Mini-symposium

Multidisciplinarity in drug development

12 February 2025, Institute for Medical Research and Occupational Health, Zagreb, Croatia

Organised by

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BioMol4ND

HrZZ-IP-2020-02-9343

Development of bioactive molecules for neurodegenerative diseases treatment – BioMol4ND

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PROGRAMME

8:30−9:00	**Welcome, introduction, and registration**
9:00−9:20	**Anita Bosak** Development of bioactive molecules for neurodegenerative disease treatment
9:20−9:40	**Marija Bartolić** Multi-target approach to the treatment of Alzheimer's disease: revival of amyloid theory
9:40−10:00	**Ana Matošević** Development of carbamate-based compounds as potential cholinesterase inhibitors for the treatment of Alzheimer's disease
10:00−10:20	**Katarina Komatović** Adamantane derivatives of 4-aminoquinoline as inhibitors of hAChE and hBChE: design and synthesis
10:20−10:40	**Antonio Zandona** Cytotoxicity of aminoquinoline- and carbamate-based inhibitors of cholinesterases
10:40−11:00	**Suzana Žunec** Antioxidant testing in development of multifunctional drug candidates for neurodegenerative diseases
11:00−11:20	**Sandra Šegan** QSAR Insights in 4-aminoquinoline derivatives as AChE and BChE inhibitors
11:20−11:40	**Danijela Barić** Computational modelling of BChE carbamylation
11:40−12:20	**Coffee break**
12:20−12:40	**Silva Katušić** Endolysosomal dysfunction − a common initiator of Alzheimer's disease and Niemann-Pick type C disease
12:40−13:00	**Tomica Hrenar** Deep reinforcement learning protocol for quantum-chemical multiple ligand docking to cholinesterases
13:00−13:20	**Nikolina Maček Hrvat** Reducing sarin-induced gliosis in mice by oxime therapy
13:20−13:40	**Anamarija Knežević** Supramolecular assemblies in water for organic synthesis and other applications
13:40−14:00	**Tena Čadež** Targeting organophosphorus poisoning by pseudocatalytic detoxification system
14:00−14:10	**Anita Bosak** Looking ahead
14:10	**Lunch break and discussion**

Mini-symposium:

Multidisciplinarity in drug development

The Mini-symposium "Multidisciplinarity in drug development" was held at the Institute for Medical Research and Occupational Health in Zagreb on February 12, 2025. The main goal of the Symposium was to share and discuss the results obtained within the project HrZZ-IP-2020-02-9343. Organization of the Symposium was supported by the Croatian Science Foundation (grant no. HrZZ-IP2020-02-9343, BioMol4ND) and by the Institute for Medical Research and Occupational Health. Also, the organization was sponsored by the companies Shimadzu, Biovit, Crux, and Medic. A total of 35 participants attended the Symposium, including PhD students and postdoctoral researchers, as well as experienced scientists, experts in their specific fields.

The scientific programme of this mini-symposium was divided into two sessions where the outcomes of four-year research project were presented. The presentation topics covered drug design, most effective routes of synthesis of tested compounds, biological evaluation of a compound's activity towards targets included in Alzheimer's pathophysiology, and *in silico* evaluation of ligand-enzyme interactions including molecular modelling, QM and MM, as well as QSAR analysis. The presented results gave the participants opportunity for fruitful discussion and opening new possibilities for collaborations and ideas for future studies. We were very pleased to host eminent scientists Silva Katušić, Danijela Barić, and Anamarija Knežević from the Ruđer Bošković Institute, Zagreb, as well as Prof Tomica Hrenar from the University of Zagreb Faculty of Science, who delivered inspiring lectures presenting new and interesting findings in areas of neurodegenerative diseases, application of QM/MM in evaluation of results obtained by enzyme kinetic, possibilities of application of supramolecular assemblies for drug delivery, and use of machine learning as tool for analysis of chemical multiple ligand docking. The Symposium has been a rewarding scientific and personal experience for us. We express our sincere thanks to all of the speakers and participants for sharing their research, and thereby contributing to the excellence of our scientific program. We thank the Croatian Science Foundation and the Institute for Medical Research and Occupational Health for their support, and the sponsors whose contribution was invaluable to the overall success of the Symposium.

SYMPOSIUM ABSTRACTS

Development of bioactive molecules for neurodegenerative disease treatment (BioMol4ND)

Anita Bosak
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Project "Development of bioactive molecules for neurodegenerative disease treatment", financed by the Croatian Science Foundation, started in 2021. The primary goal of the project was the development of compounds with the potential to alleviate symptoms and/or slow down the progression of neurodegenerative diseases, particularly Alzheimer's disease. Realization of the project called for a multidisciplinary approach and within the project we designed and synthesised compounds, determined their biological activities towards distinct hallmarks of AD, and conducted a comprehensive structure-activity analysis using *in silico* methods such as molecular modelling, QSAR and machine learning. The project team successfully completed all of the tasks set in the work plan. Until now, project results were published in 10 scientific papers, 26 symposium abstracts, 1 doctoral thesis, 2 diploma thesis, and one leaflet intended for the general population. Team members presented project results with one plenary, 4 invited and 11 lectures on international and domestic meetings or workshops. Young team members were the recipients of several valuable domestic and international grants (FEBS, EMBO, CCS), and in 2023 doctorand M. Bartolić received an Annual award for young medicinal and pharmaceutical chemists. During the course of the project, team members participated in numerous specialized trainings to obtain new knowledge and skills, but they also transferred their knowledge by mentoring student interns and bringing science closer to the wider population. The presented results show the success of this project, and taken all together, we have obtained a strong background giving us the needed input for further research.

Acknowledgements: this work was supported by the Croatian Science Foundation under project no. HrZZ-IP-2020-02-9343.

Multi-target approach to the treatment of Alzheimer's disease: revival of amyloid theory

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Alzheimer's disease (AD) is a progressive neurodegenerative disease that predominantly affects people over 65 years of age, which, due to the rapid growth and general aging of the world population, requires the development of new treatments that could prevent or slow down the onset of the disease, as well as alleviate the symptoms of cognitive decline. The design of multi-targeted ligands (MTDLs) that have the ability to target several hallmarks of AD, most often the lack of the neurotransmitter acetylcholine, the production and self-aggregation of amyloid peptides and hyperphosphorylated tau protein, the imbalance of biometal concentrations and/or the production of reactive oxygen compounds, proved to be a promising answer. In our study, we investigated the potential of three structurally different groups of compounds (aminoquinolines, *O*-alkyl oximes and hydrazones) to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) which breakdown acetylcholine, BACE1, an enzyme involved in the production of amyloid peptides, and self-aggregation of amyloid peptides. Aminoquinolines were shown to be the most potent AChE and BChE inhibitors, with inhibition constants K_i in low micromolar and nanomolar range, while hydrazone-based compounds exhibited considerable ability to inhibit both BACE1 and amyloid self-aggregation in micromolar concentrations suggesting that hydrazone-based compounds present promising scaffold for the development of drugs aimed at amyloid aggregation. According to results, a combination of hydrazone moiety with either aminoquinoline or oxime moiety could be a promising strategy for development of MTDLs targeting both cholinesterases and amyloid production.

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Development of carbamate-based compounds as potential cholinesterase inhibitors for the treatment of Alzheimer`s disease

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Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder characterized by a decline in cognitive functions. It is ranked as the seventh leading cause of death and is the most common cause of dementia among older population. Despite the multifactorial nature of AD, current treatments primarily aim to restore cognitive functions by increasing the concentration of the neurotransmitter acetylcholine (ACh) through the inhibition of acetylcholinesterase (AChE) and, to a lesser extent, butyrylcholinesterase (BChE). Carbamates have become a desirable structural moiety in the drug design due their good pharmacological properties, and they represent an important structural motif in drugs currently or previously in use for AD treatment, where they act as cholinesterases inhibitors and displayed significant positive effects on cognitive decline symptoms. In our study, we designed and synthesized twenty-five 3,5-(2-amino-1-hydroxyethyl) phenol biscarbamates, fourteen 3-(2-amino-1-hydroxyethyl) phenol carbamates and thirteen quinuclidine-based carbamates and determined their inhibitory potential toward both cholinesterases and their inhibition selectivity. The ability of carbamates to cross the blood–brain barrier by passive transport, their cytotoxic profile, their antioxidant capacity and their ability to chelate biometals were also evaluated. Our results confirmed that carbamates are promising candidates for further development as drugs for AD treatment.

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Adamantane derivatives of 4-aminoquinoline as inhibitors of hAChE and hBChE: design and synthesis

Katarina Komatović[1], Nataša Terzić-Jovanović[2], Mario Zlatović[1], Ana Matošević[3], Nikola Maraković[3], Dejan Opsenica[2], and Anita Bosak[3]
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4-Aminoquinolines (4AQ) are the most prominent family of compounds derived from quinoline heterocycles with a broad spectrum of biological activities, among which is the inhibition of cholinesterases in the central nervous system due to their structural similarity to tacrine, an anticholinesterase drug for the treatment of Alzheimer's disease. Based on the previous findings that 4AQ with adamantyl group (Ad) expressed promising activity against hAChE and hBChE, we designed and synthesized a series of 4AQ-Ad derivatives. We combined different synthetic strategies for obtaining 4AQ-Ad compounds. In general, we gradually built side chains and coupled the adamantyl group in the final step via reductive amination reaction. Alternatively, the previously synthesized side chain containing Ad was used in the coupling reaction with the corresponding 4-chloroquinoline. Among the derivatives with diverse linker structures, between 4AQ and Ad subunits, in terms of length, conformational flexibility, steric requirements, basicity and electronic density, the compound possessing the *n*-octylene group was singled out as the most active and motivated a new set of 4AQ-Ad compounds with this preferred linker. First, derivatives differing in the encirclement of the terminal amino group were synthesized by linking benzyl or various heterocyclic structures via reductive amination. Also, Ad moieties functionalized with substituents with different physicochemical properties, such as hydroxy, bromine or acetamido groups were introduced.

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Cytotoxicity of aminoquinoline and carbamate cholinesterase inhibitors

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The development of bioactive molecules targeting neurodegenerative diseases, particularly Alzheimer's disease, has gained significant attention due to the increasing prevalence of these conditions. Cytotoxicity of over 100 synthesized aminoquinoline and carbamate-based cholinesterase inhibitors was determined in three human cell lines: HEK293 (embryonic kidney), HepG2 (hepatocarcinoma), and SH-SY5Y (neuroblastoma). The aim was to evaluate the therapeutic potential of these compounds while ensuring minimal off-target cellular toxicity. Cytotoxicity was assessed using IC_{50} values, representing the concentration at which 50% of cell viability was reduced. Results demonstrated that the IC_{50} values of the tested inhibitors were generally 10−1000 times higher than the concentrations required to achieve approximately 80% inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity. This indicates a favourable therapeutic index, suggesting that these compounds can effectively inhibit cholinesterase activity without exerting significant cytotoxic effects at therapeutic doses. The findings highlight the potential of aminoquinoline and carbamate derivatives as promising candidates for further preclinical and clinical studies aimed at treating Alzheimer's disease. Future research will focus on optimizing selected structures for enhanced selectivity and reduced toxicity. These efforts contribute to the broader goal of developing novel treatments to address the unaddressed medical challenges of neurodegenerative disorders.

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Antioxidant testing in development of multifunctional drug candidates for neurodegenerative diseases

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Neurodegenerative diseases, such as Alzheimer's and Parkinson's, are characterized by progressive neuronal loss and are strongly associated with oxidative stress and inflammation. These conditions lead to cellular damage and exacerbate disease progression, creating a feedback loop that leads to neurological decline. Given that endogenous antioxidants serve as biological response modifiers by regulating oxidative stress within the cellular redox cycle, the supplementation of exogenous antioxidants become a wide spread practice to the prevalence of oxidative stress-mediated diseases. Therefore, utilizing the multitarget-directed ligands (MTDLs) approach in drug design, we focused on developing multifunctional molecules with antioxidant properties, presenting a strategic avenue for addressing the complex and multifaceted pathology of neurodegenerative diseases. In term of structure characteristics usable in MTDL approach, ligands with high potential for cholinesterase inhibition activity were identified as the most promising starting pharmacophores. Aminoquinoline-based adamantanes, 4-aminoquinoline derivatives with an *n*-octylamino spacer and derivatives of amodiaquine were tested for their *in vitro* antioxidant activity using Ferric reducing antioxidant power (FRAP) assay. The reducing capacity of novel derivatives was determined for compound concentrations selected according to the inhibition constants (K_i) determined for acetylcholinesterase and butyrylcholinesterase, and compared to standard antioxidants Trolox and butylated hydroxytoluene (BHT). Almost all of the tested compounds exhibited measurable antioxidant activity in a homogeneous *in vitro* system, contributing to the expansion of their biological profile.

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QSAR insights in 4-aminoquinoline derivatives as AChE and BChE inhibitors

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Using reversed-phase thin-layer chromatography, lipophilicity parameters were experimentally determined for 38 investigated 4-aminoquinolines and compared with *in silico* lipophilicity values (logP) and adsorption/distribution parameters (HIA, WS, Caco-2, MDCK, BBB, QPlogKhsa, PPB, Vd, Fu) *via* principal component analysis (PCA), revealing a strong correlation. PCA was also performed to explore the grouping of compounds based on their structural characteristics and biological activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition. The score plot revealed the clustering of compounds according to their structural features, while the loading plot highlighted the structural descriptors responsible for this clustering. In a specific region of the graph (negative PC1 and positive PC2 values), the inhibitory activity of the compounds against AChE and BChE was positioned alongside topological descriptors. Partial least squares (PLS) regression was conducted using experimentally determined lipophilicity descriptors and calculated structural descriptors (physicochemical, quantum-chemical, constitutional, and topological parameters) as independent variables, while the inhibition activities of AChE and BChE, expressed as log1/C, were used as dependent variables. From an initial set of 123 structural descriptors, only the most relevant ones were retained for the final model. The resulting PLS models for AChE and BChE inhibition demonstrated strong statistical performance: $R^2Cal = 0.82$, $R^2CV = 0.70$, $R^2pred = 0.59$ for AChE, and $R^2Cal = 0.78$, $R^2CV = 0.56$, $R^2pred = 0.72$ for BChE. These mathematical models emphasize the critical role of topological descriptors in determining the biological activity of the studied compounds.

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Computational modelling of BChE carbamylation

Danijela Barić[1], Ana Matošević[2], Nikola Maraković[2], Alexandre Igert[3], Xavier Brazzolotto[3], and Anita Bosak[2]
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Neurological disorders like Alzheimer's disease (AD) are characterised by diminished cholinergic activity due to reduced levels of the neurotransmitter acetylcholine (ACh), leading to dementia. The pharmacological treatment of AD involves the controlled, moderate inhibition of two serine hydrolases – acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) – to sustain adequate ACh levels in the brain. In advanced stages of AD, AChE levels decline while BChE levels rise significantly, making the selective inhibition of BChE an effective approach. Carbamates have emerged as a potent class of selective BChE inhibitors, capable of covalently binding to the enzyme's active site. Here, we investigated the mechanism of the initial step in the covalent binding of a selected biscarbamate compound from a set of 13 previously synthesised and validated selective BChE inhibitors (1). These biscarbamates demonstrated inhibitory activity against wild-type human BChE but also against one of the clinically relevant variants of the enzyme, atypical BChE with Asp70Gly mutation. For both enzyme variants, computational modelling provided insights into the structures of the non-covalent enzyme-inhibitor complex, the transition state leading to the covalently bound biscarbamate, and the unstable and short-living tetrahedral intermediate before the final product of carbamylation occurs. The quantum-mechanical calculations on model systems comprising the enzyme's active site and ligand revealed the reaction energetics, enabling the interpretation of experimentally observed data and elucidating the influence of Asp70 in the active site on reaction kinetics.

1. Matošević A, et al., Pharmaceuticals 2022;15:1220.

Endolysosomal dysfunction – a common initiator of Alzheimer's disease and Niemann-Pick type C disease

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Niemann-Pick type C disease (NPC) is a rare inherited lipid and lysosomal storage disorder caused by mutations in NPC1 or NPC2 gene. It is characterized by accumulation of cholesterol in late endosomes / lysosomes leading to progressive neurodegeneration (of primarily Purkinje neurons in the cerebellum) and neuroinflammation. It is intriguing that this rare monogenic disease shares features with the most common neurodegenerative disorder, Alzheimer's disease (AD), including accumulation of amyloid-β peptides, hyperphosphorylation of tau and activation of microglia and astrocytes. The focus of our work is: 1) to elucidating molecular details that are shared between AD and NPC, especially related to endolysosomal dysfunction, 2) to identify the earliest changes that lead to neurodegeneration and/or neuroinflammation and 3) to investigate the interrelationship between the two processes in both NPC and AD. Our results suggest that dysfunction of the endolysosomal pathway may be a key factor involved in AD-like phenotype in NPC disease causing enhanced proteolysis by a key Alzheimer's protease BACE1 (ß-secretase). Inhibition of BACE1 in NPC1 primary mouse neurons and in NPC1 mouse organotypic brain slices as well as its genetic depletion in NPC1 mouse model ameliorated Purkinje cell loss, lysosomal dysfunction and activation of astrocytes. Our analysis of NPC1 mouse astrocytes (GFAP) and microglia (CD68) suggests that neuroinflammation likely precedes neurodegeneration in NPC disease. Proteome profiling of acutely isolated microglia and astrocytes identified differentially expressed markers, suggesting the cell-specific functions of NPC1 protein in different brain cells. Our findings suggest a multi-targeted approach to combat NPC disease.

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Deep reinforcement learning protocol for quantum-chemical multiple-ligand docking to cholinesterases

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This docking study aims to find the best inhibitor of BChE as well as the best common inhibitor of AChE and BChE by quantum-chemical multiple-ligand simultaneous docking (MLSD). Instead of docking single (but complex) molecules as potential inhibitors, we changed the paradigm of investigation by docking multiple smaller molecular scaffolds which subsequently can be combined into a larger molecule by smart organic synthesis. Since MLSD complexity is proportional to the total number of small molecules docked (translational, rotational, and conformational degrees of freedom), systematic search was not feasible, and we needed a refreshed approach to this problem. Hence, we developed a parallelized Monte Carlo algorithm for sampling the big-data configurational spaces. This procedure, which included simultaneous docking of an arbitrary number of ligands using the smart structure generator was used for pre-screening and building of inputs. The electronic energies of the system (complete enzyme and all docked ligands) were calculated using the time-consuming quantum chemical methods. To accelerate this segment of the study, potential energy surfaces for the configuration spaces were gradually described by trained deep neural networks using the deep reinforcement learning algorithm and afterward used to calculate binding energies. Coverage of the active site was monitored on-the-fly during the docking simulations.

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Reducing sarin-induced gliosis in mice by oxime therapy

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Organophosphate compounds (OP) like the nerve agent sarin induce gliosis and a loss of neuronal cell viability, leading to neuroinflammation. Acetylcholinesterase inhibition by OPs results in accumulation and a prolonged residence time of the neurotransmitter acetylcholine, which in turn induces electroencephalographic seizures followed by motor convulsions that can quickly progress to status epilepticus and even more severe conditions, all of which contribute to mortality and brain damage in survivors. In this study, we investigated the effect of centrally acting oxime therapy on reducing gliosis and preserving neuronal viability in mice. We examined the levels of specific proteins expressed in glial and neuronal cells in the brains of sarin-exposed mice, in mice treated with oxime RS194B or pyridinium oxime 2PAM following sarin exposure, and compared them to untreated control mice. The microglial response was assessed through the expression of the ionized calcium-binding adapter molecule 1 (IBA-1), while astrogliosis was evaluated by measuring the levels of glial fibrillary acidic protein (GFAP). Neuronal viability was indicated by immunoreactivity to the neuronal nuclei antigen (NeuN). The results showed that RS194B therapy in mice reduced sarin-induced neurotoxicity, indicating the neuroprotective potential of RS194B oxime.

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Supramolecular assemblies in water for organic synthesis and other applications

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Over the past few decades, the self-assembly of amphiphilic molecules has emerged as a powerful strategy for designing advanced functional nanomaterials, with applications ranging from smart materials for drug and gene delivery to bioimaging, environmental remediation, and surface coating. In particular, supramolecular assemblies in water have gained significant attention due to their ability to form dynamic, well-organized structures through noncovalent interactions such as hydrogen bonding, π-π stacking, and hydrophobic effects. Among amphiphiles, non-ionic amphiphilic systems offer advantages such as biocompatibility, stability, and tunability, making them promising candidates for advanced technological applications. In aqueous media, amphiphilic molecules spontaneously self-assemble into nanostructures with hydrophobic interior, such as micelles, vesicles, and nanofibers, which can function as molecular reaction chambers, mimicking enzymatic catalysis, or as reservoirs for various drugs. These self-assembled systems enhance drug solubility, protect therapeutic agents from degradation, and enable controlled and targeted drug release. Thus, supramolecular assemblies provide versatile platforms for biomedical applications and responsive materials. This lecture will present our recent findings on the design and synthesis of a new generation of non-ionic amphiphilic molecules and their self-assembled supramolecular structures in aqueous environments. It will cover the synthesis of these non-ionic amphiphiles, along with the characterization of their supramolecular assemblies using spectroscopic, calorimetric and microscopic techniques. Furthermore, the development of supramolecular pH-responsive systems, the formation of micellar and tubular systems, and their applications in organic synthesis will be discussed, highlighting their potential for innovative and sustainable chemical processes.

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Targeting organophosphorus poisoning by pseudocatalytic detoxification system

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Organophosphorus nerve agents (NA) induce toxicity by inhibiting acetylcholinesterase (AChE) and leading to overstimulation of cholinergic system. Related enzyme butyrylcholinesterase (BChE) acts as a bioscavenger, reducing NA availability and mitigating AChE inhibition. In this study, a library of over 100 oximes was screened, identifying several pyridinium-based compounds with high BChE reactivation efficiency, including one with a reactivation rate of 34,120 M^{-1} min^{-1} against cyclosarin-inhibited BChE. Ex vivo blood analysis demonstrated that the pseudocatalytic detoxification system, combining exogenous BChE with identified pyridinium-based oximes, enabled rapid OP degradation, restored cholinesterase activity, and preserved neural cell viability in vitro. Additionally, a dual-reactivator approach, in pseudocatalytic system, targeting both BChE and AChE further enhanced detoxification efficacy in whole human blood supplemented with NA. These findings support the development of bioscavenging BChE-oxime therapy as a promising strategy for counteracting NA exposure.

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Looking ahead

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In the near future, at least six more scientific papers reporting the project's results should be published, three of them in 2025., and two more doctoral theses are planned to be defended by the end of 2025. Analysis of the overall results of the project pointed out few compounds as very promising candidates for further evaluation as multi-targeting drugs for the treatment of Alzheimer's disease. Also, the comprehensive SAR and QSAR analysis, together with molecular modelling as visualization tool gave us valuable, results-driven guidelines for rationally design of dual site binding cholinesterase's inhibitors (acting on improving the acetylcholine level in brain and on Aβ aggregation) and their use as a starting point for MTDL design by adding an additional pharmacophore in single molecule entity able to act on other hallmarks of the Alzheimer's disease. Some specific results regarding structural scaffolds of compounds in this project gave us very solid base for further structural improvement and addition of new pharmacophores acting on additional targets involved in pathophysiology of Alzheimer's disease. To secure funding for future research, the project team plan to submitted a project proposal to Croatian Science Foundation or/and available European Union funds of interest. At the end of the Symposium, the principal investigator Anita Bosak, PhD expressed her gratitude to the research team, all collaborators and colleagues from the Institute, and other institutions who in any way helped to achieve the project's goals.

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