement of cholinergic neurotransmission, achieved mainly by inhibition of acetylcholinesterase (AChE), still the most effective therapy for AD. In the brain of healthy adults, AChE is responsible for 80% of the ACh activity, nearly 1013-fold more active than BChE. However, since during AD progression, AChE activity levels decline by up to 85% and the BChE/AChE ratio can change from 1:5 to 11:1, selective inhibition of BChE has emerged as a prudent strategy to elevate the ACh levels within the brain, improving

the cognitive and memorial functions of AD patients at mild and moderate stages. In our project to discover ChE inhibitors with additional action on other AD hallmarks, new selective BChE inhibitors were identified. Three groups of compounds with diverse structural and functional cores were evaluated: a group with carbamates structural similar to bambuterol, a prodrug of bronchodilator terbutaline, and two groups of ligands whose structure resemble that of tacrine (derivatives of 4-aminoquinolines), and compounds with oxime moiety (quinuclidinium-based O-alkyloximes). Interactions with amino acids from the BChE active site and structural features important for selective inhibition of BChE were analyzed by molecular docking, SAR analysis, and crystal structure of its complex with human BChE.

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OR-17

Bifunctional compounds serving as versatile cholinesterase reactivators

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Protection against the most toxic chemical weapons of mass destruction is still inadequate. Current causal antidotes are based on reactivation of acetylcholinesterase (AChE), which is covalently inhibited by these organophosphorus compounds. Most clinical and experimental reactivators consist of two parts. One part has an oxime group responsible for reactivation and the other part serves as an anchor to the enzyme. These properties are essential for sufficient antidotal efficacy. However, such molecule also acts as a "bipolar" agent and can have either a productive or non-productive orientation. In our work, we decided to prepare several series of hybrids with two reactivating moieties. These were designed to be active against both cholinesterases, AChE and butyrylcholinesterase. As a result, we also have an active ingredient that would be effective against a variety of organophosphate inhibitors. We used specific fragments known for their different selectivity and activity and followed different synthetic approaches to cover as many structural possibilities as possible.

We will discuss the synthesis of seven symmetric uncharged bis-oximes, eight asymmetric uncharged bis-oximes, and five asymmetric permanently charged compounds as reactivators to counteract organophosphorus poisoning. The structure-activity relationship is presented in detail, showing in vitro reactivation capabilities on both cholinesterases. We have also evaluated our compounds in human, mouse and rat enzymes. This has allowed us to better correlate in vivo experiments with potential human applications. In addition, our lead molecules were also evaluated for A-agents ("novichok") inhibition. Finally, we will present our latest in vivo data on several highlighted candidates, including MTD-estimated toxicity, pharmacokinetics, and pharmacodynamics in sarin and VX-poisoned mice. Our lead candidate has demonstrated superior efficacy over standard clinical antidotes both in vitro and in vivo according to our results.

OR-18

Attenuating organophosphate-induced neuroinflammation in mice by oxime therapy

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Therapy in case of organophosphate (OP) exposure requires the use of an oxime reactivator of OP-inhibited cholinesterases (ChE). However, not all approved oximes are equally effective in reactivating both ChE or, for different OPs. Additionally, they do not penetrate the blood-brain barrier (BBB) in adequate concentrations to reactivate synaptic acetylcholinesterase (AChE). Therefore, novel centrally active oximes such as RS194B have been developed to find a more effective therapy. Herein we investigated the effect of the therapy with the uncharged, but ionizable oxime RS194B that crosses the BBB and reactivates OP-inhibited synaptic AChE on attenuating the neuroinflammation in mice exposed to sarin. The levels of specific proteins expressed in glial and neuronal cells were determined in the cortex and diencephalon of sarin-exposed mice, mice treated with oxime RS194B or pyridinium oxime 2PAM after sarin exposure, and untreated control mice. Microglial response was detected with the level of ionized calcium-binding adapter molecule 1 (IBA-1), and astrogliosis with the glial fibrillary acidic protein (GFAP) level, whereas neuronal cell viability was determined following neuronal nuclei antigen (NeuN) immunoreactivity. The results indicate the neuroprotective potential of RS194B oxime, demonstrating that RS194B therapy in mice reduces sarin-induced neurotoxicity, particularly within 1.5 hours after sarin exposure.

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OR-19

Design and Evaluation of Acetylcholinesterase Reactivators: A study of reactive oxime- and peripheral site-moieties

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Organophosphorus nerve agents (OPNAs) and organophosphorus pesticides (OPPs) are toxic chemicals that covalently inhibit the neurotransmitter-regulator acetylcholinesterase (AChE), thereby causing a rapid shutdown of the nervous system and ultimately leading to death if not treated.

Current treatment of intoxications caused by OPNAs and OPPs include antidotes that engage in a chemical reaction with the phosphorus atom of the adduct of inhibited AChE, liberating a functional enzyme. Current clinical reactive antidotes, so-called reactivators, include pyridinium oximes such as 2-PAM, HI-6 and obidoxime. The efficiency of these reactivators is limited. They lack a broad-spectrum potency, meaning that they are not able to reactivate AChE independently of OPNA-species, and, due to their permanent charge, they have a low ability of penetrating the blood-brain barrier to reach AChE in the central nervous system.

In our lab we recently designed and evaluated a new reactivator candidate (1) that showed promising indications of broad spectrum activity. Candidate 1 was designed with an aromatic moiety that interacts with AChE's peripheral site (PS), a linker, and a reactive oxime moiety interacting with AChE's catalytic site. In this work we have used 1 as a starting point in a rational design of new reactivators and here we present the design and evaluation of these analogues. 26 compounds were designed, synthesised and biochemically characterised for their potential as reactivators against OPNA-inhibited hAChE.

The main findings from our work:

1) Our study highlights the effective application of classic medicinal chemistry principles, such as matched molecular series, in guiding the design of reactivators.

2) We found that alteration of the PS-

binding moieties exert distinct influence on the structure-activity relationship of AChE reactivators, and represent a critical building block for potency.

3) We have identified matched antidote pairs (i.e. neutral/charged) that allow us to investigate reactivator potency and distribution in animal treatment models.

Session V - Paraoxonase, butyrylcholinesterase and phosphotriesterase role in detoxication, biotechnology and diseases

Tuesday - Morning Lectures
Sep 17, 8:30 - 10:30

KN-05

The thin line between substrates or inhibitors: OP-enzymes interactions for biology, toxicity, diagnosis or applications.

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Keynote Lecture

Behind the classical simplified concepts of A-, and B-esterases that N Aldridge defined to discriminate the activity measured with p-nitrophenyl acetate that is inhibited by paraoxon or DFP (B) from that activity which is not sensitive (A), and later demonstrated to be able to hydrolyze paraoxon, there is a complex and diverse different mechanisms and consequences of the OP-interaction with proteins either as substrate or inhibitors. For example, in the transcriptome of glioblastoma cells, we found that about 11.000 genes are expressed, from which more than 2300 sites were detected with the consensus sequence for serin-esterases [G-x-S-x-G], and therefore potential candidates to be phosphorylated by OPS. For how many of them potential xenobiotic substrates or inhibitors have been identified? Other example: about 90% of the phenyl valerate esterase activity of the soluble fraction of chicken sciatic nerve a time progressive inhibition by paraoxon was observed by Estévez et al, and partly is due to BuChE, and simultaneously it is also time progressive reactivated, therefore paraoxon might be considered as a slow substrate of theses B-esterases. Have them a role in detoxication in situ (the target tissue of toxicity)? Similarly, M Sogorb described that serum albumin was able to hydrolyze some phosphoramidates and paraoxon by a mechanism of a transient phosphoryl-protein stage in the same catalytic site of the p-nitrophenyl butyrate, by a mechanism of type B-esterases. Moreover, Monroy et al have described that, in the presence of Cu or Zn, avian albumin hydrolyzes the same compounds at a faster rate, as an A-esterase. The complexity and diversity of potential interactions, either in their biological function or in artificial properties, open our interest of research and offer possibilities of development in diagnosis, therapy and biotechnological applications. Work partly supported by Asociación Biotox, Spain.

OR-20

The influence of single nucleotide polymorphisms and substrate type on individual enzyme-kinetic rate constants for human plasma PON1

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The kinetic parameters of human PON1 have seldom been investigated: only a handful of articles report Km and kcat values for the most common substrates, and a single article reports the influence of the rs662 (Q192R) polymorphism on Km. In a recent study, calculating kinetic parameters and determining genotype for blood plasma samples of 161 participants allowed us to additionally cla-

rify the relations between PON1 genotype and PON1 phenotype. For each plasma sample, we measured lactonase activity with dihydrocoumarin (DHC) and arylesterase activity with phenylacetate (PA), as well as PON1 concentration with ELISA and mass spectroscopy, and calculated Km, Vmax, kcat and kcat/Km for both substrates. We then used the the Kruskal-Wallis test to calculate correlations between genotype and phenotype, and calculated Pearson correlations between different kinetic parameters. We showed that the SNP rs854560 (L55M) also has an influence on Km for both substrates, although considerably weaker than that of rs662. kcat is also strongly influenced by rs662, but not influenced at all by rs854560. For both activities, we found that rs854560 influences the rate constant k1, i.e. the formation of the ES complex, but not k2, i.e. the breakdown of the complex into E and P. rs662 has the opposite effect, it influences breakdown into E and P (k2), but not ES complex formation (k1). We also acquired some insight about the differences between PA and DHC hydrolysis: k1 is more closely correlated between PA and DHC than k2, meaning that the ES complex formation stage is more similar for both substrates than breakdown into E and P. Previous similar studies on PON1 kinetics worked with enzyme from a handful of participants or a single individual; we demonstrate the usefulness of measuring kinetic parameters for each participant in larger clinical studies.

OR-21

Albumin-copper complex hydrolyses chiral organophosphate compounds as a true A-esterase

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O-hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) and other phosphoramidate analogs were designed to elucidate the neurotoxic acute effects of insecticide methamidophos. Studies in hens demonstrated their delayed neuropathic effect in hens by inhibition the neuropathy target esterase (NTE) in nerve tissues. Experimental assays with HDCP enantiomers showed that the R(+)-HDCP is the molecule that induces the inhibition and aging of NTE. However, calcium-dependent hydrolysis studies with domestic mammal serum including human sera showed that the S(+)-HDCP is the isomer hydrolyzed. The racemic HDCP and others chiral organophosphorus (OPs) have been a tool to identify new A-esterase activities in biological tissues. A first tests showed a copper-dependent HDCPase activity in chicken serum, which is around 20-fold higher than its calcium-dependent activity. This activity was stereoselective, opposite to the calcium-dependent activity observed in vertebrate tissues, for this reason, it was called "antagonistic stereoselectivity". Hydrolysis assays with different chiral OPs, divalent cations and metalloproteins allowed to identify the chicken (CSA) and turkey serum albumin (TSA) as the proteins responsible for this copper-dependent hydrolysis of HDCP and trichloronate (chiral OPs). Subsequent studies with animal serum albumins showed that the highest levels and stereoselectivity of this A-esterase are in the avian albumin (chicken, turkey and goat). While mammal serum albumins had low levels (10%) HDCPase activity and were non-stereoselective. Except for goat serum albumin (GSA), which hydrolyzes trichloronate at similar levels to TSA. The preincubation of CSA with aminoacid modifier compounds (DFP, DTNB, zinc, paraoxon, and others) and incubation with HDCP racemic plus copper in physiological conditions, and the species specificity, suggests the N-terminal sequence of avian albumin (DAEHK) as a required sequence, while the trichloronate hydrolysis by GSA and other recent computational and ex-

perimental data, suggest the catalytic participation of other site in the copper-dependent A-esterase activity of albumin.

OR-22

Special behavior of cholinesterase interactions among thiocholine and phenyl carboxyl ester as substrates and inhibitors: Implication for research applications.

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The kinetic studies of the competition between substrates supply relevant information about the interaction between phenylvalerate (PV) or phenylacetate (PA) and acetylthiocholine (AtCh) in cholinesterases, suggesting that other(s) site(s) different to the cholinesterase active site could be involved in the PVase and PA-esterase activities. In silico and kinetic experiments in butyrylcholinesterase show a site (PV-site) that interacts with PV and this is related to the Asn298 residue, which is far from the catalytic site. Furthermore, the thiocholine released at the active site of recombinant human acetylcholinesterase, cause alterations in PVase activity and PA-activity, where AtCh could act as a Trojan horse.

New studies show that the thiocoline interaction in the active site is permanent, suggesting differences between recombinant cholinesterases and purified cholinesterases from biological samples. Recombinant cholinesterases would interact with the acetylcholine or another substrate for the first time as nascent protein, however, the purified cholinesterases would interact as a veteran protein. These differences could have implications in the use of recombinant and purified cholinesterases in biotechnological applications.

OR-23

PON-1 arylesterase activity in older patients with mild cognitive impairment, late onset Alzheimer's disease or vascular dementia

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Background: A wealth of evidence suggests that paraoxonase-1 (PON-1) may confer protection against inflammation and oxidative stress, which might be involved in the pathogenesis of late onset Alzheimer's disease (AD) and vascular dementia (VAD), as well as of the prodromal phase of dementia, the so-called mild cognitive impairment (MCI). Here we extended previous findings with the aim of evaluating whether serum PON-1 arylesterase activity might be associated with dementia and/or MCI, and to observe possible correlations with other biological markers of inflammation and oxidative stress.

Methods: Serum PON-1 arylesterase activity was assessed in subjects with mild cognitive impairment (MCI, n = 511), Late-Onset Alzheimer's Disease (AD, n = 381), vascular dementia (VAD, n =100), mixed dementia AD-VAD (MIXED, n =153), other dementia subtypes (n = 30) and in older normal cognitive controls (n = 403).

Results: Compared to controls, arylesterase activity was significantly lower in MCI, LOAD, and MIXED ($p<0.01$ for all comparisons), whereas it was comparable in VAD and in other dementia subtypes. In the whole samples, there was significant and negative correlation between arylesterase activity and serum Myeloperoxidase (MPO) activity ($r= -0.100$, $p<0.013$), which was driven by Controls ($r=-0.171$, 0.016). In addition, we found a significant and negative correlation between arylesterase and serum homocysteine in MCI ($r=-0.161$, $p<0.007$) and MIXED group ($r=0.099$, $p<0.029$).

Conclusions: Overall, our results confirm that a decreased PON-1 arylesterase activity is an early feature of dementia-related diseases. Further longitudinal exploration of the role of this enzyme in the onset and progression of these disorders are required.

OR-24

Regulatory mechanism and functional impact of post-translational modifications on human paraoxonase 2

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The Human Paraoxonase (PON) family encompasses three highly conserved lactonases: PON1, PON2 and PON3, with PON2 exhibiting the highest lactonase activity [1].
Predominantly located on the plasma membrane, PON2 plays a critical role in innate immunity, serving as a primary defense against infections. Previous studies have highlighted several Post Translational modifications (PTMs) of PON2 including Glycosylation, Ubiquitination and ADP ribosylation which occur on opposite sides of the molecule. Notably, the rapid decrease in PON2 activity in extract from HeLa cells exposed to the bacterial quormone 3-Oxo-dodecanoyl Homoserine Lactone (3OC12HSL) has been attributed to a ubiquitination at lysine 144 [2].
Our recent analysis leveraging advanced Proteomic data analysis tools, have revised the previously identified ADP-ribosylation site [3] from D124 to R101 [4]. Site-directed mutagenesis, followed by western blot analysis confirmed R101 as the putative ADP-ribosylation site. Current investigations are focused on identifying the Poly ADP-Ribose Polymerases (PARPs) involved in PON2 ADP-ribosylation, with ART5 emerging as a potential interactor from interactome databases with its correct substrate specificity for
Arginine ADP-ribosylation [6]. Additionally, we are also examining the impact of ADP-ribosylation on PON2's lactonase and antioxidant activities in response to pyocyanin and H2O2 treatments. Furthermore, our research aims to elucidate the ubiquitination of PON2, identify the E3 ligases involved, and explore potential crosstalk between ADP-ribosylation and ubiquitination. Our findings are poised to significantly enhance the understanding of PON2 regulation and its multifaceted roles in cellular defense mechanisms. These novel insights may pave the way for novel therapeutic strategies targeting PON2 PTMs to modulate its activity in disease contexts.

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OR-25

Validation and application of two AChE-targeted environmental neurotoxic pollutants detection systems

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Introduction: Acetylcholinesterase (AChE) is an enzyme with catalytic activity for the hydrolysis of acetylcholine. It plays a crucial role in cholinergic neurotransmission, controlling motor function across various species, and regulates numerous advanced brain functions in mammals. AChE not only has important physiological functions, but it is also a target of public-concerned organophosphorus (OP) pesticides. Therefore, AChE has been used as a biomarker for monitoring the contamination of OP pesticides and screening out emerging contaminants with potential neurotoxic effects. Methodology: In this study, two ready-to-use enzymatic colorimetric assay systems were established and validated for electric eel AChE (eAChE) and human recombinant AChE (hAChE) to study the species differences of selected known AChE inhibitory compounds. We evaluated the repeatability and reproducibility of the system using BW 284c51. Using this detection system, the concentration-effect curves of phosalone, chlorpyrifos, fenamiphos, methamidophos and ethoprophos on eAChE and hAChE were plotted, and IC50 values were obtained and compared. Finally, eight dioxin-like (DL-) and non-dioxin (NDL-) polychlorinated biphenyls (PCBs) were screened for the effects of hAChE activity using the validated detection system. Results: The system has good repeatability and reproducibility. We obtained IC50 values for 5 OP pesticides and found subtle species differences in their inhibitory effects on eAChE and hAChE. We also screened out three NDL-PCBs with mild inhibition of hAChE including PCB52, PCB138, and PCB153 that can be considered as novel environmental AChE ligands for further neurotoxicology studies.Conclusions: We have established reliable eAChE and hAChE detection systems that have the potential to study species differences among AChE disruptors/ligands, and can be used to discover new AChE inhibitors or ligands in emerging environmental contaminants.

Session VI - Translational research of inhibitors of cholinesterases and paraoxonases

Wednesday - Morning Lectures
Sep 18, 8:30 - 10:20

KN-06

Pharmaceutical development of plasma butyrylcholinesterase: a breakthrough in the treatment of nerve agent intoxications

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Keynote Lecture

Organophosphorus nerve agents (OPNA) irreversibly inhibit cholinesterases, leading to an accumulation of acetylcholine and an acute cholinergic crisis. This can cause death if untreated. Therapies and prophylaxis based on pharmacological organic molecules exist but have various efficacy against the broad spectrum of OPNA. Decades of research in different animal models have shown that injectable human butyrylcholinesterase (BChE) can neutralize OPNA and prevent or limit endogenous cholinesterase inhibition. It also improves the fate of victims if used as a post-exposure treatment for OPNA with a delayed toxicokinetic profile. However, BChE has never been fielded to date. We decided to remedy this situation by developing plasma-derived

human BChE for the advanced treatment of OPNA intoxication.

The pharmaceutical development of this countermeasure embraces the GMP production of plasma BChE, preclinical safety and pharmacokinetics animal studies, preclinical efficacy studies to justify the therapeutic dose, and a Phase I clinical trial for safety assessment for granting a special-use authorization by the health authorities.

GMP batches of highly purified plasma hBChE at the g-scale were obtained from the Cohn fraction IV-4 of human plasma fractioning using a Hupresin™ affinity chromatography. The preclinical and efficacy studies were performed on Göttingen minipigs, a standard large animal model in OPNA intoxication and countermeasures studies. Evidence of effectiveness was shown against supralethal percutaneous doses of VX, with a 1-h delayed IV bolus of BChE associated with late RSDL® decontamination but without any additional pharmacological therapy. The clinical trial Phase I on human volunteers demonstrated the safety of the pharmaceutical product at the anticipated human therapeutic dose with a blood half-life of 10 days.

OR-26

Selective butyrylcholinesterase inhibition induces antidepressant, pro-cognitive, and antianhedonic effects in a genetic animal model of depression: Role of acetylcholine, ghrelin, and dopamine

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Introduction: Despite new treatments becoming available, major depressive disorder (MDD) poses significant challenges due to a delayed onset of action, adverse effects, and the limited overall effectiveness of current treatments. Addressing MDD-associated anhedonia and cognitive deficits are especially problematic. Consequently, innovative approaches and new neurobiological targets for antidepressants are needed. Pre-clinical findings describing the role of acetylcholine (ACh) and ghrelin in MDD have been promising. However, it is their interaction with one another and the role of butyrylcholinesterase (BChE) that warrants investigation. We studied the possible antidepressant-like behavioural and neurobiological effects of a novel butyrylcholinesterase inhibitor (BChEI) versus a reference antidepressant, the selective serotonin reuptake inhibitor (SSRI), escitalopram.

Methodology: Using a genetic rat model of MDD, the Flinders Sensitive Line rat, and doses of BChEI (30, 60mg/kg) and escitalopram (10, 20mg/kg), despair-related behaviour and locomotor activity, anhedonia, and cognitive function were assessed in the forced swim test (FST), open field test (OFT), sucrose preference test (SPT) and novel object recognition test (NORT), respectively. In addition, cortico-hippocampal levels of ACh, monoamines, brain derived neurotrophic factor (BDNF), as well as serum acyl and desacyl ghrelin, growth hormone, and BChE, were assayed using liquid chromatography-mass spectrometry (LC-MS) or enzyme-linked immunosorbent assay (ELISA).

Results: Both BChEI and escitalopram demonstrated significant and comparable antidepressant-like effects in the FST. Moreover, the BChEI significantly reduced anhedonic behaviour in the SPT and improved cognition in the NORT, surpassing that of escitalopram. BChEI-treated rats exhibited elevated acyl-to-desacyl ghrelin and ACh-to-choline ratios. These ratios positively correlated with antidepressant-like and cognitive-enhancing effects in a dose-dependent manner. Moreover, increased ghrelin positively correlated with increased dopamine concentrations, known for its association with reward and/or hedonic behaviour.

Conclusions: The investigated selective BChEI shows promising, dose-dependent antidepressant-like actions that involve ACh, ghrelin, and dopamine. It is especially its antianhedonic and pro-cognitive qualities that warrant investigation.

OR-27

Leveraging template effects of acetylcholinesterase for the development of new anti-Alzheimer drug candidates with multiple mechanisms

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The particular architecture of the catalytic gorge of acetylcholinesterase (AChE) and its composition, rich in aromatic residues, make that multisite inhibitors, designed to hit both the active and peripheral aromatic sites (CAS, PAS), are structurally well suited to hit other biological targets of interest for Alzheimer's disease (AD) treatment. They include soluble epoxide hydrolase (sEH), another enzyme with a large active site cavity, involved in neuroinflammation, and the aggregation of amyloidogenic proteins with key pathogenic roles in several neurodegenerative diseases.
Here we show the structure-activity and structure-DMPK property studies of a new class of compounds that were designed as multisite inhibitors of AChE and sEH. These compounds display very potent inhibitory activity on human AChE, sEH and butyrylcholinesterase (BChE) as well, with IC50 values in the nanomolar or subnanomolar range. They also inhibit the aggregation of β-amyloid peptide (Aβ42) and tau protein in intact Escherichia coli cells that overexpress these proteins, with potencies in the low micromolar range. TAR DNA-binding protein (TDP-43) is another aggregation-prone protein, which has a key pathogenic role in amyotrophic lateral sclerosis and frontotemporal dementia, and also in AD, in which it aggravates Aβ42 and tau pathologies. Interestingly, several compounds of this family have been found to inhibit the ethacrynic acid-induced aggregation of TDP-43 in SH-SY5Y cells and in the CL6049 strain of Caenorhabditis elegans, which overexpresses human TDP-43. A lead compound (ASP45) with very interesting activity profile, brain permeability, favorable aqueous solubility and microsomal stability, and devoid of neurotoxicity, has been selected for further preclinical studies.

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OR-28

A novel identification of anti-hypertensive drug spironolactone as an inhibitor of paraoxonase-2 lactonase activity

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Introduction: Paraoxonase 2 (PON2) is an antioxidant lactonase showing hydrolytic activity against 3-oxo-C12 homoserine lactone (HSL). Previous report demonstrated PON2 expressed in distal nephron in kidneys, where PON2 may play a role on blood pressure regulation through epithelial sodium channel (ENaC). Recently, we have discovered that PON2 in rat renal cortex is upregulated at sodium restricted condition in parallel with ENaC activation. These regulations are dependent on steroid hormone aldosterone since pretreatment with mineralocorticoid receptor antagonist spironolactone prevented these effects of sodium restriction (unpublished data). Since widely-used anti-hypertensive drug spironolactone contains a lactone residue, we hypothesized there might be a direct interaction between spironolactone and PON2 which may subsequently affects PON2 function.

Methodology: Human recombinant PON2 (rPON2) was expressed in E.coli and purified from inclusion bodies. PON2 kinetic parameters were measured using 3-oxo-C12 HSL as substrate in the presence of spironolactone as putative inhibitor. In order to integrate with kinetic data, molecular dockings using PON2 model with spironolactone and 3-oxo-C12 HSL were performed.

Results: Kinetic measurements utilizing purified rPON2 revealed that spironolactone inhibits PON2 lactonase activity. In the presence of 250 M spironolactone, Vmax of PON2 decreased by approximately 45 %, while KM showed 4-fold enhancement. These results indicate the inhibition of rPON2 activity by spironolactone may be a mixed type enzyme inhibition, with different binding of spironolactone to free PON2 with respect to PON2-substrate complex. Docking analyses demonstrated that binding sites for the inhibitor and substrate are partially overlapping in the proximity of active site, which may indicate a competition between two compounds.

Conclusions: Our study demonstrated that anti-hypertensive drug spironolactone inhibits PON2 lactonase activity presumably through binding near the active site of PON2. Further study is required to confirm the type of inhibition and unveil the role of PON2 in blood pressure regulation involving aldosterone, ENaC and spironolactone.

OR-29

Development of pleiotropic prodrugs to treat Alzheimer's disease: from conception to in vivo evaluation

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Introduction: Alzheimer's disease (AD) is a multifactorial disease which involved several pathogenic pathways. Some treatments slow the progression of AD, but the development of effective drugs remains a challenge. Thus, to develop news drugs against AD, the Multi-Target Directed Ligands (MTDLs) approach seems promising. In this context, the design of novels pleiotropic prodrugs, capable of inhibiting cholinesterases (AChE or BuChE) according to the same mechanism as rivastigmine and then releasing an active metabolite to modulate 5-HT4R, is a promising strategy to treat AD.

Methodology: Preliminary studies have led

to the discovery of an original benzisoxazole core with partial agonist activity on 5-HT4R. Subsequently, we synthesized a series of novel carbamylated prodrugs on this specific scaffold. Biological evaluations were performed on ChE, 5-HT4R and cellular model. Then, the modification of the carbamate substituents has enabled us to perform a first SAR study aiming to increase the selectivity on BuChE.

Results: Twelve carbamylated prodrugs were synthesized and tested. Some have shown interesting activity, in the nM order, selective on BuChE over AChE and 5-HT4R. The inhibition mechanism of cholinesterases and the effect of prodrugs on cellular model were evaluated. Permeability and druggability parameters were also determined to identify prodrugs administration route.

Conclusions: These studies provided initial structure-activity relationship (SAR) data for the three targets, enabling us to identify the most promising candidate for in vivo studies in mice through intranasal administration.

OR-30

The selective butyrylcholinesterase inhibitor UW-MD-95 shows symptomatic and neuroprotective effects in a pharmacological mouse model of Alzheimer's disease

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Alzheimer's disease (AD) is a devastating dementia characterized by extracellular amyloid-β (Aβ) protein aggregates and intracellular tau protein deposition. Clinically available drugs mainly target acetylcholinesterase (AChE) and indirectly sustain cholinergic neuronal tonus. Butyrylcholinesterase (BChE) also controls acetylcholine (ACh) turnover and is involved in the formation of Aβ aggregates and senile plaques. UW-MD-95 is a novel carbamate-based compound acting as a potent pseudo-irreversible BChE inhibitor, with high selectivity vs AChE, and showing promising protective potentials in AD. We characterized the neuroprotective activity of UW-MD-95 in mice treated intracerebroventricularly with oligomerized Aβ25-35 peptide using behavioral, biochemical and immunohistochemical approaches. When injected acutely 30 min before the behavioral tests (spontaneous alternation in the Y-maze, object recognition or passive avoidance), UW-MD-95 (0.3-3 mg/kg) showed anti-amnesic effects in Aβ25-35-treated mice. When injected once-a-day over 7 days, it prevented Aβ25-35-induced memory deficits. This effect was lost in BChE knockout mice. Moreover, the compound prevented Aβ25-35-induced oxidative stress (assessed by lipid peroxidation or cytochrome c release), neuroinflammation (IL-6 and TNF levels or GFAP and IBA1 immunoreactivity) in the hippocampus and cortex, and apoptosis (Bax level). Moreover, UW-MD-95 significantly reduced the increase in soluble Aβ1-42 level in the hippocampus induced by Aβ25-35. UW-MD-95 appeared as a potent neuroprotective compound in the Aβ25-35 model of AD, with potentially an impact on Aβ1-42 accumulation that could suggest a novel mechanism of neuroprotection.

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

Wednesday - Late morning Lectures
Sep 18, 10:45 - 12:35

KN-07

Multi-target-directed ligands for potential symptomatic and disease-modifying treatment of Alzheimer's disease

Svit Ferjančič Benetik, Damijan Knez, Urban Košak, Aleš Obreza, ***Stanislav Gobec***

University of Ljubljana, Faculty of Pharmacy, Slovenia

Keynote Lecture

The enzymatic activity of butyrylcholinesterase (BChE) in the brain increases with the progression of Alzheimer's disease, making BChE a promising target for the treatment of advanced stages of the disease. In addition to cholinergic hypofunction, neuroinflammation is a characteristic pathological change in Alzheimer's disease. However, the simultaneous targeting of both pathologies by a single molecule is not addressed by any of the drugs currently in use or in clinical trials, highlighting a critical gap in therapeutic approaches. We propose that the simultaneous targeting of BChE and mitogen-activated protein kinase p38α (p38α MAPK) by dual-acting small molecule inhibitors represents a novel therapeutic strategy to combat Alzheimer's disease. This hypothesis is based on a series of findings from cell and animal studies as well as in silico modelling showing that it is possible to target both enzymes simultaneously. The pro-inflammatory microglial response triggered by amyloid beta (Aβ) plaques leads to overactivation of p38α MAPK, which in turn enhances Aβ synthesis, hyperphosphorylation of tau and alteration of synaptic plasticity. Crucially, overactivation of microglia exacerbates neuroinflammation, which worsens along with cholinergic degeneration, eventually culminating in overall cognitive impairment. Examination of the binding sites of BChE and p38α MAPK revealed a structural similarity between the hinge and hydrophobic region I of p38α MAPK and the acyl-binding pocket and peripheral aromatic site of BChE. This formed the starting point for the development of dual BChE/p38α MAPK inhibitors using structure-based drug discovery approaches. Two series of multifunctional inhibitors will be presented that have the potential not only to alleviate the symptoms of Alzheimer's disease but also to address its underlying aetiology.

OR-31

Advancing neocopride: a preclinical candidate multitarget directed-ligand (MTDL) for neurodegenerative disease intervention

Christophe Rochais, Patrick Dallemagne

Université de Caen Normandie, Centre d'Etudes et de Recherche sur le Médicament de Normandie (CERMN), Caen, France

The targeting of multiple molecular causes implicated in the pathogenesis of Alzheimer's disease (AD) with a single drug is now regarded as a priority by numerous scientists. One strategy in polypharmacology is the development of "multi-target-directed ligands" (MTDLs), which have the potential to benefit multiple targets implicated in the complex AD. In this context, we have developed pioneering work in this field, which involved the modulation of a RS67,333, a reference 5-HT4R partial agonist, which possesses moderate acetylcholinesterase (AChE) inhibition properties. This resulted in the delivery of the first example of a dual agent, named Donecopride.

This lead compound has demonstrated both precognitive and antiamnesic effects in several animal models of AD. In the continuation of the study, we will present an undisclosed modulation that led to Neocopride, our preclinical candidate, which has been validated by a complete drug development plan.

OR-32

Cholinesterase-based inhibitors as multitarget small molecules for the therapy of Alzheimer's disease

José Marco Contelles[1], Francisco López-Muñoz[2]

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Introduction. Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory decline, affecting various cognitive domains such as attention, language comprehension, and problem-solving skills. Despite dedicated efforts to understand the causes of AD, there is still an urgent need to uncover the origin of this disease in detail and to advance the development of new therapeutic interventions.

Methodology. Based on the therapeutic strategy of "one molecule-multiple targets" for the development of drugs to treat multifactorial diseases such as AD, we have discovered Contilisant as a multifunctional ligand that inhibits cholinesterases (ChEs) increasing the level of neurotransmitters such acetylcholine, in clear deficit in the brain of AD patients.

Results. Contilisant has been investigated in vitro and in vivo in several biological targets involved in the progress and development of the disease, and whose pharmacological inhibition and/or modulation it is expected to produce satisfactory results in order to recover from the cognitive impairment, showing the following results:

hAChE (IC50 = 0.53 μM); hBuChE (IC50 = 1.69 μM);

hMAO-A (IC50 = 0.145 μM); hMAO-B (IC50 = 0.078 μM);

hH3R (antagonist), Ki = 10.8 nM;

hS1R (agonist) Ki = 65.2 nM)]

Conclusions. Contilisant is a neuroprotective, non-toxic, antioxidant, permeable ligand, able to cross the blood-brain-barrier, showing satisfactory in vitro pharmacological properties on selected biological targets (hChEs, hMAOs, hH3R, and hS1R) involved in the progress of Alzheimer's disease (AD), being able to restore the cognitive impairment in appropriate in in vivo AD animal models, comparing very favorably with donepezil, a drug in the clinics for AD patients tretament. Thus, these data suggest that Contilisant is a new "lead-compound" for AD therapy, ready to enter in the pre-clinical phase.

OR-33

Discovery of novel BChE inhibitors for cognitive improvement

Baichen Xiong, Yuanyuan Wang, Weiting Zhang, *Haopeng Sun*

School of Pharmacy, China Pharmaceutical University, Nanjing, China, China

Butyrylcholinesterase (BChE) has been considered as a potential therapeutic target for Alzheimer's disease (AD) because of its compensation capacity to hydrolyze acetylcholine and its close association with Aβ deposit. In our study, hierarchical virtual screening protocols were applied, and several kinds of potential BChE inhibitors with different skeletons were selected as a lead compounds. Based on the binding model of compounds and BChE protein predicted by computer simulation, activity and druggability optimization was conducted. Two classes of compounds, one containing benzimidazole-aminofurazan structures and the other N-benzyl benzamide structures, have been identified as candidate compounds due to their promising inhibitory activity and cyto-safety. Candidate compounds (S11-1014, hBChE IC50 = 0.08 nM; S11-1031, hBChE IC50 = 0.039 nM and S06-1064, hBChE IC50 = 45.2 nM) possessed blood–brain barrier penetrating ability, a long T1/2, and low intrinsic clearance. BChE inhibitors exhibited neuroprotective effects and the ability to improve cognition in APP/PS1 transgenic mouse

model, by benefiting cholinergic system, reducing the total Aβ amount and increasing the ghrelin content. Simultaneous modulation in the center and periphery greatly improves the efficiency of BChE inhibitors. Thus, candidate BChE inhibitors are promising compounds with drug-like properties for improving cognitive dysfunction, providing a potential strategy for the treatment of AD.

OR-34

Development of dual pharmacophore models of acetylcholinesterase and human soluble epoxide hydrolase based on local cosolvent molecules distribution

Weronika Bagrowska, Artur Góra
Tunneling Group, Biotechnology Centre, Silesian University of Technology, Poland

The complicated nature of Alzheimer's disease (AD) involves several pathways in which dysregulation can stimulate its progression. Modulation of multiple targets may provide benefits during therapy. The main cognitive impairment in AD patients results in a cholinergic deficit in the central nervous system. Acetylcholinesterase (AchE) is the main enzyme involved in cholinergic signalling through the hydrolysis of acetylcholine (Ach). Another problem is neuroinflammation, caused by high concentrations of pro-inflammatory cytokines, which gradually causes neuronal damage. This can be effectively reduced by epoxyeicosatrienoic acids (EETs) metabolised by human soluble epoxide hydrolase (hsEH). Increased levels of ACh may promote the metabolism of arachidonic acid to EETs, reinforcing anti-neuroinflammatory effect. For this reason, new drugs targeting both enzymes may prove more effective in treating the AD.
The search for new drugs targeting a specific molecular target often begins with the design of a pharmacophore model - matrix from which potential inhibitors are designed. Here, we aim to propose new pharmacophores models that are compatible for both enzymes. We used a novel technique using pharmacophore design with molecular probes. For both enzymes, we performed classical molecular dynamics simulations and simulations in a water-cosolvent system containing mixed solvents. The use of other molecules than water treated as specific molecular probes allowed us to predict the preferred location of functional groups with different physicochemical properties. Defining the points of highest probe entry density allowed the creation of a map of potential inhibitor-protein interactions, which combined between enzymes creates consistent pharmacophore model.
We gratefully acknowledge Poland's high-performance Infrastructure PLGrid (ACK Cyfronet AGH) for providing computer facilities and support within computational grants' no. PLG/2023/016344, PLG/2023/016484, PLG/2024/017171

OR-35

Pleiotropic prodrugs for both symptomatic and disease-modifying treatment of Alzheimer's disease

Anže Meden[1], Neža Žnidaršič[2], Damijan Knez[1], Yuanyuan Wang[3], Ziwei Xu[3], Huajing Yang[3], Weiting Zhang[3], Anja Pišlar[1], Andrej Perdih[1], Simona Kranjc Brezar[4], Neža Grgurevič[2], Stane Pajk[1], Haopeng Sun[3], Stanislav Gobec[1]
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[4]Department of Experimental Oncology, Institute of Oncology, Slovenia

Alzheimer's disease (AD) complexity and failed clinical trials have spiked the interest of the community in multifunctional ligands that target at least two key macromolecules in AD pathology. Here we report on a focused series of pleiotropic N-carbamoylazole prodrugs with dual mechanism of action. Pseudo-irreversible inhibition of the first therapeutic target, human butyrylcholinesterase (hBChE), enhances cholinergic transmission,

and thereby provides symptomatic treatment. Simultaneously, this represents metabolic activation that liberates a nanomolar selective α2-adrenergic antagonist atipamezole, which blocks pathological amyloid β (Aβ)-induced and noradrenaline-dependent activation of GSK3β that ultimately leads to hyperphosphorylation of tau, thus achieving a disease-modifying effect. Lead compound 8 demonstrated long-term pseudo-irreversible hBChE inhibition, metabolic activation in human plasma, blood-brain barrier permeability, and oral availability in mice. Multi-day in vivo treatment with 8 in an Aβ-induced AD murine model revealed a significant alleviation of cognitive deficit that was better or comparable to rivastigmine, the current drug of choice for AD therapy, and also a decrease in tau phosphorylation. This surpasses the symptomatic-only treatment with cholinesterase inhibitors, as it directly blocks an essential pathological cascade in AD. Therefore, these multifunctional α2-adrenergic antagonists–butyrylcholinesterase inhibitors, exemplified by lead compound 8, present a potentially revolutionary small molecule disease-modifying treatment for AD.

Session VIII - Functions of cholinesterases in different tissues

Wednesday - Afternoon Lectures
Sep 18, 14:00 - 16:00

KN-08

Acetylcholinesterase regulates inflammatory responses and pigmentation in skin epidermis

Karl Wah-Keung Tsim

The Hong Kong University of Science and Technology, Hong Kong

Keynote Lecture

The cholinergic system presented in skin is proposed as “skin synapse”, where the cholinergic traits are found in the epidermis consisting of melanocytes and keratinocytes, and acetylcholine (ACh) mediates signal transduction between the epidermal cells. Ultraviolet B (UVB) serves as a “switch” controlling the release of ACh from epidermal keratinocytes to trigger the event. The expression of acetylcholinesterase (AChE) in keratinocytes, both in vivo and in vitro models, was suppressed following exposure to UVB irradiation: this regulation was mediated by an upregulation of miR-132 and miR-212. Under the low level of AChE, i.e., AChE inhibition or AChE knockdown, the overflow of ACh led by UVB irradiation caused a promoted pro-inflammatory response in the skin epidermis, demonstrated by increased secretion of cytokines IL-1 and IL-6, as well as COX-2. The activation of α7 nicotinic ACh receptor (nAChR) in keratinocytes is responsible for the ACh-induced inflammatory response. In melanocytes, the challenge of ACh regulates the production of melanin, as well as the release of melanin synthesizing organelle melanosome, via M2/M4 muscarinic AChRs (mAChRs). In parallel, the activation of α7 nAChR in keratinocytes plays a role in regulating the uptake of released melanosome through phagocytic activity. This phenomenon was markedly enhanced by applying ACh, AChE inhibitor and α7 nAChR agonist. Besides, the intracellular Ca2+ mobilization in keratinocytes, triggered by UVB exposure, is attributed to initiation of phagocytosis, while the blockage of Ca2+ influx with BAPTA-AM, or α7 nAChR antagonist, terminates the event completely. The findings provide insights into the development of novel therapeutic strategies for photoprotection, tackling inflammation and hyper-pigmentation caused by UVB irradiation.

OR-36

Characterizing Cardiac and Vascular Cholinesterases: From Molecular Insights to Therapeutic Prospects

Dominika Dingová, Kristína Szmicseková, Matej Kučera, Lenka Bies Pivačková, Tibor Hodbod, Parsa Shafieikazerooni, Peter Křenek, Eric Krejci, ***Anna Hrabovska***

Faculty of Pharmacy, Comenius University Bratislava, Slovakia

Autonomic imbalance is a well-documented feature of cardiovascular diseases. For the past six decades, the adrenergic system became a significant pharmacological target. Recent studies in animal models and human patients have highlighted the beneficial effects of enhanced cholinergic signaling in the heart, prompting interest in cholinesterase (ChE) inhibitors. Our research aims to characterize ChE in the cardiovascular system. Through a series of biochemical, microscopic, physiological, and pharmacological experiments in rats and mutant mice lacking different ChE forms, we analyzed the molecular composition, localization, physiological functions, and pharmacological responses of ChE in the heart and blood vessels. Both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are present in the heart, with the highest activities in the sinoatrial and atrioventricular nodes. Their expression levels are similar in the atria, but BChE predominates in the ventricles. Only anchored AChE is present in the heart where it colocalizes with neurons. AChE participates in heart rate control and provides the bradycardic effect of ChE inhibitors. AChE expression in blood vessels is limited. BChE, detected as a precursor monomer in the heart, is localized intracellularly. BChE knockout mice exhibit a more pronounced bradycardic response to ChE inhibitors, prolonged recovery after muscarinic blockade, and reduced sensitivity to acetylcholine under adrenergic stimulation, indicating adaptations in acetylcholine release and muscarinic receptor signaling. In BChE knockout mice, basal hemodynamic parameters are preserved, but their response to adrenergic stimulation is diminished. In mice, aortic BChE expression is negligible; however, in rats, there is high BChE expression in rat aorta, primarily in the smooth muscle, possibly compensating for very low BChE activity in serum. BChE inhibition enhances acetylcholine-induced relaxation of rat isolated aorta. In conclusion, AChE mediates the bradycardic effects of cholinesterase inhibitors, while BChE plays a significant role in cholinergic signaling in the heart and blood vessels, potentially offering therapeutic benefits in cardiovascular pathologies like chronic heart failure. Supported by APVV-22-0541 and VEGA 1/0283/22.

OR-37

Mice with inactive cholinesterases: new tools to evaluate cholinergic and non-cholinergic functions of AChE

Eric Krejci

université Paris cité, CNRS centre Borelli, France

Organophosphate (OP) pesticides, nerve agents or drugs have dramatic immediate and long-term effects on various physiological functions (respiration, cardiovascular system, digestion, inflammation, brain functions (seizures)...). Inhibition of acetylcholinesterase (AChE) is a key target of these highly reactive molecules. Since AChE terminates synaptic transmission at the neuromuscular junction (NMJ) as well as at other cholinergic synapses in the central and autonomic nervous systems, the canonical explanation for these effects is related to an excess of ACh at the synapses. AChE KO mice survive because butyrylcholinesterase (BChE) compensates for AChE, presumably because BChE hydrolyses acetylcholine. However, BChE is not localized in the synaptic cleft. For example, BChE is abundant at the mouse NMJ, anchored by PRiMA to the surface of the terminal Schwann cells, where BChE limits the activation of an $\alpha 7$ receptor signaling pathway that reduces

ACh release. Thus, inhibition of BChE reduces ACh release while toxicity results from more ACh or, if not, from unrelated effects of the inhibitors. To know whether the toxicity is related to an excess of endogenous ACh (cholinergic function) or to non-enzymatic functions of the protein (non-cholinergic function), we generated mice with a point mutation in the AChE or BChE gene. As a result, two mutants, WAChE and SBChE mice, produce AChE and BChE proteins, respectively, that are correctly localized but inactive. We used the growth of the mice as a marker of toxicity. I will show how different combinations of mutations affect the growth and viability of the mice, supporting a severe toxicity of ACh during postnatal development. Unexpectedly, these genetic approaches reveal that skeletal muscle is a major source of toxic ACh. I will discuss how the hydrolysis of ACh by AChE and BChE may explain the pleiotropic effects of ChE inhibitors.

OR-38

New perspectives on the effects of dioxin-like pollutants on acetylcholinesterase

Heidi QH Xie, Guanglei Yang, Ruihong Zhu, Jiahui An, Yangsheng Chen, Li Xu, Bin Zhao

Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, China

Introduction: Acetylcholinesterase (AChE, EC3.1.1.7) plays an important role in cholinergic neurotransmission and has been widely recognized as a biomarker for monitoring organophosphate and carbamate pesticide contamination and poisoning. Dioxins are newly discovered AChE environmental disruptors. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is representative of this group of compounds and inhibits AChE activity by disrupting its expression in cultured neurons and muscles. The role of aryl hydrocarbon receptor (AhR)-dependent pathway has diverse and species-specific roles in the suppression of AChE expression by TCDD. In human-derived neuroblastoma cells, AhR mediates transcriptional and post-transcriptional regulation of AChE by TCDD. Whereas, in rodent-derived models, AhR-independent mechanisms are involved in TCDD-induced AChE dysregulation.

Methodology: C2C12 is a well-established mouse model for myogenic differentiation studies and is a sensitive system for assessing the effects of dioxins on AChE. In light of the growing global concern about the health impacts of emerging pollutants, we employ the C2C12 model to investigate the potential interference effects of several emerging dioxin-like pollutants on AChE.

Results: We found that certain brominated dioxins and polyhalogenated carbazoles (PHCZs) were able to downregulate AChE gene expression, that was in parallel with their inhibitory effect on myogenous differentiation in C2C12 cells. Similar to classical dioxins, AChE dysregulations caused by these emerging dioxin-like pollutants are mediated by AhR-independent mechanisms.

Conclusions: The emerging dioxin-like pollutants are new AChE disruptors, which act by interfering with the expression of the AChE genes.

OR-39

The cholinergic enigma of pigmented and Müller glial cells

Paul G. Layer[1], Gesine Bachmann[1], Alex Bausch[1], Nicola Coronato[1], Gopenath Thangaraj[2]

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Cholinergic mechanisms are not restricted to neural functions. Using in vivo and in vitro models, the developmental appearance of cholinergic components in vertebrate eye structures allows to analyse cholinergic functions both in a synaptic and non-synaptic context. For proper development of the inner retina, a particular cholinergic amacrine cell type together with radial glial cells (Müller cells, MCs) is crucial. Less well understood are possible cholinergic interac-

tions of the outer retina, i.e., at the interface of photoreceptors (PRs) and retinal pigmented epithelium (RPE). Our retinal organoid and explant work has established that retinal lamina and network formation depends both on RPE and on MCs (or, growth factors derived from them). But what does „cholinergic" mean within the triad PRs, RPE and MCs? Literature data have shown that ACh is produced in, and released from PRs, while functional α7-nAChRs located on the apical surface of RPE cells receive the ACh signal. Furthermore, MCs of adult mice retinae provide a source for adult mammalian retinal regeneration (tradit. thought impossible). Notably, these MCs are triggered by cholinergic communication via α7-nAChR onto RPE cells and from there back onto MCs. Thus similar to cancer cells, MCs have preserved some transitory state (as indicated by their BChE expression), which - via cholinergic actions - can be (re)activated to turn into proliferative progenitor cells. The cholinergic enigma at the retinal-pigmented-glial triad bears eminent biomedical relevance, by far not restricted to eye structures (e.g., cancer biology, tissue regeneration and engineering, etc.). How cholinergic stimulation of MCs leads them to divide, is at the heart of stem cell biology.

OR-40

Acetylcholinesterase Reliefs Beta-Amyloid Plaque Burden via Enhancing Glial Activation in the Brain of 5×FAD Mouse

Yingjie Xia, Xiaoyang Wang, Maggie Suisui Guo, Ran Duan, Tingxia Dong, Karl Wah-Keung Tsim
The Hong Kong University of Science and Technology, Hong Kong

Acetylcholinesterase (AChE) has been reported to have additional functions in neuroinflammation, i.e., AChE regulates inflammatory responses via being involved in cholinergic anti-inflammatory pathway (CAP). In our previous in vitro study, AChE was upregulated in LPS-induced microglia/macrophage, and contrarily potentiated the inflammatory responses. However, how AChE-regulated neuroinflammation affects the pathology of Alzheimer's disease (AD) is still unclear. Here, we applied two mouse models, conditional AChE knock-in mouse (cKI), in which AChE is specifically overexpressed in myeloid cell linkage, and 5×FAD mouse. By using the cKI mice, the LPS-induced neuroinflammation, including the expressions of proinflammatory cytokines and the activation of both microglia and astrocytes, was aggravated in the brain of mice having overexpression of AChE. Transcriptomics analysis confirm the severer inflammation in the AChE overexpressing mice than the wildtypes after LPS administration. Subsequently, the effect of AChE overexpression on AD pathology was explored by crossbreeding the AChE cKI mouse with 5×FAD mouse. Amyloid beta (Aβ) plaque is one of the hallmarks of AD, which is co-localized with activated glial cells in the brain. Surprisingly, the AChE overexpression significantly relieved the Aβ burden in the brain of AD mice. Microglia and astrocytes are primarily responsible for Aβ clearance in the AD brain. The Aβ plaques were closely surrounded by activated glial cells and were reduced significantly in the AChE cKI mice, as compared with wildtypes. Notably, there were more activated glial cells, i.e., astrocytes and microglia, around Aβ aggregates in the AChE cKI mice. Thus, the high expression of AChE could relief Aβ plaque burden via stimulating the neuroinflammation and subsequently enhancing the glial activation in the brain of AD mice.

POSTER PRESENTATIONS' ABSTRACTS

Poster Session

Monday - Poster Session
Sep 16, 18:05 - 19:30

PO-01

Target-guided synthesis of novel butyrylcholinesterase inhibitors

Ines Primožič, Alma Ramić, Toni Divjak, Tomica Hrenar

University of Zagreb, Faculty of Science, Croatia

In this work, target-guided synthesis of compounds was combined with quantum chemical methods and extensive machine learning protocol to find new inhibitors of cholinesterases for possible use in the treatment of Alzheimer's disease. Compounds with chemical scaffolds based on α-acylamino amides were prepared by multicomponent reaction to obtain novel inhibitors of butyrylcholinesterase (BChE). Several different ketones (acetone, carbonyldiimidazole) and/or aldehydes (formaldehyde, imidazole-2-carbaldehyde), amines (benzamine, isobutylamine), isocyanides (tert-butyl, para-toluenesulfonylmethyl) and carboxylic acids (acetic, benzoic) were chosen as components. Compounds were prepared in a conventional manner and reaction was conduct in the presence of the enzyme for in situ selection of reaction components (as a proof-of-principle). The impact of substituents on the inhibition potency was analyzed using the quantum chemical flexible simultaneous multiple ligands docking scheme. Using the docking simulations, characterization of optimal binding modes within the binding site and estimation of standard Gibbs binding energies were determined for all synthesized compounds. The relationship between measured binding affinities (IC50 values) and theoretical data was established using machine learning multivariate linear regression. Experimentally obtained inhibition data were regressed on theoretically calculated potential energy surfaces sampled from the ab initio molecular dynamics. The best possible regression models for BChE inhibition/theoretical data based on various statistical parameters will be presented and utilized for future smart design of new inhibitors based only on the in silico simulations. ACKNOWLEDGEMENTS: This work was financially supported by the Croatian Science Foundation (Grant IP-2022-10-9525).

PO-02

Capitalizing on human BChE-ligand complex structures for the design of BChE-specific reactivator against nerve agent intoxication

Damijan Knez[1], Masa Zorman[1], Anne-Julie Gastellier[2], Charlotte Courageux[2], Janek Bzdrenga[2], José Dias[2], ***Xavier Brazzolotto***[2]

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Among the potential treatment strategies studied against nerve agent poisoning, the use of a nerve agent bioscavenger in combination with a specific reactivator - i.e., the pseudo- catalytic bioscavenger strategy, should provide the best of both worlds, by reducing the quantity of the expensive bioscavenger. To this aim, determining multiple structures of cholinesterases in complex with different ligands is a powerful tool to enrich the structural database required to rationally design new reactivators of nerve-agents poisoned cholinesterases.

In the last few years, multiple crystal structures of human butyrylcholinesterase (BChE) were solved in complex with a family of BChE-specific ligands originally designed as potential anti- Alzheimer's disease drugs. Among the different structural motifs composing these ligands, a remarkable cycloheptyl-based pattern emerged due to its specific arrangement into human BChE. After verification by molecular docking, such motif arrangement would be possible into OP-inhibited BChE and

some molecules sharing both the cycloheptyl motif and the reference reactivator, pralidoxime, were synthesized. Despite the relative structural simplicity of these molecules, a molecule presented noticeable reactivation potency towards VX- and sarin-inhibited BChE compared to the reference oximes pralidoxime and HI-6, especially by increasing the affinity towards the enzyme.

PO-03

Monoquaternary analogues of double charged K-oximes (K027, K048 and K203) are less effective reactivators of cholinesterases inhibited by organophosphates

Zuzana Kohoutova, Rudolf Andrys, Kamil Musilek, David Malinak

University of Hradec Kralove, Department of Chemistry, Hradec Kralove, Czech Republic, Czech Republic

Irreversible inhibition of acetylcholinesterase (AChE) by organophosphorus compounds (OPs) such as nerve agents or pesticides can be life threatening. On the other hand, inhibition of butyrylcholinesterase (BChE) has no adverse effects and therefore BChE can be used as bioscavenger of OPs. Reactivators of cholinesterases (ChEs) cleave the OP moiety from the active site of the enzyme by making a covalent bond to form a phosphyloxime and restore the activity of ChEs. There are five clinically used reactivators of AChE (e.g. asoxime or pralidoxime), but they have certain drawbacks. The promising results were obtained for K-oximes K027, K048, K203 and their halogenated analogues, especially oxime K868, which showed very promising reactivation ability for both ChEs. The aim of this research was synthesis and in vitro evaluation of monoquaternary analogues of K-oximes K027, K048 and K203 with or without halogen substituents. Their stability and pKa values were determined and were found to be analogous to bisquaternary oximes. Similarly, no significant change was observed in the inhibitory effect of novel compounds for both ChEs compared to their bisquaternary oximes. The reactivation ability of novel monoquaternary compounds for OP-inhibited ChEs was found to be similar or lower compared to bisquaternary K-oximes. These results highlight the importance of a second charge for binding into OP-inhibited cholinesterase and for the reactivation. This work was supported by the University of Hradec Kralove (no. SV2111-2024) and Czech Science Foundation (no. GA21-03000S).

PO-04

Halogenated pralidoxime analogues are efficiently reactivating cholinesterases

Sara Rademacherova, Karolina Knittelova, Adela Fuchsova, Rudolf Andrys, Kamil Musilek, David Malinak

University of Hradec Kralove, Hradec Kralove, Czech Republic

Organophosphorus compounds (OP) are inhibiting cholinesterases, disrupt cholinergic functions and leading to the development of an acute cholinergic crisis. Treatment of OP poisoning involves symptomatic antidotes and causal oximes, such as pralidoxime, which act as reactivators of inhibited cholinesterases. However, the efficacy of charged oximes is limited due to their hydrophilic character, resulting in low permeability through the blood-brain barrier or an inability to reactivate "aged" acetylcholinesterase in vivo. Additionally, none of the commercially available oximes is universally effective against multiple structurally variable OPs.

This study proposes halogenated oxime reactivators, when the various halogens were positioned to pralidoxime scaffold. The presence of a halogen atom in a close proximity of oxime group could lower its pKa value and increase their reactivation efficiency. The reactivators were synthesized, their stability and oximate forming properties were evaluated. They were challenged to OP surrogate inhibited acetylcholinesterase and butyrylcholinesterase with promising results. Overall, the C3

positioned halogens demonstrated a higher reactivation ability compared to pralidoxime and confirmed the importance of higher oximate formation for better reactivation.This work was supported by the University of Hradec Kralove (no. SV2111-2024) and Czech Science Foundation (no. GA21-03000S).

PO-05

Brominated oxime nucleophiles are efficiently reactivating cholinesterases inhibited by nerve agents

Eliska Prchalova[1], Rudolf Andrys[1], Jaroslav Pejchal[2], Zuzana Kohoutova[1], Kamil Musilek[1], Jana Zdarova Karasova[2], David Malinak[1]

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Cholinesterases (ChEs) are essential for maintaining function of nervous system. Unfortunately, they are targets of organophosphates (OPs) and can be inhibited which leads to cholinergic crisis and in serious cases death. The causal antidotes for OPs poisoning are reactivators of ChEs. To date five commercially available reactivators are used. However, they have several limitations; i.e. inability to reactivate "aged" acetylcholinesterase (AChE) in vivo, low permeability through blood-brain barrier (BBB) and none of the commercial reactivators is universal for all OPs [1]. Six novel brominated bis-pyridinium oximes were designed and synthesised with the aim to increase their nucleophilicity and reactivation ability of phosphorylated AChE and butyrylcholinesterase (BChE) [2]. Bis-brominated oximes showed decreased stability in a buffer solution as well as their previously prepared fluorinated and chlorinated analogues [2, 3]. Their degradation proceeds through isoxazole formation to the formation of nitriles. The brominated reactivators showed increased reactivation ability to non-halogenated analogues, confirming that an electron withdrawing group in a close proximity to the oxime group can be valuable tool for design of more potent reactivators. The most promising brominated oxime was tested in vivo on sarin- and VX-poisoned rats. This brominated oxime showed interesting CNS distribution and significant reactivation effectiveness in blood and resulted with the best protective index for VX-poisoned rats.

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PO-06

Importance of the shape of the linker between two quaternary pyridinium rings on reactivation process in oximes – in vitro and in silico study

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Acetylcholinesterase (AChE) reactivators are commonly employed as antidotes for the treatment of nerve agent intoxications. According to recent literature, oxime K203 is among the drug candidates exhibiting promising reactivation efficacy both in vitro and in vivo. In this study, we examined three oxime reactivators in vitro, each differing slightly from oxime K203. The sole structural distinction lies in the connecting chain between the two quaternary nitrogen atoms in their molecules. Traditionally, the connecting linker is believed to play a passive role in the reactivation process.

However, our findings suggest that the connection linker is a significant structural element influencing the efficacy of reactivators. These conclusions are bolstered by our in vitro findings and molecular modeling studies.

PO-07

Synthesis and in vitro assessment of the reactivation profile of clinical oximes on the acetylcholinesterase model inhibited by A-230 and A-242 nerve agents' surrogates

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The risk of use of toxic chemicals for unlawful acts has been matter of concern for different governments and multilateral agencies. The Organisation for the Prohibition of Chemical Weapons (OPCW), which oversees the implementation of the Chemical Weapons Convention (CWC), considering recent events involving chemical warfare agents, has recently expanded the CWC "Annex on Chemicals" by including some organophosphorus compounds that are regarded as cholinesterase inhibitors, acting similarly to G- and V-series of nerve agents, the A-series. Hence, knowledge on the activity of the pyridinium oximes, the sole class of clinically available acetylcholinesterase reactivators so far, is clearly warranted. Taking this into account, our Research Group at IDQBRN, the only OPCW Designated Laboratory in the GRULAC Area (environmental samples, 2023-2024) synthesized surrogates for A-230 and A-242 nerve agents and employed a modified Ellman's assay in order to evaluate their ability to inhibit the acetylcholinesterase from Electrophorus eel, and if the clinically available antidotes are able to rescue the enzyme activity. Our experimental data indicated that pralidoxime and trimedoxime were the most efficient oximes for reactivation of acetylcholinesterase inhibited by A-230 and A-242 surrogates, respectively.

PO-08

Synthesis, modeling and in vitro assessment of the reactivation profile of monocationic isatin-oximes hibrids on the acetylcholinesterase model inhibited by nerve agents' surrogates

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The risk of using toxic organophosphorus (OP) compounds, potent cholinesterase inhibitors, as methods of warfare or for unlawful actions is an ongoing concern. Despite the availability of medical countermeasures, such as pyridinium oximes, these chemicals do not exhibit a broad spectrum and have poor pharmacokinetic properties that prevent them from crossing the blood-brain barrier, hindering the rescue of enzymatic activity in the central nervous system. Therefore, research on novel and more effective reactivators is of utmost importance. Taking this into account, our Research Group at IDQBRN, the only OPCW Designated Laboratory in the GRULAC Area (environmental samples, 2023-2024), in partnership with the Military Institute of Engineering (IME) – Rio de Janeiro/Brazil and Concordia University – Montreal/Canada, synthesized a series of monocationic isatin-oxime hybrids aiming to test their reactivation profile towards acetylcholinesterase from Electrophorus eel (EeAChE) inhibited by surrog-

ates for nerve agents VX, A-230, and A-242 in comparison to pyridinium oximes already approved for clinical use, such as pralidoxime, trimedoxime, and obidoxime. Simultaneously, molecular modeling studies will be performed on computational models of the AChE/OP complexes to investigate the reactivation performances of the hybrids. This comprehensive approach aims to develop more effective countermeasures against OP poisoning, enhancing both safety and therapeutic outcomes. The interdisciplinary nature of this research, combining chemical synthesis, biochemical testing, and computational modeling, underscores its potential impact on public health and safety.

PO-09

Triazoles as potential reactivators of human acetylcholinesterase inhibited by the nerve agents VX and Novichok A-242

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Introduction

Nerve agents are organophosphate compounds and irreversible inhibitors of the enzyme acetylcholinesterase (AChE), considered Chemical Warfare Agents. Traditional treatment includes quaternary pyridine oxime compounds administration, but they are not universal antidotes, because they are not efficient to all types of neurotoxicants2. Studies show that structural modifications that increase lipophilicity can provide better permeation through the blood-brain barrier (BBB) and it may turn the compounds better reactivators of AChE. In this work, 12 new triazoles compounds that differ in lipophilicity were proposed and evaluated by molecular docking studies on the enzyme inhibited by VX and Novichok A-242.

Methodology

Tridimensional structures of 12 proposed compounds were constructed, had partial charges and geometry optimization performed using software Spartan08. The systems of AChE inhibited by VX and A-242 were constructed from the crystallographic structure obtained on Protein Data Bank, and validated with Ramachandran graphics. Docking studies of selected compounds on the enzyme were performed using Molegro Virtual Docker and results were evaluated by Near-Attack Conformation (NAC), total energy of interaction, residues of interaction profiles criteria.

Results

Docking studies point to an neutral proposed compound 2 as better reactivator for AChE inhibited by VX. These results suggest that the proposed modification in the structure can increase reactivation, considering that penetration in BBB is better for uncharged compounds. For A-242, charged compounds presented better results. All the ligands presented interaction with Ser203, a residue of catalytic triad, and also with residues located on the peripheral anionic site (PAS). These results point that all the proposed compounds present stabilization and affinity for the sites of the enzyme.

Conclusion

For both systems, the most of the ligands presented better results than reference oxime HI-6, according to all evaluation criteria. In sequence, molecular dynamic calculations will be performed to corroborate docking results.

PO-10

Beyond carbamates: N-substituted piperidine ureas as butyrylcholinesterase inhibitors

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Alzheimer's disease (AD) is the leading cause of dementia worldwide. The cognitive decline in AD is associated with deficient cholinergic transmission in the central nervous system (CNS). In healthy individuals, acetylcholinesterase (AChE) is the primary enzyme that hydrolyzes acetylcholine (ACh), while butyrylcholinesterase (BChE) plays a supporting role. However, as AD progresses, BChE becomes the main enzyme deactivating ACh, providing a rationale for BChE inhibitors to be used as a symptomatic treatment of AD.
Our group has developed several efficient N-substituted piperidines as BChE inhibitors of different structural classes: from amides over sulphonamides to carbamates. To further investigate the structure-activity relationship (SAR) and expand the chemical space of our CNS library, a series of N-alkyl piperidine ureas as inhibitors of BChE was designed, synthesized end evaluated.

A library of 95 compounds was prepared and their activity against AChE and BChE was assessed in vitro using Ellman's test. Most compounds demonstrated good selectivity for BChE over AChE. The most active compound showed an IC50 value of just under 100 nM, and its crystal structure was obtained. The best compounds were tested in vitro for cytotoxicity and were found to be non-cytotoxic on the BV-2 cell line.

The SAR of N-substituted piperidine ureas was determined. Based on in vitro activity and cytotoxicity profiles, a candidate for in vivo studies using the scopolamine model will be selected. Additionally, our in-house library of BChE inhibitors has been expanded, providing a robust foundation for further activity screening on various CNS targets in the quest for multifunctional ligands as potential treatments for neurodegenerative conditions like AD.

PO-11

Piperidine-carboxamides, -sulfonamides and -carbamates as selective butyrylcholinesterase inhibitors

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Introduction: Butyrylcholinesterase (BChE) is a nonspecific cholinesterase involved in neurotransmission, (drug) metabolism, embryonic neural development, inflammation and cellular differentiation. This serine hydrolase is a potential drug target for treating Alzheimer's disease (AD), multiple sclerosis and substance abuse and selective BChE inhibitors could thus be used to treat these diseases.
Methodology: We first used virtual screening to discover our hit compound, a novel 1,3-disubstituted piperidine-based nanomolar selective human (h)BChE inhibitor. To explore the chemical space around our hit compound we utilized ligand-based and structure-based drug design for hit-to-lead optimization and designed novel carboxamide, sulfonamide and carbamate derivatives which we then synthesized and biologically evaluated.
Results: We synthesized and evaluated close to 1000 compounds and obtained over 30 crystal structures of inhibitor-hBChE complexes. The most potent piperidinecarboxamide (IC50 ≥ 1 nM) we developed is one of the most potent BChE inhibitor ever reported. It improves memory, cognitive functions and learning abilities of mice with scopolamine-induced AD-like symptoms. Our most prom-

ising piperidinesulfonamide (IC50 = 19 nM) also has procognitive effects in mice with scopolamine-induced symptoms of AD and improves cognitive functions and quality of life of dogs suffering from canine cognitive dysfunction (CCD), which has many similarities with AD in humans. We showed with our piperidinecarbamates that not all carbamates are covalent BChE inhibitors and that we can produce multi-target-directed ligands simply by placing the right group onto the piperidine nitrogen.
Conclusions: The piperidine-carboxamides, -sulfonamides and -carbamates we developed are some of the most potent and selective BChE inhibitors ever reported and could be used to treat and/or develop new drugs for treating CCD, Alzheimer's disease, multiple sclerosis and substance abuse.

PO-12

In vitro reactivation screening of A-234-inhibited human recombinant acetylcholinesterase and butyrylcholinesterase

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The A-series agent A-234 belongs to a new generation of nerve agents that directly inhibit human acetylcholinesterase (HssAChE) and butyrylcholinesterase (HssBChE). Although

A-agents were synthesized during the Cold War, studies devoted to their therapeutic countermeasures are nonexistent. Symptomatic treatment is the mainstay of therapy; however, there is no evidence causal antidotes are ineffective. Using a modified Ellman's method, we carried out extensive in vitro screening of oxime and non-oxime compounds to reactivate

A-234 inhibited by HssAChE and HssBChE in the search for potential antidotes. Only one compound showed limited recovery of HssAChE activity, but none of them could reactivate HssBChE inhibited A-234. Subsequent experiments confirmed that compound is also a mild inhibitor of HssAChE. This compound could serve as a starting molecule for the design of new, more potent antidotes against A-agents.

PO-13

Computational investigation of hardwickic acid-derived amides using molecular docking and prediction of ADME/Tox properties as potential inhibitors of cholinesterase enzymes

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Alzheimer's disease (AD) is the leading cause of dementia worldwide, characterized by progressive cognitive decline. Pharmacological treatment involves cholinesterase inhibitors targeting human acetylcholinesterase (hAChE) and butyrylcholinesterase (hBuChE). Current drugs have low therapeutic efficacy, diverse side effects, and reduced patient adherence over time. Developing inhibitors that target multiple targets could enhance therapeutic efficacy at lower doses. Historically, most marketed drugs were natural products or their analogs. Many drugs for various diseases feature amide bonds in their bioactive compounds, a crucial group due to their role in forming peptide bonds in proteins. Considering the importance of amide bonds in medicinal chemistry and the use of natural products to synthesize new drugs, this study investigated amides derived from hardwickic acid amides as inhibitors of hAChE and hBuChE. Thirteen compounds were sketched and optimized using GaussView 5.0.8 with the DFT method, employing the B3LYP/6-31G basis set, to visualize molecular electrostatic potential maps (MEP) and frontier orbitals (HOMO and LUMO). In addition, pharmacokinetic and toxicological properties were studied using the

online servers PreADMET and SwissADME. Molecular docking was performed against 3D structures of acetylcholinesterase (AChE; PDB ID 4M0E) and butyrylcholinesterase (BuChE; PDB ID 4BDS) prepared with the biopolymer module in SYBYL-X 2.0. The results indicated similar profiles in surface maps and molecular orbitals for the nitro substituent group. Pharmacokinetic predictions demonstrated that all 13 hardwickic acid amide derivatives showed significant values for blood-brain barrier (BBB) penetration, classifying them as active in the central nervous system (CNS), a crucial pathway for AD treatment. Intermolecular interactions between the compounds and targets showed that benzyl amide derivative J had the best binding energy at hAChE binding site (-10.1 kcal/mol) and hidroxy amide derivative M for hBuChE binding site (-9.7 kcal/mol). These findings can guide future enzymatic assays of hardwickic acid amide derivatives as AChE and BuChE inhibitors.

PO-14

Modeling studies and experimental evaluation of the reactivation potential of oximes K027, K048, K170 and K203 against the nerve agent A-242

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Despite being known since the 1970s, the nerve agents belonging to the A-series remained out of the headlines and were not listed in Schedule 1 of the Annex on Chemicals of the Chemical Weapons Convention (CWC) (https:/www.opcw.org/chemical-weapons-convention/annexes/annex-chemicals/schedule-1) until 2018, when agent A-234 was used in assassination attempts in England. These agents are considered more toxic than the nerve agents of the G and V series, and as far as we know, there is no reported antidote yet capable of reactivating acetylcholinesterase (AChE) inhibited by them. Aiming to contribute to mitigating this situation, our research group has developed and used protocols to search for potential reactivators against AChE inhibited by surrogates of the A-series nerve agents. Moving forward in this direction, we investigated the reactivation profiles of oximes K027, K048, K170, and K203 towards acetylcholinesterase from Electrophorus eel (EeAChE) inhibited by a surrogate of agente A-242. A modified Ellman's assay was employed to assess the reactivation rate in comparison to the commercial oximes pralidoxime, trimedoxime, and obidoxime. Simultaneously, molecular modeling studies were performed on computational models of the AChE/A-242 complex to investigate the reactivation performances of the oximes and correlate them to the experimental data.

PO-15

Novichok A-232: basic knowledge of biochemical and toxicological properties

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In the last few years, CBRN threat research institutes have intensively studied the topic of fourth-generation nerve agents, code-named "A-agents", called novichoks. There has never been an official unveiling of the chemical structures of these extremely toxic organophosphorus compounds. Vil Mirzayanov published possible formulas of the substances in his book in 2009, but they do not correspond to the structures published by Hoenig.

We evaluated novichok A-232 in our laboratory and compared its physical-chemical and bio-

logical properties to well-known structures of nerve agents such as sarin or VX. Before starting the experiments, we observed the stability of the substance in an aqueous solution. In the next step, we determined the in vitro inhibition kinetics of human acetylcholinesterase and butyrylcholinesterase. We also evaluated toxicity in vivo on an animal model using WISTAR rats by estimating the LD50 value. Further, we assessed the ability of standard oxime nerve agent antidotes to reactivate both inhibited cholinesterases.

These findings are preliminary data in our research of fourth-generation nerve agents and should be useful for developing effective antidotes and possible subsequent therapy.

PO-16

Reactivation potency of GB, VX and A-234-inhibited human recombinant acetylcholinesterase in vitro and in silico

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The A-series agents, also known as 'Novichoks', are the latest generation of chemical warfare nerve agents (CWAs) that directly inhibit human acetylcholinesterase (HssAChE). Although these agents were synthesized during the Cold War, studies on their therapeutic countermeasures are rare. Traditional cholinesterase reactivators have proven ineffective against these agents.

In the search for potential antidotes, we conducted an in vitro reactivation assay and in silico study to evaluate five commercially available oximes and a few compounds as new potential reactivators of recombinant HssAChE inhibited by nerve agents GB (sarin), VX and A-234. We performed a reactivation assay using modified Ellman's protocol to obtain the percentage of reactivation potency. Next, we utilized computational methods such as molecular docking and molecular dynamics simulations to calculate the average distance between the oxygen atom of the oxime and the phosphorus atom of the nerve agent. Additionally, we computed the interaction energies, composed of short-range Coulombic energies and short-range Lennard-Jones energies, between the oxime and the active site of the inhibited enzyme, between the oxime and nerve agent, and between nerve agent and the enzyme. The nerve agent-HssAChE complex appears to be highly stable, forming stabilizing hydrophobic and electrostatic interactions at the choline-binding site. Furthermore, we calculated the partial charges on the oxygen atom of the oximate group to estimate the nucleophilicity strength of each oxime.

This research has enhanced our understanding of the behavior of individual reactivators in interaction with the inhibited enzyme. Our research attempted to contribute to explanation of right usage of in silico methods in the discovery of new reactivators. Future studies combining molecular dynamics and quantum mechanics could provide a more comprehensive understanding of the reactivation mechanisms.

PO-17

Exploring drug modality switch from in situ assembly to reversibility: reversible modulators of choline O-acetyltransferase activity

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Research on the development of new treatments of intoxications by organophosphorus nerve agents (OPNAs) is largely focused on cholinergic receptors and acetylcholinesterase, the enzyme that is the primary target of OPNAs. Currently available antidotes are limited in their clinical applicability and new alternative targets are needed. Choline acetyltransferase (ChAT) catalyzes the synthesis of the neurotransmitter acetylcholine in cholinergic neurons, and drugs

targeting ChAT could potentially be used for broad-spectrum symptomatic treatment of intoxications caused by OPNAs.

We have recently discovered that one of the most studied classes of ChAT inhibitors, the arylvinylpyridiniums (AVPs), act as substrate in an in situ synthesis in ChAT yielding an adduct that is the actual inhibitor of the enzyme. In this study, we used structure-based drug design to change from the reactive inhibition mechanism of AVPs to compounds that act through a reversible inhibition mechanism. With the crystal structure of an in situ formed inhibitor as a template, the objective was to design chemically stable, reversible inhibitors and investigate their potency, selectivity and binding mode using a combination of biochemical, biophysical and structural techniques.

By comparing the thermal stabilization provided by reactive inhibitors, we found a stabilization in ChAT that was not found in the related carnitine-O-acyltransferase (CrAT), indicating that AVP-like scaffolds could potentially be used to gain selectivity for ChAT over CrAT. A set of reversible molecules was synthesized and evaluated for inhibition of ChAT and CrAT activity. Furthermore, analysis of X-ray crystal structures of binary complexes with ChAT confirmed the expected binding site of the reversible inhibitors.

To conclude, we have switched modality from in situ assembly to reversibility and present insights to the selectivity and potency of the first class of ChAT inhibitors with a confirmed reversible mechanism, offering valuable insights for future efforts of developing ChAT inhibitors.

PO-18

Kinetic and structural evidence for specific DMSO interference with reversible binding of uncharged bis-oximes to hAChE and their reactivation kinetics of OP-hAChE.

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The structural basis of the inhibitory effect of the solvent dimethyl sulfoxide (DMSO) on the kinetics of reversible binding of novel uncharged, heterocyclic bis-oximes to human acetylcholinesterase (EC 3.1.1.7; hAChE) and on rates of reactivation of inactive organophosphate (OP)-hAChE conjugates by bis-oximes was studied. The reversible inhibition constant of DMSO for hAChE in 0.1 M phosphate buffer pH 7.4 at 22 °C, was Ki= (0.257 ± 0.048) % (or 33 ± 6 mM), competitive with acetylthiocholine (K(S)=0.176 ± 0.062 mM), evaluated from the Hunter & Downs analysis. The Ki of the bis-oxime LG-703 for hAChE was ~ 4-fold larger in 1% DMSO, consistent with direct competition between LG-703 and DMSO. The X-ray structure of the LG-703*hAChE complex (PDB ID: 6U3P) shows both DMSO and LG-703 bound to individual hAChE monomers. In the chain A monomer, LG-703 extends along the gorge axis from its opening to the base without DMSO, and in the chain B monomer DMSO molecule binds at its base, without LG-703. Since co-crystallization of the LG-703*hAChE was in ~ 2% (or ~0.28 M) DMSO (originating from the LG-703 stock) and with ~1.5 mM LG-703 final concentration, both small molecules were present at a similar excess over their corresponding Ki values for hAChE (7.8-fold for DMSO and 6.5-fold for LG-703). The formation of two different complexes (DMSO*hAChE

and LG-703*hAChE) of otherwise competing ligands, in the same crystal, is thus consistent with competitive kinetics of their reversible inhibition of hAChE. Furthermore, rates of reactivation of paraoxon-inhibited hAChE (POX-hAChE) were reduced 2 – 3-fold in 1% DMSO, consistent with observation of DMSO molecules in POX-hAChE structures obstructing the access to the conjugated P atom.

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PO-19

Outlining the A-series of organophosphorus compounds – cholinesterase inhibition, reactivation, cytotoxicity

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The A-series has been known for quite some time now but gained widespread attention after the UK incident in 2018. These phosphoramidates inhibit cholinesterases (ChE), potentially leading to death, but little else is known about them. This study aimed to investigate the rate of ChE inhibition by five OP compounds from the A-series and compare it to the G-series and VX. The most potent inhibitors were A-230, A-232, and A-234, with a phosphorylation rate comparable to cyclosarin and soman. We also tested standard oximes, 2-PAM, HI-6, obidoxime, TMB-4, and MMB-4, as reactivators of ChE inhibited by the A-series compounds. Our results identified MMB-4 and HI-6 oxime as the most promising reactivators, especially of the A230-AChE conjugate, restoring AChE activity in 2 and 4 hours, respectively. Furthermore, we modeled a near-attack conformation of oxime MMB-4 and HI-6 in phosphorylated acetylcholinesterase, providing guidelines for further structural improvements of the oxime and the design of more potent reactivators. The liver is the major organ responsible for detoxification, so we evaluated the effect of the investigated organophosphates on cell death in a hepatic cell line. Some of the tested compounds exhibited toxic effects independently of their effects on cholinergic transmission. Overall, this study contributed to a better understanding of these poorly characterized phosphoramidates by determining their kinetic and cytotoxic properties. Although the A-series posed challenges for reactivation, important insights were gained, and the reported results will contribute to and accelerate the progress in developing therapy.

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PO-20

Evaluation of resveratrol compounds as therapeutics in organophosphorus poisoning

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The dephosphorylation of acetylcholinesterase (AChE), a crucial enzyme in the hydrolysis of the neurotransmitter acetylcholine, remains insufficiently addressed by current medical countermeasures, especially when inhibited by organophosphorus (OP) compounds such as A-series agents. The ability of butyrylcholinesterase (BChE) to bind OP compounds lowering their concentration in the circulation and preventing the inhibition of AChE represents a basis for potential therapy for OP poisoning. This approach calls for the development of effective oximes to address positive outcomes in circulation for reactivating inhibited BChE. In our study, we investigated

heterostilbene derivatives as potential drugs for OP poisoning focusing on their ability to reactivate cholinesterases, mostly circulating BChE in blood. These compounds were designed as analogs of resveratrol, a polyphenol known for its antioxidant, anti-inflammatory, and neuroprotective properties, which have shown promise in treating various neural disorders. Oximes were screened for oxime-dependent reactivation of sarin-, cyclosarin-inhibited human BChE as well as BChE inhibited with A-series agents. We also estimated the AChE and BChE binding affinity for heterostilbene oximes in terms of the oxime-enzyme dissociation constants. Among the tested compounds, 10 showed potential as selective inhibitors of cholinesterase enzymes, with dissociation constants (Ki) ranging from 7 to 68 M. A comprehensive in vitro analysis of enzyme kinetics, enabled detection of possible more efficient reactivators of phosphorylated BChE compared to standard oximes. Encouragingly, some compounds achieved up to 80% recovery of inhibited enzyme activity. Furthermore, we evaluated the effects of these oximes on cell death in hepatic cell lines. Remarkably, oximes did not induce significant toxicity in cells within 24 hours suggesting their potential for further investigation and development as therapeutic for OP neurotoxicity.

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PO-21

Neuroprotective role of CNS-active uncharged bis-oxime antidotes in mice exposed to organophosphate compounds

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Oxime antidotes are used in standard medical treatment of organophosphate (OP) nerve agent and pesticide poisoning, but the search for novel antidotes with the capacity to cross the blood-brain barrier (BBB) is an ongoing pursuit. The cationic pyridinium oximes do not cross the BBB in sufficient concentrations for reactivation of AChE in the brain due to their permanent charge. Cholinergic receptor overstimulation can therefore trigger neuroinflammation and lead to permanent brain damage. In this work we analysed antidotal potency of a new class of uncharged but ionizable bis-oxime antidotes, which exhibited efficient in vitro reactivation of both AChE and BChE inhibited by sarin, cyclosarin, VX, paraoxon, and phosphoroamidate insecticide fenamiphos. All three tested bis-oximes were effectively comparable to 2-PAM, and the reversible inhibition constants (Ki) of bis-oxime-ChE complexes were 150 to 800 M. Cytotoxicity assay showed that bis-oximes did not affect the viability of neural (SH-SY5Y) and hepatic (HepG2) cell lines up to 1.6 mM concentration within 24 hours. Analysis of cholinesterase activity in blood and brain of mice exposed to sarin and bis-oxime demonstrated 30-40% recovery of ChE activity within 30 minutes after poisoning. We further investigated bis-oxime neuroprotection capacity in the brain of mice exposed to a sublethal dose of sarin w/o bis-oxime by assessing the change of expression in neuroinflammation markers GFAP (glial fibrillary acidic protein) and IBA1 (ionized calcium-binding adapter molecule 1). The results showed that both GFAP and IBA1 levels were in the control range and significantly lower in mice which received treatment compared to mice exposed to sarin only. The results of this study provide evidence for in vivo effectiveness, low toxicity and good neuroprotective efficacy in mice, and can serve as basis for further in vivo research.

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PO-22

Synthesis of broad-spectrum antidotes to organophosphorus neurotoxins

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Organophosphorous nerve agents (NOPs) are phosphorus containing compounds that can be found as pesticides or chemical warfare agents. In case of intoxication, NOPs will phosphylate the catalytic serine of acetylcholinesterase (AChE), an enzyme that plays a major role during the nerve impulse transmission in the brain and at neuromuscular junctions, which ends up with an irreversible inhibition of this enzyme. After AChE inhibition, acetylcholine neurotransmitter (ACh) released at cholinergic synapses are no longer eliminated, resulting in its accumulation in the synaptic cleft, and thus an overstimulation of post-synaptic receptors (muscarinic and nicotinic ACh Receptors and a cholinergic crisis, with deadly consequences. As medical countermeasures, research is focusing on the synthesis of new compounds called reactivators of AChE as antidotes against organophosphorus nerve agents through dephosphylation of the serine residue, associated with an anticonvulsant and a muscarinic AChR antagonist. Along the years, research groups have tested a large variety of compounds with different functions and structures. Our new strategy is to obtain a broad-spectrum antidote with two different targets and roles within one molecule. On one hand, our antidote aims to dephospylate AChE leading to elimination of abnormal concentration of ACh in the synaptic gap. On the other hand, the molecule should also act as antagonist of post-synaptic nicotinic AChR in order to limit the continuous nerve impulse and limit the cholinergic crisis.

PO-23

Bis-pyridinium mono-aldoxime K203: a promising prophylactic cholinesterase reactivator for organophosphate poisoning.

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Introduction

Oximes like acetylcholinesterase reactivators (AChR) are used therapeutically to revive inhibited acetylcholinesterase (AChE) by organophosphorus compounds (OPs). OPs are potent inhibitors of AChE, constituting a wide variety of structurally different compounds with lethality ranging from extremely poisonous like warfare chemicals to extreme, moderate and mild poisonous OPs used as pesticides. With increasing threats of terrorist activities and wartime scenarios, finding an effective pretreatment compound for organophosphorus poisoning is of utmost importance. Currently, pyridostigmine is the only FDA-approved drug in the USA for prophylactic treatment, but it has shown limited efficacy and significant side effects.

Methodology

This study aimed to identify a compound that could be used effectively and efficiently as a pretreatment for organophosphate poisoning and tentative attack. An in vitro study on human blood was conducted using oximes K-203, K-027, and pralidoxime, comparing them with the pretreatment drugs pyridostigmine and eserine against the highly toxic organophosphorus compound paraoxon-ethyl (POX). The oximes and drugs were administered at 1/05, 1/10, and 1/20 of their IC50 values at 0, 15, 20, and 30 minutes before the application of the IC70 concentration of POX.

Results

The results demonstrated that K-203 is efficacious, and more effective than K-027 and

pralidoxime oxime, followed by the standard compounds pyridostigmine and physostigmine (eserine) as prophylactic treatment. It is concluded that K-203 could be a promising pretreatment drug for organophosphate and organophosphonate poisoning. Further research on organophosphonates is suggested.
Conclusion
K-203 may be a candidate drug for pretreatment against organophosphorus/OP warfare chemicals. However, efficacy against different OP warfare chemicals is suggested.

PO-24

A-agents: more resistant than expected? Biomarker detection in biological matrices

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When almost all countries adhere to the Chemical weapons convention and have their stockpiles destroyed, the phantom of the Cold war started to emerge in what became to be known as A-agents family or „Novichoks", very potent, irreversible, acetylcholine esterase inhibitors. They came from dangerous yet almost forgotten chemical curiosity to the most watched theme in the late 20' of the 21st century when several people were poisoned by compounds that probably came from this family. With this renewed interest the methods of their detection as well as their biomarkers and their effects in the body and environment became interesting research topic because the determination of the correct substance can be vital in administering the right treatment.
In our work we focus on the detection of residues, biomarkers and unreacted parent molecules of several A-agents after exposure in rats as well as human plasma. To separate the compounds from the biological matrix several methods are employed including enzymatic cleavage, regeneration with fluoride and purification using SPE. The samples are then separated via high performance liquid chromatography and detected using mass spectrometry via total ion current and parallel reaction monitoring using orbitrap mass spectrometer.
Up – to-date we were able to detect A230, A232 and A234 as tyrosine adducts broken from albumin and their acidic remnants, which could be found for example in urine. On the other hand, when trying to regenerate compounds from plasmatic butyrylcholinesterase with fluorine and derivatize them, mixed results were obtained. Nevertheless, we were able to detect original compounds. The preparation of samples and their regeneration afterwards showed distinct differences between A230 and A232 in reactivity and regenerability while A232 regeneration went as expected, A230 binded to plasma rather slowly, however proved hard to regenerate.
This project is supported by Ministry of the Interior (OPSEC VK01020171).

Poster Session

Tuesday - Poster Session
Sep 17, 11:00 - 12:35

PO-25

PON1 gene polymorphisms and inflammatory markers in organophosphate pesticides cohorts from Cameroon and Pakistan

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Background and objectives: The detrimental effects of organophosphates (OPs) on human health are thought to be of systemic, i.e., irreversible inhibition of acetylcholinesterase (AChE) at nerve synapses. However, several studies have shown that AChE inhibition alone cannot explain all the toxicological manifestations in prolonged exposure to OPs. Predisposition to population heterogeneity

and irregularities in various biochemicals like paraoxonases and inflammatory biochemicals are the possible affects of OPs long term exposure that may lead to sequels of diseases and are less addressed in literature. The study was aimed to assess the cholinergic enzymes (AChE and BChE), PON1, and inflammatory markers (IL1β, IL6, TNFα, CRP, Apo AI, Apo B) and determine the toxicogenetics association of PON1 gene to chronically OPs exposed groups from Pakistan and Cameroon.

Materials and methods: AChE, BChE and PON1 were measured by colorimetric method using spectrophotometry. Inflammatory markers were determined by Elisa assay. PCR-restriction fragment length polymorphism (PCR- RFLP) using salting out method was employed for SNP genotyping.

Results: The results revealed the significant ($p \leq 0.05$) inhibition of cholinergic enzymes PON 1 was found to be 6.91 ng/mL±1.03 and 2.84 ng/mL±1.40 (mean ±SD) in Pakistan and Cameroon groups respectively. IL6, TNFα, CRP were increased and Apo AI was less while Apo B was increased in OP exposed groups in both population groups. SNPs analysis of PON1 showed significant differences in allelic and genotype frequencies of OPs exposed and non-exposed groups.

Conclusions: PON1 was noticeably less in Cameroonian than Pakistani, albeit both groups have significant decrease in PON1 actity. In addition, the study concludes that OPs induce low grade inflammation, an aetiology of many diseases. Selected PON1 SNPs analysis showed a significant toxicogenetics association with OPs exposure marker enzymes. The results of this study may help in regulation of usage of OPs anticholinesterases in different populations. The study will further open new avenues in toxicogenetic and exploration of SNPs based strategies on organophosphate intoxication.

PO-26

Screening and Characterization of Inhibitors for the Recombinant Variant of Paraoxonase 1

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Paraoxonase 1 (PON1) is a serum enzyme primarily associated with high-density lipoproteins (HDL). While its physiological function is not completely understood, PON1 displays great substrate promiscuity, acting on lactones, aryl esters, and organophosphate paraoxon. Additionally, PON1 plays a significant role in protecting against oxidative stress by contributing to the antioxidant properties of HDL and potentially preventing atherosclerosis and several other diseases. One of the best-known inhibitors of PON1 is 2-hydroxyquinoline (2HQ), but searching understanding of the inhibitory mechanisms of PON1 is crucial to interpreting its physiological roles, particularly its protective functions against oxidative stress and its potential role in preventing atherosclerosis. By identifying and characterizing other inhibitory compounds, we could better understand how PON1 activity is affected within the human body.

This study aims to screen 20 organic molecules for their inhibitory effects on the recombinant G2E6 variant of paraoxonase 1 (rePON1). We aim to enhance the understanding of rePON1 activity and inhibition mechanisms, providing further valuable insights into the PON1 physiological roles.

Time-courses of product formation (progress curves) were measured spectrophotometrically at 270 nm for the enzyme-catalyzed hydrolysis of dihydrocoumarin (DHC) by rePON1, and kinetic models for inhibited enzyme reactions were fitted to the progress curves by using ENZO software. This method provided quantitative data on the inhibitory effects of the various molecules on rePON1, allowing us

to assess enzyme activity and inhibitor impact. For inhibitors with high absorbance at 270 nm, we used thio-phenylacetate as substrate which coupled with Ellman's reagent enabled time-product measurements in visible spectrum at 412 nm.

The screening of 20 various organic molecules revealed several inhibitors of rePON1 activity. These findings can enhance our understanding of the promiscuous nature of PON1. The kinetic analysis provided quantitative data on the inhibitory effects and helped to determine the mechanisms of inhibition for the studied compounds.

PO-27

Exploring the Impact of Lanthanide (III) Ions on the Function of Paraoxonase 1 (PON1)

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Paraoxonase 1 (PON1) is a metallohydrolase that enables to protect the human against oxidative stress and detoxifies harmful organophosphates, such as pesticides and nerve agents. It is primarily associated with high-density lipoprotein (HDL) in the bloodstream, enhancing HDL's antioxidant and anti-atherosclerotic properties. PON1's activity varies significantly among individuals due to genetic polymorphisms affecting its expression and functionality. The enzyme's activity relies on two calcium (II) ions for structural integrity and catalytic functions. Hence, the specific roles of calcium (II) ions in PON1's function are essential for understanding the enzyme's biological mechanisms. In this context, the lanthanide (III) ions, which share similar ionic radii and coordination chemistry with calcium (II) ions, offer a unique opportunity to probe these roles and explore potential modulation of PON1 activity.

This study aims to investigate the effects of the entire line of lanthanide (III) ions on the structure and function of recombinant PON1 (rePON1). By comparing the binding affinities, thermodynamic interactions, and kinetic activities of rePON1 in the presence of these ions, we seek to gain deeper insights into the specific contributions of calcium (II) ions to the enzyme's functionality.

We conducted a comprehensive kinetic analysis and Isothermal Titration Calorimetry (ITC) to examine the interactions between rePON1 and various lanthanide (III) ions. The kinetic analysis utilized dihydrocoumarin as a substrate to assess the inhibitory effects of lanthanide ions on rePON1 activity. The binding affinities and thermodynamic parameters of interactions between rePON1, calcium, and the lanthanide ions were measured by using ITC.

Our study revealed distinct interactions between rePON1 and each lanthanide ion. The results indicated that all lanthanide ions can reversibly replace both calcium (II) ions from the protein backbone of rePON1 as the process is driven spontaneously, and a reversible loss of enzymatic activity was observed.

PO-28

PON1 plasma activity in the aftermath of bariatric metabolic surgery: the benefits of investigating more than one substrate

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Previous research has indicated a connection between PON1 and both obesity and type 2 diabetes, but most studies only investigated PON1 with a single substrate, and no study investigated kinetic parameters. Bariatric metabolic surgery (BMS) is used to treat severe obesity and usually results in significant weight loss as a result of reduced calorie consumption and metabolic adaptation. We wanted to investigate a BMS-related change

in patients' weight would correlate with a change in the patients' PON1 activity. For a sample of 69 patients who underwent BMS, we acquired blood samples on the day of surgery and used three different substrates to measure three parameters of PON1 activity in blood plasma: rate of hydrolysis (substrates DHC, PA, and PX), Km and Vmax (substrates DHC and PA). We showed that the rate of hydrolysis for PX does not correlate with those for PA and DHC, but it does correlate with Km for both, which stresses the importance of measuring activity for at least two substrates and calculating kinetics in similar studies. For a subsample of 19 patients, we acquired follow-up blood samples and clinical data and compared PON1 and clinical parameters between operation and follow-up. Of the 7 enzyme-activity parameters, all correlated significantly between operation and follow-up; the correlation was especially strong for PX rate of hydrolysis (Pearson CC = 0.846, p = 3.62×10-5). We calculated the difference between the values at both time-points for each parameter and checked the correlations between these differences: notably, the only significant correlations were between all three rates of hydrolysis, and there was no significant correlation of any PON1 parameter with changes in BMI or weight. Our research thus suggests that PON1 activity changes in the aftermath of BMS in a way that is not clearly connected to weight loss.

PO-29

Recombinant human paraoxonase-1 variants depict hydrolyzing capabilities of A-series nerve agents in vitro

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Chemical warfare nerve agents (CWNA) are potent neurotoxicants, acting by rapid inhibition of the acetylcholinesterase (AChE) pool of the intoxicated individual, ultimately leading to death. The threat posed by these chemicals has recently attracted renewed attention after nearly two decades of silence. Not only sarin was used during the Syrian conflict in 2013 and 2017, but a new generation, a.k.a Novichok or A-series nerve agents, emerged in 2018 and 2020, like a remnant of the past.

Even though medical countermeasures have been a part of the scientific landscape for several decades now, no technology has emerged as a credible alternative to the historically available emergency treatment (i.e combination of atropine, an oxime, and an anticonvulsant) for both military and civilians.

To prevent AChE inhibition in vivo, injectable enzymes were considered as CWNA bioscavengers and constitute an already well-studied field. Despite numerous engineering efforts on several target candidates, catalytic scavengers are not yet part of the therapeutic arsenal, but their development is still ongoing, with promising candidates such as PTEs.

In this context, several mutants of human paraoxonase-1 (PON-1) were assessed as catalytic bioscavenger candidates against A-230, A-232 & A-234. The enzymes consist of variants of the human PON-1, fused to a half-life extension partner (HLEP) to enhance their in vivo pharmacokinetics, as previously reported in a prophylaxis study against pesticides. Here, we describe the evaluation of these recombinant PON-1 to hydrolyze the Novichok agents in vitro, using butyrylcholinesterase inhibition as a reporter in complement to LC-MS analysis to directly assess compounds hydrolysis and their degradation products.

PO-30

Copper-dependent stereoselective hydrolysis of O-hexyl O-2,5-dichlorophenyl phosphoramidate by recombinant serum albumins

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A-esterases are enzymes that hydrolyze organophosphorus (OP) compounds. These enzymes are metalloproteins that are identified in bacteria and mammalian tissues including human serum. In our laboratory, a Cu2+-dependent hydrolyzing activity of chiral OPs, such as O-hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) and O-ethyl O-2,4,5-trichlorophenyl ethylphosphonothioate (trichloronate) has been identified in serum albumin of birds and mammals. On the other hand, it is well known that albumin is the protein that transports 15% of the copper in the bloodstream to the tissues and organs in vertebrate animals. Physicochemical studies have revealed that the N-terminal site of the serum albumin of these animals is one of the sites with the highest affinity for copper. It is different with respect to its sequence among mammals, birds and reptiles. Therefore, has been suggested the participation of this sequence in the A-esterase catalytic site of this protein. In order to identify the involvement of the N-terminal sequence of albumin as the catalytic site responsible for the Cu2+-dependent A-esterase activity (HDCPase and trichloronatase), an aliquot of 400 M racemic HDCP was incubated with 200 g of pentapeptide Ala-Glu-His-Lys (DAEHK) and the tetrapeptide Asp-Ala-His-Lys (DAHK), peptides derived from the N-terminal site of CSA and HSA, respectively, as well as recombinant albumins of CSA and HSA. The HDCP hydrolysis was quantified by UV-Vis spectrophotometry and chiral chromatography. The results show a lack of hydrolysis of HDCP by the peptides, while the recombinant chicken albumin (truncated in the N-terminal sequence (DAEHK)) show hydrolysis levels (120 M) of HDCP very similar to its respective wild albumin. This same lack of activating effect of Cu2+-dependent HDCPase activity was also observed between the rHSA (DAEHK) with glutamic acid inserted in position 3 and itś corresponding HSA wild protein (DAHK). Both proteins showed 12-15% of HDCPase activity. These results suggest that the N-terminal sequence of these animal albumins does not participate in the A-estrasic catalytic center of albumin.

PO-31

Update of ESTHER, the database and server dedicated to the analysis of protein and nucleic acid sequences within the superfamily of cholinesterase relative

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For IT security reasons, the ESTHER database, which is dedicated to ESTerases and alpha/beta Hydrolase Enzymes and Relatives headed by the cholinesterases, switched to a new operating system in April 2024. After 30 years of using the ACEDB database system, we had to start again from scratch to warrant the quality of the transition. Thanks to the robustness of the model and data structure recovered as text files, the procedure was relatively smooth, and most of the data and tools are now operational again.

ESTHER is growing at fast pace: nowadays it contains ca. 70.000 gene-proteins, grouped in 247 families. In 2024, the number of 3D structures available in the RCSB-PDB reached 3000, corresponding to 739 distinct proteins validated as superfamily members. There are 143 families in which at least one 3D structure is known, and new families are regularly added to the superfamily. For the human genome, 120 genes belong to 59 fam-

ilies described in the database. A 3D structure is available for only 51 proteins encoded by these genes. Mutations associated with a disease are found in 35 of human genes. The 768 published mutations in the genes of Block C proteins (Carboxylesterase, Cholesterolesterase, Cholinesterase, Neuroligin, Thyroglobulin...) correspond to 341 residue positions in the protein sequence of Torpedo (Tetronarce californica) acetylcholinesterase used as a reference. 112 distinct mutations in acetylcholinesterase or carboxylesterase genes where described in insecticide-resistant populations of 43 arthropod species.
The new version of the web server with improved layout and new tools is available at http:/bioweb.supagro.inrae.fr/ESTHER. Feedbacks from the users on this rejuvenated ESTHER will be most welcome. Acknowledgement: INRAE Phase department.

PO-32

In silico evaluation of the anticholinesterase activity of triazole fungicides

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Triazoles are compounds with various biological activities, including fungicidal action. Triazoles are currently the most used class of antifungals in agriculture and medicine. Unwanted cross-reactivity with human cytochrome P450s enzymes forced the development of safer azoles and it was shown that voriconazole inhibited fewer human P450s. Mefentrifluconazole (Ravystar®) is the first isopropanol triazole fungicide, developed to overcome fungus resistance in plant disease management, which like voriconazole inhibits about 1 800-fold fewer human P450s. Evaluation of the anticholinesterase effect of the triazole fungicide mefentrifluconazol,e together with structurally related fungicides was performed using in silico methods including CHARMm-based scoring function Cdocker interaction energy used in a molecular docking study. Triazole scores were compared to the scores obtained for anticholinesterases. Additionally, the physicochemical properties of the tested fungicides were compared to the properties of CNS active drugs to estimate the blood-brain barrier permeability. As mefentrifluconazole is commercially available individually or in a binary fungicidal mixture with the carbamate pyraclostrobin (Ravycare®), a kinetic study was performed on both compounds and showed that mefentrifluconazole reversibly inhibited human acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) with a 7-fold higher potency toward AChE (Ki = 101 ± 19 μM), while pyraclostrobin inhibited AChE and BChE progressively with a rate constant of ki = 6.6 . 103 M-1 min-1 and ki = 9.2 . 103 M-1 min-1, respectively. The approach in which in silico activity/property evaluation and a kinetic study are combined may help in the future development of biologically active compounds where in silico methods can be used to test the possible unwanted biological activity toward non-primary biological targets, already characterized by drug design and QSAR regression models including ChEs.

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PO-33

Cholinesterase monitoring for nerve agent exposure

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In our organisation, research scientists work with organophosphorus (OP) compounds such as nerve agents for defensive purposes, for example for the development of medical countermeasures. Blood cholinesterase (ChE) activity measurements have been monitored in numerous individuals for over 40 years. This

method is particularly suited for routine monitoring in laboratory situations because collection of an effective baseline measurement is possible. In a military setting where exposure risks are greater, continuous wearable monitoring could be of significant benefit. This would enable pre-symptomatic exposure warning and subsequently early administration of medical countermeasures in addition to supporting the medical management of prognosis by monitoring recovery of ChE and treatment effectiveness. These are either not possible or are difficult and labour intensive with the current available in vitro methods.
Our expertise in measuring ChE activity is being used to help develop a real-time microfluidic assay with collaborators at Imperial College London, UK. This system uses microdialysis sampling to measure choline resulting from acetylcholine hydrolysis, detected using microelectrode biosensors housed in a 3D-printed chip. Although still in proof-of-concept phase, we have shown that changes in ChE activity can be continuously detected and quantified following OP exposure by measuring the pick-up of choline via microdialysis.
These assays and technologies provide options to those at risk of exposure to OPs in the military setting which can be utilised at various points in the medical management chain, from mobile units with limited options for equipment, to field hospitals back at base.

PO-34

Immobilization of cholinesterases on magnetic microparticles for enhanced stability and biosensing applications

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According to substrate specificity, tissue distribution, or inhibition sensitivity, two human cholinesterases can be distinguished: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Many compounds interact with human cholinesterases. Organophosphorus nerve agents (e.g. VX, sarin, or tabun) and pesticides (e.g. carbofuran or bendiocarb) present significant threats to civilian and military populations [1]. On the other hand, some compounds are irreplaceable helpers in alleviating the symptoms of neurodegenerative disorders such as Alzheimer's disease. Precise determination of the mechanism of interaction between these substances and cholinesterases is thus of fundamental importance for civilian and military use [2].
The main challenge when working with enzymes is their cost, limited stability, possible interference with analytical methods, and inability to reuse. In this project, human recombinant AChE and BChE were non-covalently immobilized on the surface of porous and non-porous magnetic microparticles to overcome these limitations. By attaching ChEs to magnetic solid support, a powerful, cost-effective, stable, recyclable, and easy-to-use tool for studying enzyme kinetics, and inhibition mechanisms has emerged [3]. Moreover, the resulting immobilized biocatalysts were used to investigate the reactivation potential of a series of novel oximes. By utilizing immobilized enzymes, we were able to observe pure reactivation potential without the confounding effect of oxime inhibition. Additionally, the immobilized ChEs facilitated the study of phosphorylated-oxime intermediates, which are crucial byproducts of the reactivation reaction. This work was supported by the Czech Science Foundation (no. GA22-14568S), International mobility for research activities of the University of Hradec Kralove II (no. CZ.02.2.69/0.0/0.0/18_053/0017841), University of Hradec Kralove (Faculty of Science, no. SV2106-2023, Excellence project PrF UHK 2201/2024-2025.

PO-35

Highly potent and selective butyrylcholinesterase inhibitors for cognitive improvement and neuroprotection

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Butyrylcholinesterase (BChE) has been found to regulate the cholinergic system, neuroinflammation and energy metabolism in the brain of advanced Alzheimer's disease (AD) patients. Therefore, BChE has been more and more attractive for treating neurodegenerative diseases. However, there is still a lack of BChE inhibitors with high selectivity and activity, and the biological function of BChE in AD has not been clarified. In this study, a hierarchical virtual screening protocol was applied, and a potential BChE inhibitor 8012-9656 was selected as a lead compound. We have conducted three rounds of structure-activity relationship studies. Based on the results of inhibiting activity and cyto-safety evaluations, compound S06-1064 (hBChE IC50 = 45.2 nM) has been selected as candidate. Candidate compound S06-1064 possessed outstanding safety and pharmacokinetic profiles. As the results of pharmacological evaluations, candidate compound exhibited potent antioxidant activity and neuroprotective effects against Aβ-, glutamate- and H2O2-induced cytotoxicity at very low concentrations (1, 2, 5 μM). They could also decline the production of ROS caused by LPS and Aβ, exerting an anti-inflammation profile. Furthermore, the analogs S06-1064 were able to improve the cognition and learning in APP/PS1 transgenic mouse model, without leading to body weight loss, verifying the efficacy and safety of BChE inhibition. Additionally, we demonstrated that BChE inhibitors can up-regulate ghrelin levels in vivo and in vitro. Simultaneously enhancing ghrelin level in the center and periphery greatly improves the application efficiency of BChE inhibitors. In conclusion, a series of BChE inhibitors with nanomolar IC50 values and above 1000-fold selectivity was designed and synthesized. BChE inhibitors exhibited neuroprotective effects and the ability to improve cognition in several AD mouse models. BChE inhibitors were proved to up-regulate ghrelin levels. Simultaneous modulation in the center and periphery improves the efficiency of drug.

PO-36

Detection of butyrylcholinesterase signal peptide in human brains

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The pathogenesis of Alzheimer's disease (AD) is attributed to extracellular aggregates of amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) in the human brain. Butyrylcholinesterase (BChE) has also been reported to be associated with Aβ plaques and NFTs in the brains of AD patients. We previously found that a substitution in the 5' untranslated region (5'UTR) of BChE resulted in an in-frame N-terminal 41-amino acid extension of the BChE signal peptide. The resulting variant with a 69-amino acid signal peptide, designated N-BChE, may play a role in AD development. We recently reported that the BChE signal sequence, when produced in an extended version of 69 amino acids, can self-aggregate and form seeds that enhance amyloid fibril formation in vitro in a dose-dependent manner and generate larger co-aggregates. Based on these observations, we hypothesized that a similar phenomenon of an extended version of 69 amino acids expression could be observed in the brain. Our recent experiments confirm the presence of the BChE signal peptide in human brains. We have detected this signal peptide using specific antibodies by western blotting and observed different aggregation forms of it in the brain samples as well as in the purified protein

in vitro. The exact role of this signal peptide in AD requires further investigation.

PO-37

Impact of Type 1 Diabetes Mellitus on Butyrylcholinesterase Expression and Activity in Rats

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Serum butyrylcholinesterase (BChE) activity is altered in pathologies associated with impaired metabolism, such as diabetes mellitus (DM). Increased activity has been observed in both animal models and human patients. Although BChE is an abundant enzyme throughout the body, information about its expression and activity in tissues other than blood is sparse. The main aim of the project was therefore to investigate changes in BChE expression and activities in major tissues in an animal model of type 1 DM. Twelve-week-old male Wistar rats were used in the experiment. DM was induced by administering a single-dose intraperitoneal injection of streptozotocin (STZ, 55 mg/kg) dissolved in 0.1M citrate buffer. Hyperglycemia was confirmed three days after STZ administration by measuring glucose concentration in blood collected from tail vein. Four weeks after DM induction, the animals were sacrificed in CO2 chamber, and blood, liver, lungs, spleen, pancreas, hypothalamus, and heart (dissected into its functional compartments) were collected and flash-frozen in liquid nitrogen. The relative expression of BChE mRNA was determined by RT-qPCR. BChE activity was measured using a Ellman method. Plasma lipid profile and ALT and AST activities were assessed in a certified laboratory. Successful induction of DM was confirmed by increased fasting glycemia, elevated levels of VLDL and triglycerides in plasma. The development of DM was accompanied by increased plasma BChE activity. In the liver, the main synthetic organ of BChE, there was an increase in both BChE expression and activity. Interestingly, increased ALT and AST enzyme activities, clinically used markers of liver injury, were also detected. Others collected tissues showed no changes in relative expression or activity of BChE. In conclusion, the development of STZ-induced type 1 DM led to an increase of BChE activity in plasma and an increase in BChE expression and activity in liver but not in other studied organs. Importantly, increased BChE levels were observed despite increased markers of liver damage; thus, plasma BChE should not be used in clinical practice to diagnose liver injury.

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PO-38

Expression of cholinesterases in rats

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Introduction: Wistar rats are suitable model for studying various pathological states in pharmacological research. Cholinergic system plays an important role in many pathologies, but information about cholinergic system across individual tissues in rats is sparse. Therefore the goal of this research was to study the expression of cholinesterases in major organs of rats.

Methodology: Nineteen-week-old male Wistar rats were used. After euthanasia with CO2, brain, liver, lungs, spleen and heart (dissected to its functional compartments) were swiftly removed, flash frozen in liquid nitrogen, and stored at -80°C until further analysis. Total RNA was isolated using TRI-Reagent, quantified and checked for quality and reverse transcribed. RT-qPCR was conducted with SYBR Select Master Mix. Gene expression was normalized to ActB, and $\beta 2\mu$G. Results were analyzed relative to a pool of liver cDNA from Wistar rats.

Results: Rat mRNA relative expression patterns, considering liver cDNA pool as a

baseline, differed for AChE and BChE. For AChE, the highest expression was observed in brain and the lowest in the lungs: brain (88 x) > right atrium (8x) > left atrium (4x) > left ventricle and septum (3x) > right ventricle (2x) > liver (baseline) > spleen (1 x) > lungs (0.1x). For BChE, the highest expression was observed in right atrium and the lowest in spleen: right atrium (9x) > left ventricle and septum (7x) > right ventricle (6x) > left atrium (5x) > brain (2x) > lungs (0.2x) > spleen (0.1).

Conclusion: In rat, the expression of AChE in the brain is dramatically higher than in other studied tissues, while the expression of BChE was highest in heart compartments. The expression patterns of AChE and BChE in rats differs from the ones reported for mice or humans.

This research was funded by APVV-22-0541 and VEGA 1/0283/22 grants.

PO-39

Investigating the Link between Butyrylcholinesterase and Pulmonary Vascular Disease in Rats

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Introduction: Recent research suggests a link between butyrylcholinesterase (BChE) and pulmonary vascular disease, underscored by inflammation, metabolic dysregulation, vascular dysfunction, and oxidative stress. The aim of this project was to study this link in monocrotaline(MCT)-induced pulmonary vascular syndrome in rat, which is characterized by proliferative pulmonary vasculitis, pulmonary hypertension, and cor pulmonale (i.e., structural and functional pathological changes in right ventricle).

Methods: Male Wistar rats aged 10-12 weeks were used in experiments. Pulmonary vascular syndrome was induced by MCT injection at dose of 60 mg/kg, s.c. (n=11). Controls were injected with 0.9% NaCl, s.c. (n=10). The animals were weighted weekly. Vital functions (heart rate, hemoglobin oxygen saturation, and breathing frequency) were recorded by non-invasive pulse oximetry four weeks after injection. Animals were euthanized 24 h later, and plasma, lungs, pulmonary artery, and right and left heart ventricles were collected, flash frozen and store at -80°C until further analysis. Cholinesterase mRNA relative expression was determined by RT-qPCR, and activities were measured by Ellman's activity assay.

Results: We observed a progressive decrease in body weight and an increase in heart rate and breathing frequency following MCT application. Post-mortem gravimetry confirmed hypertrophy of the right ventricle. There was a decrease in plasma BChE activity in MCT- treated rats as compared to controls. BChE expression and activity were also decreased in pulmonary artery and left and right ventricles. BChE activity and mRNA expression in the lungs was, however, comparable between the two experimental groups.

Conclusion: Plasma BChE activity is decreased in MCT-induced pulmonary vascular syndrome but due to high variability in the values, it is not a reliable biomarker. Decreased BChE levels in pulmonary artery may suggest endothelial dysfunction, and decreased BChE levels in ventricles are consistent findings from heart failure research.

This project was supported by APVV-22-0541, APVV-19-0458 and VEGA 1/0283/22.

PO-40

Acetylcholinesterase and muscarinic receptors control the ultraviolet-mediated release of melanosomes in cultured melanoma

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Melanosome is an intracellular organelle

responsible for melanin synthesis and storage, functioning in determining the skin color. In the epidermis, mature pigmented melanosomes are released by melanocytes, and subsequently internalized by surrounding keratinocytes via phagocytosis, leading to skin pigmentation. This serves as a protective mechanism for the epidermis, forming a nuclear melanin shield and preventing the cells from ultraviolet (UV) radiation. The idea of "skin synapse" describes the interaction between epidermal keratinocytes and melanocytes, mediated by acetylcholine (ACh). Here, we aimed to investigate how the release of melananosomes could be regulated in the "skin synapse". Cultured mouse melanoma B16F10 were deployed as the cell model. Narrow-band UVB light, acetylcholinesterase (AChE) inhibitor, agonists and antagonists targeting muscarinic ACh receptors (mAChRs) were applied as the drug treatments. The extracellular melanin present in culture medium was collected and quantified. Calcium indicator Fluo 4-AM was used to measure the change of intracellular Ca2+ level following the treatments. The phosphorylation of protein kinase C (PKC) was assessed by western blotting; while the expression of tethering complex genes (i.e. synaptotagmin, Sec8, Exo70 and Rab11b) related to melanosome exocytosis was studied by western blotting and RT-qPCR. The application of ACh and AChE inhibitor was able to increase the amount of released melanosomes, as well as the intracellular Ca2+ level. The antagonists of M1 and M3 could reverse the effects, showing the role of M1 and/or M3 mAChRs in mediating the UVB-induced melanosome release. Moreover, the expressions of tethering complex for exocytosis were also controlled in a similar manner. In conclusion, M1/M3 mAChRs could be involved in regulating melanosome release following the UVB radiation.

PO-41

Trehalose restores the tacrine-induced endoplasmic reticulum stress in cultured neuronal cells

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Alzheimer's disease (AD) is known as the most common dementia with progressive loss of cognitive functions. Acetylcholinesterase (AChE) inhibitors have been approved as conventional pharmacotherapies for AD. Tacrine was the first AChE inhibitor introduced into clinical use as a therapy for AD; however, it was withdrawn from the usage in 2013 due to safety concerns. In the brain, AChE is associated with a proline-rich membrane anchor (PRiMA), producing the tetrameric globular (G4) form and anchoring to the membrane. In cultured neurons, tacrine was found to induce endoplasmic reticulum (ER) stress and finally lead to apoptosis. This mechanism potentially explains part of the adverse effects associated with tacrine.

In PRiMA-linked G4 AChE overexpressed NG108-15 cells, C/EBP homologous protein (CHOP) was used to measure the level of ER stress. The immunofluorescence staining of AChE was employed to visualize the effect of tacrine on AChE protein expression and localization. The ER fraction was isolated to analyze the distribution of different forms of AChE within ER by non-reducing gel electrophoresis. Tacrine could significantly increase the expression level of CHOP, which could be decreased by the co-treatment of trehalose, a known ER stress reducer. The result of immunofluorescence staining showed that trehalose could relieve the tacrine-reduced G4 AChE level on the cell surface. In non-reducing gel electrophoresis analysis of the ER fraction, the tacrine-exposed group revealed a considerable G1/G2 forms of AChE accumulation, while the treatment of trehalose decreased the G1/G2 AChE accumulation.

Thus, tacrine could affect the assembly of G4 AChE in the ER and finally lead to ER stress. Trehalose possesses the ability to relieve ER stress by promoting the proper assembly of G4 AChE.

PO-42

The Muscarinic Acetylcholine Receptor in Dermal Papilla Cells Regulates Hair Growth

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The role of the cholinergic system in hair biology remains an interesting topic with limited research, having only a few reports exploring the relationship of the complex process. Several lines of evidence support the notion of acetylcholine (ACh) playing role in hair biology. Alzheimer's patients taking oral acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitor, rivastigmine, exhibited symptoms of hypertrichosis and hair re-pigmentation. In dermal papilla cells (DPCs) treated with AChE inhibitor, norgalantamine, a stimulation of anagen's activating signalling in the growth of hair follicles has been identified. In addition, the knockout mice of M4 muscarinic receptor (M4 mAChR) showed a defect in hair growth and which failed to produce pigmented hair shafts. Here, we reported that hair growth was regulated by cholinergic signaling via mAChRs. DPCs expressed different cholinergic biomarkers. In addition, the release of ACh, induced by solar light, from DPC was determined by ELISA kit and LC–MS/MS, which thereafter activated the AChRs localized on DPCs. Inhibiting AChE or stimulating M4 mAChR in DPCs, culture vibrissae and skin epidermis promoted the activated Wnt/β-catenin signalling was inhibited by tropicamide (M4 mAChR antagonist). These results were supported by various indicative biomarkers, including pTOPflash luciferase assy, phosphorylation of GSK-3β by western blot and mRNA expression of various molecules for Wnt/β-catenin signalling by real-time PCR. Activation of Wnt/β-catenin signalling was mediated by PI3K/AKT and ERK signalling upon the stimulation of bethanechol. In addition, an increase in hair shaft elongation in mouse vibrissae and accelerate re-entry of anagen in the in vivo hair growth test was observed the upon the treatment of bethanechol. These findings shed light on the role of cholinergic system in hair growth.

PO-43

The Regulatory Role of Gut Microbiota in Expression of AChE in Epithelial Cells: a Regulator of Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a group of chronic inflammation in gastrointestinal tract, which is affecting millions of people all over the world and has a significant impact on the quality of life, as it is often accompanied by comorbid conditions, such as cardiovascular disease, neuropsychological disorders, and metabolic syndrome. Recent studies have focused on developing effective treatments and potentially finding cures. Several potential drug targets have been identified for IBD therapy, including gut bacteria and gut barrier. Studies illustrate that gut bacteria produce different kinds of neurotransmitters, e.g., acetylcholine (ACh). ACh can bind to α7 nicotinic acetylcholine receptor (α7 nAChR) on the intestinal macrophages, reducing the production of inflammatory cytokines, thereby inhibiting the intestinal inflammatory response. Besides, the gut microbiome activates the cholinergic anti-inflammatory pathway (CAP) via activating α7 nAChR in intestinal epithelial cells, as well as altering the expression level of acetylcholinesterase (AChE). Here, IEC-6 cell line

was employed as the intestinal epithelial cell model. The expression profiles of cholinergic molecules, like AChE, AChR, and choline acetyltransferase were determined in cultured IEC-6 cells. LPS/TNF-α was applied to induce inflammatory responses in the cultures. The expression and enzymatic activity of AChE were altered in LPS/TNF-α-induced cultured IEC-6 cells. The related inflammatory signaling pathways, e.g., NF-κB/CREB pathways, were involved in this process. ACh, secreted by the gut microbiome, could regulate the LPS-induced inflammatory responses, including the production of inflammatory cytokines via activating α7 nAChR. The result suggested that ACh, released by gut bacteria, could inhibit intestinal inflammation and subsequently alleviate the symptoms of IBD by activating CAP and/or regulating AChE expression in intestinal cells. The study provides potential preventive and therapeutic targets for IBD treatments.

PO-44

Studying the Expression and Regulation of AChE in Multiple Cancers Using Data-Driven Approach

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Introduction: A growing body of in vitro and in vivo studies has shown that the expression levels of acetylcholinesterase (AChE) are associated with the development of a variety of cancers. However, the association between AChE expression and cancer development and prognosis has not been revealed from a pan-cancer perspective. Therefore, in order to clarify the expression of AChE in cancer tissues to find clues for its atypical functions in cancers, it is necessary to conduct a comprehensive bioinformatics analysis of pan-cancer datasets based on a data-driven approach.

Methods: The expression data of AChE in a variety of cancers were extracted from online websites and databases such as HPA, TIMER2, GEPIA2, cBioPortal, UALCAN, STRING, and Sangerbox. Pan-cancer patient datasets were analyzed to reveal AChE expression signatures, map protein-protein interactions, and co-expression networks in cancers. Furthermore, correlation between AChE expression and cancer prognoses was also predicted by survival analysis.

Results: The pan-cancer analysis showed that AChE mRNA expression was significantly downregulated in 9 cancers including cervical cancer, acute myeloid leukemia lung squamous cell carcinoma, and ovarian cancer; while upregulated in 6 cancers including stomach cancer, pheochromocytoma & paraganglioma, kidney papillary cell carcinoma, and thymoma. By gene set enrichment analysis, co-expressing genes of AChE can be identified. Taking breast cancer as an example, the co-expression genes were found to be mainly involved in extracellular structure organization and positive regulation of cell activation and adhesion. Survival analysis showed that relatively higher AChE expression was significantly associated with better overall survival (OS) in patients with melanoma, ovarian cancer, and ocular melanoma, whereas with a poor OS in endometrioid cancer, adrenocortical cancer, and mesothelioma.

Conclusions: Our study highlights the potential of AChE as a novel biomarker for predicting prognosis across different human cancers.

PO-45

Advances in the development of new drugs against Alzheimer's disease based on tacrine scaffold

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Current symptomatic pharmacotherapy of Alzheimer's disease is primarily focused on acetylcholinesterase inhibitors and NMDA (N-methyl-D-aspartate) receptor blocking. Tacrine, a molecule with both of the above mechanism of action was withdrawn from clinical use in 2013, mainly due to drug-induced liver injury. The culprit of tacrine-associated hepatotoxicity is believed to be 7-OH-tacrine metabolite, a possible precursor of quinone methide, which binds to intracellular -SH proteins. With regard to this toxicity pathway, a new selective HPLC-MS/MS method for monitoring of metabolites (especially for 7-OH-tacrine) formed from tacrine derivatives was developed. Further, we would like to find out if rational structure modifications of tacrine derivatives are less prone to quinone methide formation. For this purpose the introduction of methoxy- or phenoxy- group to position 7 or chlorine into position 6 on the tacrine moiety was done to form 7-methoxy-, 7-phenoxy-, and 6-chloro-tacrine. These substitutions may potentially hinder the formation of toxic species.

Using our newly developed HPLC-MS/MS method we proved that tacrine and 7-methoxytacrine were primarily hydroxylated to 7-OH-tacrine, whereas 7-phenoxytacrine formed only trace amounts. Surprisingly, our study showed that 7-OH-tacrine which was predicted the most toxic according to the quinone methide hypothesis, was experimentally determined as the least hepatotoxic (7-OH-tacrine < tacrine < 7-methoxytacrine < 7-phenoxytacrine) after incubation with primary human hepatocytes. These results were corroborated by mass spectrometry, which did not identify traces of quinone methide or -SH adducts formation by cysteine quantification. Based on our results we suggests that 7-OH-tacrine and quinone methide formation is not the mechanism causing tacrine toxicity as previously hypothesized. Finally, we suggested via primary human hepatocytes, a new toxicity pathway, which could bring a new insight to further development of tacrine-based compounds.

This work was supported by the Grant Agency of the Czech Republic [No. 23-07570S], and by Charles University [Nr. SVV 260 547].

PO-46

Synthesis of a multifunctional compound targeting neuroinflammation and cholinergic deficit in Alzheimer's disease

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Neurodegenerative diseases, including Alzheimer's disease (AD), pose a serious healthcare threat with the staggering number of patients diagnosed with these disorders. The therapy is currently limited to palliative care and relief of the symptoms with numerous novel drugs in the pipeline. Among these, multifunctional ligands are focused on targeting multiple pathologies involved in the AD progression. The main aim of this study is to design and synthesized novel anti-AD agent able to simultaneously target neuroinflammation by inhibiting p38 mitogen-activated protein kinase α (p38 MAPKα or p38α) and cholinergic deficit by inhibiting butyrylcholinesterase (BChE). That means this compound should belong to a multi-target-directed ligands (MTDLs) group. Two compounds were developed for structure optimization towards designed lead molecule. First is selective p38α inhibitor (Ki = 101 nM) and second compound is selective BChE inhibitor (Ki = 11.1 pM). The newly designed compound has better physical-chemical and biological properties. For its in vivo evaluation, we have optimized synthetic routes of the lead compound by reducing the number of synthetic steps and increasing yields of target molecule. This work was supported by Czech Science Foundation

(No. GF23-42701L).

PO-47

Dual inhibitors targeting BChE and p38α MAPK: a novel strategy for Alzheimer's disease therapy

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INTRODUCTION:

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the main cause of dementia. Currently, available therapeutic options for AD remain scarce with six drugs on the market, three of which are small-molecule cholinesterase inhibitors. As the efficacy of these drugs is limited to mild-to-moderate dementia and adverse effects such as vasogenic edema, hinder the progress of novel biological treatments against amyloid beta (Aβ) in clinical trials, the development of innovative small-molecule drugs targeting key proteins involved in the early pathophysiology of AD presents a daunting task for medicinal chemists. Although numerous hypotheses attempt to elucidate the intricate mechanisms underlying pathophysiology of AD, they invariably converge on the extensively studied neuroinflammation hypothesis. According to this hypothesis, the presence of Aβ plaques alongside phosphorylated tau proteins leads to hyperactivated microglia, triggering the expression of various enzymes that exacerbate the imbalance between anti- and proinflammatory cytokines. Among the many enzymes that are overactivated p38α MAP kinase (p38α MAPK) has recently gained more attention. This ubiquitously expressed enzyme enhances the production of proinflammatory cytokines such as TNF-α and IL-1β, facilitates Aβ accumulation, and catalyzes the hyperphosphorylation of neurotoxic tau proteins (4). This in turn makes it an interesting pharmacological target to combat AD. Our aim is to develop a dual inhibitor that targets both the neuroinflammatory pathway and the traditional cholinergic hypothesis. The latter states that forebrain cholinergic neuron loss is characteristic of AD. By inhibiting the hydrolytic action of cholinesterases, in particular butyrylcholinesterase (BChE) we may augment the activity of surviving cholinergic neurons. This multifaceted approach seeks to address the complex interplay of neuroinflammation and cholinergic dysfunction, offering a promising strategy for AD intervention.

RESULTS AND DISCUSSION: First, a library of small molecules with confirmed activity against p38α MAPK was generated. The compounds were categorised into 30 clusters according to their molecular fingerprint and docked into the BChE active site gorge. Of the best docked ligands ARRY-371797, a p38α MAPK inhibitor from Pfizer Inc. showed very promising activity against BchE. This molecule was then subjected to further optimization leading to two different series of compounds.

PO-48

Berberine in comparison to 7-MEOTA for Alzheimer's treatment: a polypharmacological approach

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Introduction

Alzheimer's disease (AD) is characterized by multiple pathophysiological mechanisms like cholinergic neuronal damage, abnormal accumulation of β-amyloid forming senile plaques, tau hyperphosphorylation, neuroinflammation, and oxidative stress. The complexity of these mechanisms has hindered

the discovery of an effective treatment for AD. Phytochemicals are bioactive compounds found in fruits, vegetables, and grains, and are known to reduce the risk of major diseases. Berberine, a plant alkaloid, has been reported to be beneficial in various pathological conditions.

Methodology

This study aimed to evaluate the cholinergic inhibitory activity of berberine against human RBC-AChE and human plasma BChE. To assess the polypharmacological effects of berberine and 7-MEOTA, thirteen different receptors like AChE, BChE, MAO-A, MAO-B, Amyloid β, Apo-E, α7, α4β2, Catalase, Glutathione Reductase, β-Secretase, Superoxide Dismutase, and P38α MAPK were selected for molecular docking using standard softwares. Results were compared with 7-Methoxytacrine-adamantylamine (7-MEOTA).

Results

The results showed that berberine inhibited RBC-AChE and BChE at millimolar concentrations, with IC50 values of 0.20 mM for RBC-AChE and 7.24 mM for BChE, compared to 0.50 mM and 1.08 mM for 7-MEOTA, respectively. Computational polypharmacology offers the ability to predict the activity profile of a ligand against a set of targets using various computational approaches. Molecular docking indicated that berberine had low binding energies of -8.9 kcal/mol and -8.3kcal/mol, demonstrating higher binding affinities for AChE and BChE compared to the 7-MEOTA. Additionally, berberine exhibited good binding interactions with Apo-E, the α7-nAChR receptor, glutathione reductase, β-secretase, P38α MAP kinase, and superoxide dismutase, outperforming 7-MEOTA in these interactions.

Conclusions

Berberine is found to be better than 7-MEOTA and may be used as an adjunct in the treatment of AD. Further in silico studies with analogues of berberine and 7-MEOTA is suggested followed by experimental studies with potentially good analogues.

PO-49

Novel amiridine-based multi-target directed ligands for the Alzheimer's disease treatment

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Alzheimer's disease (AD) is a complex disorder with significant economic impact. Current treatment offers only temporary delay in disease progression. The complexity of AD and its unknown etiology are the major obstacles for development of new therapeutic treatment. As single-target therapies have proven not effective, rational specific-targeted combination into a single molecule represent more promising approach for treatment of AD. Consequently, a new class of potential drugs called multi-target directed ligands (MTDLs) has been introduced as an alternative option to counter the AD. Within our contribution, we will present new concept of MTDLs pursued by a rationally designed series of small molecules based on amiridine linked with memantine or benzothiazole scaffolds, simultaneously targeting impaired cholinergic and glutamatergic neurotransmission, along with antioxidant properties. The putative targets of these molecules are cholinesterases and NMDA receptors.

This study was supported by the Czech Science Foundation grant no. 22–24384S.

PO-50

Investigating the effects of basketball and volleyball as team sports on social cognitive function and BDNF and cholinesterase levels in young men and women

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Neurogenesis is the production of new neurons in areas of the brain such as the hippocampus. In recent years, studies have shown that exercise is one of the strongest promoters of neurogenesis in the human brain. Brain-derived neurotrophic factor (BDNF) and acetylcholine (ACh) are two regulators of synaptic plasticity, neuronal survival and differentiation. The relationship between physical activity and fitness, cognitive function and academic performance is of great interest but remains unclear. Therefore, this study further examined the effect of two of the world's most popular physical activities on cognitive development in sedentary men and women from the medical school basketball (n=16) and volleyball (n=12) teams who participated in a tournament for 6 months. Social cognitive function tests (emotion recognition test) were performed and blood samples were taken before and after the tournament. BDNF level and cholinesterase level were measured in blood by ELISA and Ellman assay, respectively. Our results showed that the BDNF level increased in male basketball players (p=0.1611) and volleyball players (p=0.0547), while it decreased significantly in female basketball players (p=0.0313). Total cholinesterase and acetylcholinesterase levels increased only in basketball players (p=0.1613), especially in male participants (p=0.0967), while no changes were observed in volleyball players. The results of the emotion recognition tests showed that there was a time effect in the recognition of sadness (F=5.004; p=0.031) and an increase in both the basketball and volleyball groups. This study showed that team sports such as basketball and volleyball altered acetylcholine (and thus cholinesterase) and BDNF levels, which are associated with cognitive function, and that this effect was particularly pronounced in men.

PO-51

Design of AChE reactivators using versatile molecular platforms and nanodiamonds

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The currently available antidotes against toxic organophosphorus compounds suffer from poor permeability across the blood-brain barrier (BBB) and are limited in their ability to restore the activity of the inhibited acetylcholinesterase (AChE) in the central nervous system (CNS). Another requirement nowadays is to target a synergy between principles of green chemistry and chemical disarmament and nonproliferation.

We report two novel approaches to addressing this requirement.

The L-Phe-derived versatile molecular platform was chosen as a prospective scaffold for design of sustainable products with different functionality. Their oxime derivatives are the sustainable AChE reactivators demonstrating remarkable activity against the AChE inhibited by VX, compared with, or exceeding those for2-PAM and obidoxime. The regularities on antidotal activity, cell viability, plasma stability, biodegradability as well as molecular docking study of the newly synthesized oximes are being analyzed for further improvement of their structures.

We designed functionalized detonation nanodiamond (ND) nanocarrier platforms to transport quaternary oxime moiety bound to a biocompatible linker covalently attached to the nanodiamonds. The ND-based AChE reactivators crossed Madin-Darby Canine Kidney (MDCK) cells and demonstrated a

dose-independent in vitro reactivation capacity towards human AChE inhibited by GB, VX, and paraoxon. Visualization of tight junctions and actin cytoskeleton in the MDCK and HUVEC assays points to a cell type-dependent internalization pathway of ND-based reactivators. The results reveal the potential of detonation nanodiamonds as a promising delivery platform for charged therapeutic agents to CNS, aimed to restore the activity of the inhibited AChE and enhance treatment outcomes in organophosphorus poisoning.

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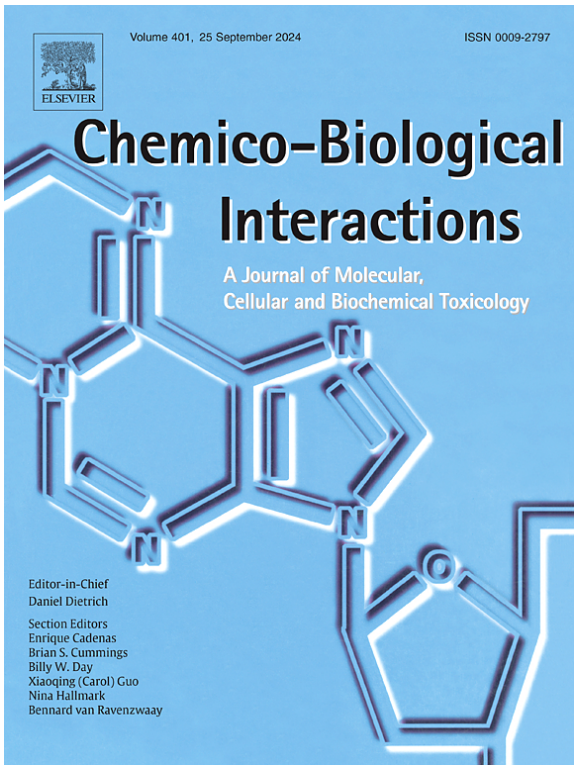

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