CROSSING THE BOUNDARIES OF MEDICINAL CHEMISTRY

BOOK OF ABSTRACTS

University of Ljubljana, Faculty of Pharmacy
September 28, 2023

ORGANISED BY

Sekcija za Farmacevtsko Kemijo
Slovenskega Farmacevtskega Društva
Section for Medicinal Chemistry
of the Slovenian Pharmaceutical Society

SECTION FOR MEDICINAL CHEMISTRY OF THE SLOVENIAN PHARMACEUTICAL SOCIETY

AND

Univerza v Ljubljani
Fakulteta za farmacijo

UL FACULTY OF PHARMACY

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PUBLISHER: University of Ljubljana, Faculty of Pharmacy

Kataložni zapis o publikaciji (CIP) pripravili v Narodni in univerzitetni knjižnici v Ljubljani
COBISS.SI-ID 169828611
ISBN 978-961-6378-95-6 (PDF)

Ljubljana, september 2023

Welcome to the Crossing the Boundaries of Medicinal Chemistry minisymposium

The Section for Medicinal Chemistry of the Slovenian Pharmaceutical Soviety, University of Ljubljana, Faculty of Pharmacy, and EATRIS (Node Slovenia) are pleased to welcome participants to the first minisymposium on medicinal chemistry and adjacent fields entitled *Crossing the Boundaries of Medicinal Chemistry*!

It has truly been our pleasure to put together the scientific programme for this minisymposium, and to hand-pick the speakers. The agenda of the minisymposium covers a wide range of topics selected to highlight the recent advancement in the field of medicinal chemistry and relevant adjacent fields. The programme is designed to provide interactive communication and exchange of experiences. We sincerely hope that you will find the symposium programme attractive and, above all, useful for your future work.

The symposium will be conducted in a live format, allowing for personal contact, face-to-face gathering and socializing. Committed to carry out the educational arm of the programme at the highest level possible, we will also do our best to encourage and support networking, teamwork and collaboration among the participants. To this end, we have planned a poster session accompanied by snacks and beverages, that we hope you will find useful and make the most of.

We wish you a warm welcome to University of Ljubljana, Faculty of Pharmacy!

Žiga and Tihomir

Timeline	Lecturer and title of talk
9.00 – 9.05	*Opening Address* **Prof. Dr. Žiga Jakopin and Prof. Dr. Tihomir Tomašič** Faculty of Pharmacy, University of Ljubljana, Slovenija
9.05 – 9.30	*From pyrroleamides to new balanced dual-targeting antibacterial compounds with limited resistance* **Prof. Dr. Lucija Peterlin Mašič** Faculty of Pharmacy, University of Ljubljana, Slovenia
9.30 – 9.55	*Battle of the E3 Ligases: Using heteroPROTACs to Induce Pan-IAP Degradation* **Assist. Aleša Bricelj, MPharm** Faculty of Pharmacy, University of Ljubljana, Slovenia
9.55 – 10.25	*Zebrafish in cancer modeling and drug screening* **Prof. Dr. Martin Distel** St. Anna Children's Cancer Research Institute, Vienna, Austria
10.25 – 10.50	*A rule of thumb for selection of compounds with viscosity-reducing effects on biopharmaceutical protein formulations* **Dr. Matic Proj** Sandoz, Slovenia
10.55 – 12.00	*Coffee break, snacks and poster presentation*
12.00 – 12.25	*Deciphering the kinetic pathways that determine aggregation and stability of therapeutic antibodies* **Prof. Dr. Jurij Lah** Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia
12.25 – 12.50	*Reaction mechanisms as a key for designing biomolecule modifications* **Assoc. Prof. Dr. Martin Gazvoda** Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia
12.50 – 13.15	*Dynamic kinetic resolution with Noyori–Ikariya asymmetric transfer hydrogenation: method development and applications* **Assist. Prof. Dr. Andrej Emanuel Cotman** Faculty of Pharmacy, University of Ljubljana, Slovenia
13.15 – 13.20	*Closing Address*

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 871096

Invited Lectures

FROM PYRROLAMIDES TO NEW BALANCED DUAL-TARGETING ANTIBACTERIAL COMPOUNDS WITH LIMITED RESISTANCE

Lucija Peterlin Mašič

University of Ljubljana, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Aškerčeva cesta 7, 1000 Ljubljana, Slovenia

Lecture in honour of Prof. Dr. Danijel Kikelj's contribution to the development of new antibacterial agents

There is a critical need for new therapies to treat deadly infections caused by Gram-negative ESKAPE pathogens—bacteria that are often resistant to available antibiotics. Only a handful of new treatments with the potential to address these serious threats are currently in development. We have developed new dual low nanomolar benzothiazole inhibitors of bacterial DNA gyrase and topoisomerase IV. The resulting compounds show excellent broad-spectrum antibacterial activities against Gram-positive *Enterococcus faecalis*, *Enterococcus faecium* and multidrug resistant *Staphylococcus aureus* strains (best compounds MICs: range, <0.03125–0.25 µg/mL), and against the Gram-negatives *Acinetobacter baumannii* and *Klebsiella pneumoniae* (best compounds MICs: range, 1–4 µg/mL). Lead compound was identified with favorable solubility and plasma protein binding, good metabolic stability, selectivity for bacterial topoisomerases, and no toxicity issues. The crystal structure in complex with *Pseudomonas aeruginosa* GyrB24 revealed its binding mode at the ATP-binding site. Expanded profiling of a lead compound showed potent antibacterial activity against over 100 MDR and non-MDR strains of *A. baumannii*, and several other Gram-positive and Gram-negative strains. Ultimately, *in vivo* efficacy in a mouse model of vancomycin-intermediate *S. aureus* thigh infection was also demonstrated (Fig. 1).

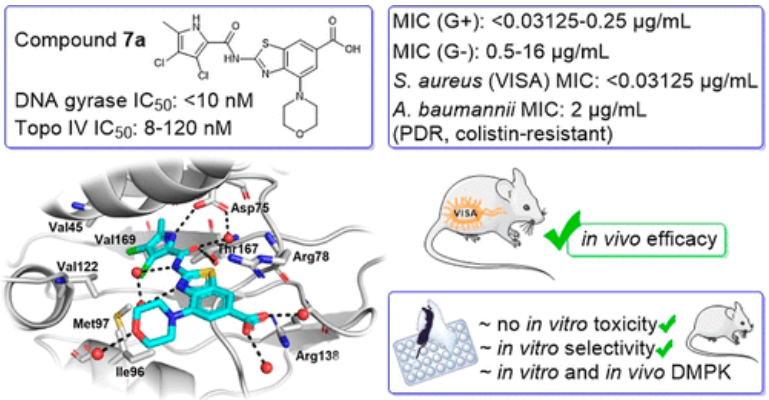

Fig. 1. New Dual Inhibitors of Bacterial Topoisomerases with Broad-Spectrum Antibacterial Activity and In Vivo Efficacy against Vancomycin-Intermediate *Staphylococcus aureus*

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BATTLE OF THE E3 LIGASES: USING HETEROPROTACS TO INDUCE PAN-IAP DEGRADATION

Aleša Bricelj[1], Yuen Dora Ng[2], Jacqueline Jansen[2], Arunima Murgai[2], Jan Krönke[2], Kirsten Peter[2], Katherine A. Donovan[3], Michael Gütschow[4], Christian Steinebach[4], Izidor Sosič[1]

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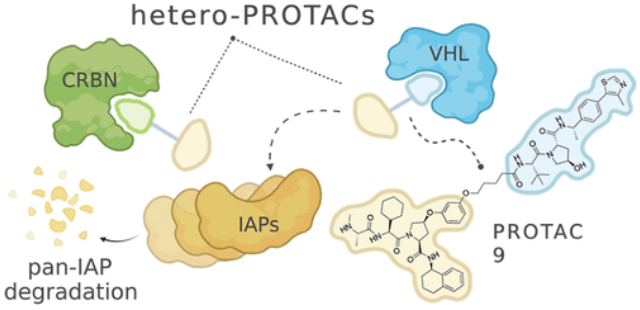
[4]*University of Bonn, Pharmaceutical Institute, An der Immenburg 4, 53121 Bonn, Germany*

Cellular IAP1 (cIAP1), cellular IAP2 (cIAP2), and X-chromosome-linked IAP (XIAP) are members of the inhibitor of apoptosis (IAP) protein family and have been extensively studied due to their crucial role in the regulation of apoptosis, where they act as proto-oncogenes by inhibiting cell death. Deregulation and overexpression of IAPs is frequently observed in various cancers and correlates with tumour progression, resistance to anticancer therapies, and poor prognosis.[1] Due to its clinical significance, numerous small-molecule mimetics of the IAP-binding motif of the endogenous IAP antagonist, second mitochondria-derived activator of caspases (SMAC), have been developed. Several monovalent and bivalent antagonists have entered clinical trials, but demonstrated low efficacy as single agents.[2] Importantly, these IAP antagonists have profound effects on cIAPs levels, as their binding leads to autoubiquitination and degradation of cIAP1 and cIAP2, whereas such effects are rarely observed with XIAP.[3]

Through significant advances in the field of targeted protein degradation, proteolysis-targeting chimeras (PROTACs) are considered as one of the most promising modalities in medicinal chemistry. Consisting of two distinct ligands connected by a linker, PROTACs can facilitate the formation of a ternary target complex protein–PROTAC–E3 ligase, followed by ubiquitination of the target protein and its subsequent degradation by the proteasome.[4] The concept has also been utilized in so-called homo- and hetero-PROTACs in which E3 ligases were directed against each other, resulting in successful depletion of cereblon (CRBN),[5,6] von Hippel-Lindau (VHL)[7], murine double minute 2 (MDM2),[8] and Keap1.[9] Encouraged by previous successful attempts and the fact that IAPs are validated anticancer targets, we systematically designed three series of bifunctional molecules by cross-linking VHL- and CRBN-targeting ligands with an IAP antagonist to apply the heteroPROTAC approach to IAP modulation. Our efforts produced compounds that resulted in strong, rapid and preferential degradation of cIAP1, cIAP2, and even XIAP and, in one case, also to concentration-dependent selective XIAP degradation. Notably, our pan-IAP degraders outperformed IAP antagonists and showed potent inhibition of cancer cell proliferation and could translate to degraders with significant therapeutic benefits in the battle against cancer.[10]



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ZEBRAFISH IN CANCER MODELING AND DRUG SCREENING

Martin Distel

Innovative Cancer Models & Zebrafish platform Austria for preclinical drug screening
St. Anna Children's Cancer Research Institute, Vienna, Austria

Zebrafish (*Danio rerio*) have served as model system for developmental biology for more than 40 years. Especially, the unique imaging opportunities enable live investigation of the development of an entire organism down to the subcellular level. A remarkable conservation of genes related to human diseases has more recently also triggered an enormous interest in zebrafish as disease model, including applications in cancer research.

We follow two different strategies for modeling pediatric cancer in zebrafish. A genetic and a xenotransplantation approach, where we transplant human cancer cells derived from cell lines (CDX model) or directly from patients (PDX model). In addition, we have established a platform for semi-automated drug screening on larval zebrafish disease models, including xenograft models, which we named Zebrafish platform Austria for preclinical drug screening (ZANDR). We applied this platform to screen small compounds and compound combinations for their anti-tumor effects against Ewing sarcoma *in vivo* in zebrafish xenografts. Ewing sarcoma is a pediatric bone and soft tissue tumor, driven by the fusion oncogene *EWSR1::FLI1* in the vast majority of cases (>85%).

We identified several small compounds with *in vivo* anti-tumor efficacy, including novel Hsp90 inhibitors. Furthermore, combinations of topoisomerase inhibitors with anti-apoptotic protein inhibitors could greatly reduce tumor size in our zebrafish model. Most strikingly, combined inhibition of anti-apoptotic proteins MCL-1 and BCL-XL eradicated all tumor cells in our larval zebrafish xenograft models and constitutes a specific vulnerability of Ewing sarcoma, which warrants further investigations towards clinical exploitation.

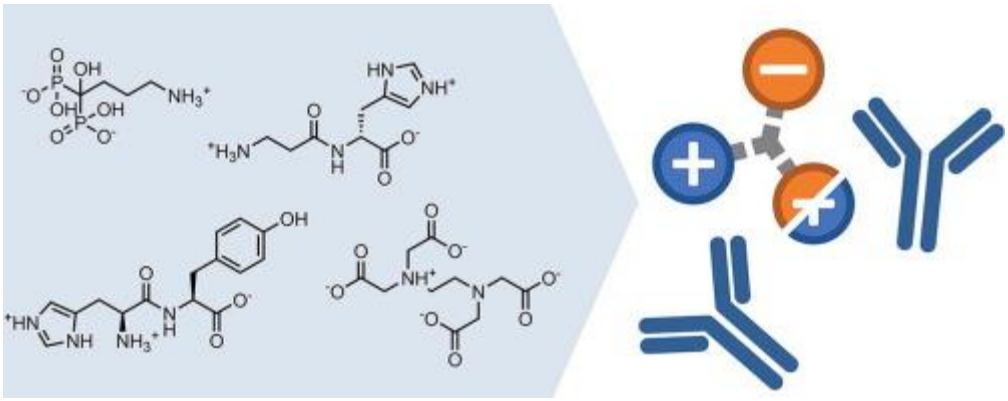
In addition, we use the zebrafish model to address a current major obstacle towards engineering genetic models for Ewing sarcoma: the unknown cell-of-origin. Here, we are following a cross-species enhancer activity analysis approach to identify candidate cell types for the Ewing sarcoma cell-of-origin in zebrafish. We will express EWSR1::FLI1 in these candidate cell types and will investigate, if this enables us to generate a targeted genetic Ewing sarcoma model. Such a model will enable a better understanding of Ewing sarcomagenesis and drug screens to develop novel therapeutic options.

A RULE OF THUMB FOR SELECTION OF COMPOUNDS WITH VISCOSITY-REDUCING EFFECTS ON BIOPHARMACEUTICAL PROTEIN FORMULATIONS

Matic Proj[1], Mitja Zidar[2], Blaž Lebar[1], Nika Strašek[1], Goran Miličić[2], Stanislav Gobec[1], Aleš Žula[2]

[1]*University of Ljubljana, Faculty of Pharmacy, Chair of Pharmaceutical Chemistry, Ljubljana, Slovenia*
[2]*Biologics Drug Product, Technical Research and Development, Global Drug Development, Novartis, Lek d.d., Slovenia*

When developing concentrated monoclonal antibody formulations for subcutaneous application, one of the major challenges is the exponentially increasing viscosity of the solutions. To mitigate the attractive protein-protein interactions, viscosity reducing agents (VRAs) are usually used as excipients. Our goal was to expand the pool of established VRAs, which is mostly limited to salts and amino acids in the literature. We applied two computational chemistry approaches: searching for fingerprint similarities to known VRAs and filtering based on physicochemical properties.[1] A total of 94 compounds were selected and experimentally evaluated on two model monoclonal antibodies. In this way, 33 new compounds with viscosity-reducing effects were identified. In addition, 11 dipeptides were discovered that can be used as dual excipients to simultaneously reduce viscosity and buffer the solution. Analysis of the results showed that the use of a simple filter that selects only compounds with three or more charge groups is a good 'rule of thumb' for selecting excipients with a high potential to reduce the viscosity of monoclonal antibody formulations. Moreover, our iterative strategy, which involves filtering compounds based on physicochemical properties, represents a simple and effective approach that can be readily used to identify novel VRAs in other monoclonal antibody formulations.



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DECIPHERING THE KINETIC PATHWAYS THAT DETERMINE AGGREGATION AND STABILITY OF THERAPEUTIC ANTIBODIES

Marko Bunc[1,2], San Hadži[1], Christian Graf[3], Matjaž Bončina[2], and Jurij Lah[1]

[1] Faculty of Chemistry and Chemical Technology, University of Ljubljana, Ljubljana, Slovenia

[2] Technical Research and Development, Global drug development, Novartis, Lek d.d., Mengeš, Slovenia

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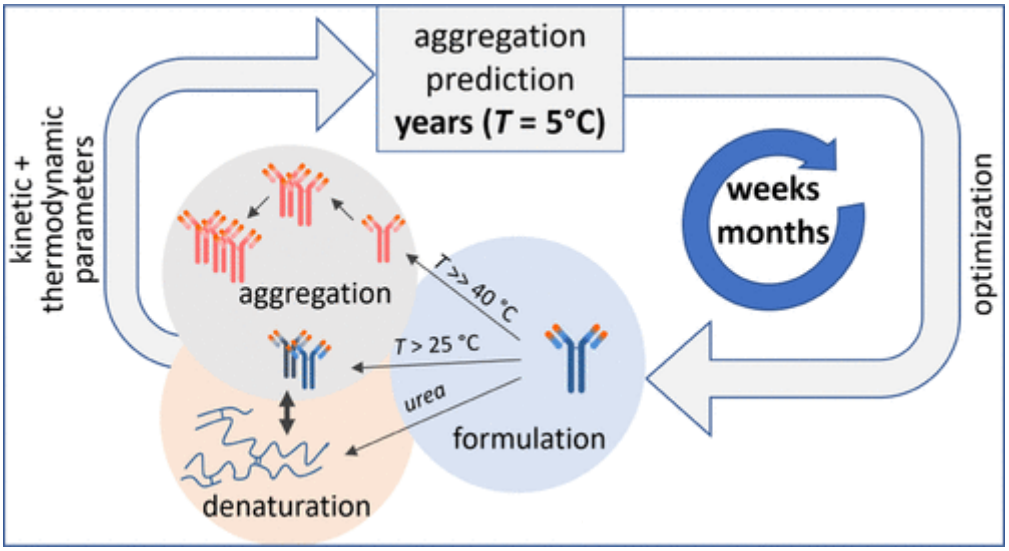
Monoclonal antibodies are the fastest growing class of therapeutics. However, aggregation limits their shelf life and can lead to adverse immune responses. Assessing and optimizing the long-term stability of antibodies is therefore a key challenge in biologic drug development. Here, we will present the analysis of temperature-dependent aggregation data that can dramatically shorten the assessment of long-term aggregation stability and thus accelerate the optimization of antibody formulations. For a number of antibodies used in the therapeutic areas of oncology, rheumatology, and osteoporosis, we obtain accurate prediction of aggregate fractions for up to three years in advance. The strategy, which combines kinetic and thermodynamic analyses (summarized in the figure) contributes to a better understanding of the molecular mechanisms of antibody aggregation and is very effective in the development and production of biological therapeutics (1).



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REACTION MECHANISMS AS A KEY FOR DESIGNING BIOMOLECULE MODIFICATIONS

Martin Gazvoda

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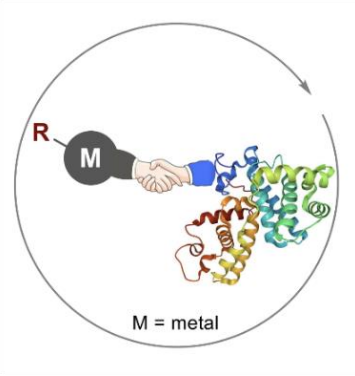
Selective modifications of biomolecules can provide an understanding of their fundamental role in biological systems, i.e., their function, dynamics, and localization in biological environments, and can also lead to increased activity and stability. However, their modification is challenging due to their highly complex structures and the need for specific reaction conditions, i.e., aqueous media, required low reaction temperatures and pH values. Transition-metal mediated reactions revolutionized the modification of biomolecules due to their unique reactivity, selectivity, and tolerance to various functional groups present in biomolecules.[1] These reactions include palladium-catalyzed cross-coupling and copper-catalyzed azide-alkyne cycloaddition (CuAAC). Understanding the mechanisms of such transformations allows the identification of reactive species that can be used to design reactions at specific sites in biomolecules. For example, palladium oxidation addition complexes, intermediates of palladium-catalyzed cross-coupling reactions, have been shown to be very efficient tools for protein bioconjugation, [2] allowing, among other things, the introduction of compounds of interest at specific sites in proteins.[2,3] We have developed a CuAAC reaction that, using hydrazoic acid as a reagent, allows the introduction of an NH triazole into peptides functionalized with an alkyne moiety.[4] The mechanistically guided development of reaction conditions for simple organic substrates led to an efficient reaction protocol that allows the application of the reaction to more complex peptide substrates with nearly the same efficiency. To this end, novel approaches to mechanistic analysis are useful, like for example dissasably approach that we recently demonstrated in an example of a monometallic dual catalytic process.[5]



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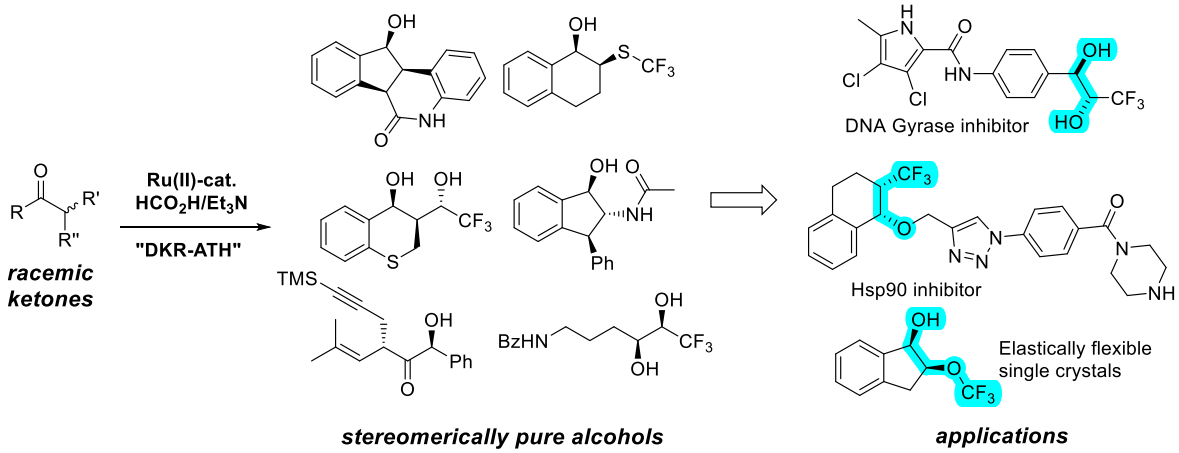
DYNAMIC KINETIC RESOLUTION WITH NOYORI–IKARIYA ASYMMETRIC TRANSFER HYDROGENATION: METHOD DEVELOPMENT AND APPLICATIONS

Andrej Emanuel Cotman

Faculty of Pharmacy, University of Ljubljana, Aškerčeva cesta 7, SI-1000 Ljubljana, Slovenia

Bioactive molecules with three-dimensional scaffolds and multiple chiral centers are more successful in transition from discovery, through clinical trials, to drugs than their easily accessible flat competitors, because of the pinpoint on-target activity and better ADME-related physicochemical properties.[1] Indeed, introduction of a benzylic chiral center to the antibacterial lead compound resulted in improved solubility and on-target selectivity.[2] Asymmetric transfer hydrogenation (ATH) of ketones using Noyori–Ikariya-type ruthenium(II)-catalysts has proved as a well-behaved and user-friendly platform for the synthesis of complex chiral secondary alcohols, where up to four contiguous stereocenters can be controlled in a single chemical operation through dynamic kinetic resolution (DKR).[3] We contributed to the field by developing new modes of DKR and elucidating the mechanism of catalyst–substrate recognition in the stereochemistry-determining hydrogen transfer step.[4,5] These synthetic tools were used in the preparation of analogs of our in-house DNA Gyrase inhibitors,[4] and a heat shock 90 (Hsp90) inhibitor with a click-triazole scaffold.[6] Furthermore, a family of fluorinated benzo-fused alcohols was discovered that form needle-shaped crystals exhibiting mechanically responsive either elastic or plastic flexibility. This is a rare and counter-intuitive behavior of crystals and opens the door to functional materials based on mechanically responsive chiral molecular crystals.[6]



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Poster Presentations

BATTLE OF THE E3 LIGASES: HETEROBIFUNCTIONAL E3 LIGASE RECRUITERS ENABLE PAN-DEGRADATION OF INHIBITOR OF APOPTOSIS PROTEINS

Aleša Bricelj[1], Yuen Dora Ng[2], Jacqueline Jansen[2], Arunima Murgai[2], Jan Krönke[2], Michael Gütschow[3], Christian Steinebach[3], Izidor Sosič[1]

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[3]University of Bonn, Pharmaceutical Institute, An der Immenburg 4, 53121 Bonn, Germany

Cellular IAP1 (cIAP1), cellular IAP2 (cIAP2), and X-chromosome-linked IAP (XIAP) are members of the inhibitor of apoptosis (IAP) protein family and have been extensively studied due to their crucial role in the regulation of apoptosis, where they act as proto-oncogenes by inhibiting cell death. Deregulation and overexpression of IAPs is frequently observed in various cancers and correlates with tumour progression, resistance to anticancer therapies, and poor prognosis.[1] Due to its clinical significance, numerous small-molecule mimetics of the IAP-binding motif of the endogenous IAP antagonist, second mitochondria-derived activator of caspases (SMAC), have been developed. Several monovalent and bivalent antagonists have entered clinical trials, but demonstrated low efficacy as single agents.[2] Importantly, these IAP antagonists have profound effects on cIAPs levels, as their binding leads to autoubiquitination and degradation of cIAP1 and cIAP2, whereas such effects are rarely observed with XIAP.[3]

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NOVEL MITOCHONDRIAL K_V1.3 INHIBITORS: DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION

Marzia Fois[1], Špela Gubič[1], Xiaoyi Shi[2], Ildiko Szabo[3], Luis Pardo[2], Lucija Peterlin Mašič[1],Tihomir Tomašič[1]

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K_V1.3 is a transmembrane protein, expressed in cellular and in mitochondrial membrane, belonging to voltage-gated potassium channel K_V1.x subfamily. K_V1.3 has become an interesting target for anticancer therapy because a correlation between its expression and the development of cancer was demonstrated. It is overexpressed in different types of tumors and its activity is involved in cell proliferation and in the process of apoptosis.[1–3]

The aim of our work is to develop new inhibitors of the mitochondrial K_V1.3 (mitoK_V1.3) channel that would induce the apoptosis of cancer cells. We recently optimized the thiophene-based inhibitors and tested their ability to inhibit the proliferation of cancer cells that overexpress K_V1.3,such as PDAC, Colo-357 and Panc-1.[4] Based on the results, we hypothesized that the inhibition of mitochondrial K_V1.3 is required for a significant anticancer activity.

To improve the anticancer potential of K_V1.3 inhibitors, we designed a series of mitoK_V1.3 inhibitors composed of the thiophene-based K_V1.3 inhibitor, a lipophilic alkyl linker and the cationic triphenylphosphonium$^+$ group (TPP^+). The anticancer activity of new compounds was investigated in Colo357 cancer cell models and mouse melanoma BF16F10 cells in which a significant toxicity was observed. The compounds were less cytotoxic in B16F10 C52 mouse melanoma cells in which K_V1.3 was stably knocked down, so it appears that the effects depend onK_V1.3 expression in mitochondria. Further biological evaluation is currently in progress.

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DESIGN, SYNTHESIS AND KINETIC CHARACTERIZATION OF OPTIMISED ALLOSTERIC EFFECTORS OF HUMAN CATHEPSINS K AND S

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Human cathepsins K and S are proteolytic enzymes which belong to the family of cysteine cathepsins. Among the members of the family, cathepsins K and S are the most closely related from an evolutionary and structural points of view. Apart from catalytic residues, their active sites are constituted of substrate binding sites that are important for the interaction with amino acid residues of their substrates. These sites are therefore important for the affinity of the enzyme for its substrates. Among them, the S2 site is the only one that can form a binding pocket. Cathepsins K and S have important physiological roles in the human body. However, their elevated enzyme activities contribute to progression of numerous diseases. One of the possible ways by which their enzyme activities can be regulated is by allosteric effectors glycosaminoglycans. In the case of cathepsin K, apart from natural effectors glycosaminoglycans, small-molecule synthetic compounds NSC13345 and NSC94914, have been characterized on the structural level as allosteric effectors [1, 2]. Based on the succinimide scaffold of the selective cathepsin K allosteric effector, methyl [(3*RS*)-2,5-dioxopyrrolidin-3-yl]glycinate, the compound [(3*R*)-2,5-dioxopyrrolidin-3-yl]-*L*-threoninate was designed, synthesized and characterized as a hyperbolic inhibitor of cathepsin S which is consistent with allosteric regulation. Cyclic derivative of the latter compound, (3'*RS*)-3-{[(1*S*,2*R*)-2-hydroxycyclohexyl]amino}pyrrolidine-2,5-dione, was shown to be more potent hyperbolic inhibitor of cathepsin S and selective over cathepsin K. According to the results obtained by enzyme kinetics, these novel cathepsin K or S effectors act via similar mechanisms of action as the known effectors of cathepsin K NSC13345 and NSC94914. According to these mechanisms, the effectors decrease affinity of the enzyme for its substrates and have minor or no effect on the catalytical properties, particularly on the turnover number of the enzyme. The determined mechanisms of action are consistent with the results obtained by screening of cathepsin K or S effectors using macromolecular substrates. Furthermore, mutant forms of cathepsin K with individual residues substituted by alanine residues in predicted allosteric pathways were designed and prepared. Using the compound [(3*RS*)-2,5-dioxopyrrolidin-3-yl]glycinate, it was shown that residues which constitute predicted allosteric pathway between allosteric site and S2 site are important for allosteric communication of cathepsin K. We assume that the information regarding the binding of the compound to the allosteric site is transmitted to the S2 site which decreases the affinity of the cathepsin K for its substrates and is consistent with determined mechanism of action of the compound.

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DISCOVERY OF FIRST-IN-CLASS TAK1 PROTACS FOR TARGETED THERAPY IN AUTOIMMUNE DISEASES AND CANCER

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TAK1 kinase (transforming growth factor-β-activated kinase 1) belongs to the protein kinase kinase kinase (MAP3K) family[1]. Its involvement in complex signaling networks, including the tumor necrosis factor-alpha (TNF-α) signaling cascade, the interleukin-1 (IL -1), Toll-like receptor (TLR) signaling cascade, and the transforming growth factor-β (TGF-β) signaling pathway, makes TAK1 a critical regulator of diverse cellular processes. Due to its role in cell proliferation and differentiation, apoptosis and immune response, dysregulation of TAK1 has been implicated in the development and progression of various cancers and autoimmune diseases[2]. An important discovery in the field of TAK1 inhibition was the identification of the small-molecule inhibitor takinib in 2017 by Totzke et al, which is a highly selective and potent inhibitor of TAK1 with IC_{50} of 9.5 nM[3].

In the last decade, there has been a significant breakthrough in the field of small molecules as modulators of pharmacologically relevant targets. Such example are PROTAC molecules (Proteolysis Targeting Chimeras; chimeric decomposers), which hijack the ubiquitin-proteasome system. These heterobifunctional molecules consist of a target protein binder, a suitable linker, and a ligand for the E3 ligase[4].

We designed, synthesized and biologically evaluated nineteen PROTACs utilizing takinib as a ligand for TAK1, different linkers and ligands for VHL, CRBN and IAP E3 ligases. The first series of PROTACs have long flexible linkers attached to takinib at two different points; at the aromatic ring of benzimidazole and at the nitrogen of benzimidazole ring. The IAP-hijacking PROTACs showed the best results, inducing degradation of TAK1 at 1 μM in MDA-MB-231 cell line. For the second series of compounds, we incorporated rigid linkers at the aromatic ring of the benzimidazole, yielding compound SRLK-5, a CRBN-hijacking PROTAC, which demonstrated depletion of TAK1 at 1 μM in MDA-MB-231 and THP-1 cell lines.

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DEVELOPMENT OF NOVEL SELECTIVE TOLL-LIKE RECEPTOR 7 AGONISTS WITH ISOXAZOLO[5,4-d]PYRIMIDINE SCAFFOLD

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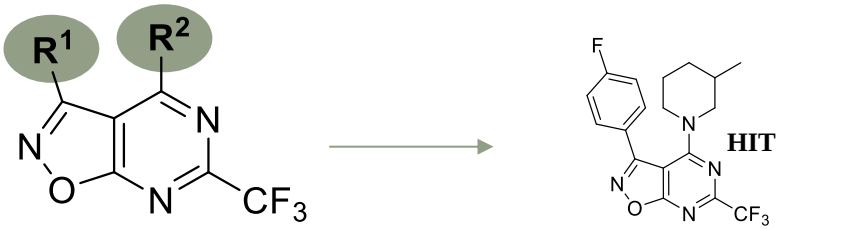
TLRs are transmembrane receptors, localized at the cellular or endosomal membrane, and they play critical role in the innate and adaptive immune system. TLRs represent a well-described family of pattern recognition receptors (PRRs) and serve to identify pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). To date, 10 members of TLRs have been identified in humans. One of them is TLR7, which is an endosomal receptor that recognizes single-stranded RNA and signal through the MyD88 pathway. Modulators of TLR7 are considered promising for the treatment of viral infections, autoimmune diseases, and cancer. [1-3]

In our work, we report a novel small-molecule TLR7 agonists with 6-(trifluoromethyl)isoxazolo[5,4-*d*]pyrimidine-4-amine scaffold. The idea for developing a new class of TLR7 agonists was derived from our group´s prior work with TLR7 agonists that had been previously published.[4] We have synthesized 45 compounds with different substituents at positions R^1 and R^2. In the first series of compounds, we synthesized compounds with different R^1 substituent. The most promising compound in this series was compound **21a**, which showed an EC_{50} value of 7.8 μM, which is similar to the EC_{50} value of the clinically approved TLR7 agonist imiquimod. Furthermore, we wanted to explore the impact of various amines on potency. Consequently, we introduced different functional groups at R^2 and it became evident that 3-methylpiperidine is crucial for activity. Compounds with isobutylamine at R^2 and compounds with cyclic amine at R^1 proved to be either inactive or cytotoxic. All compounds were biologically screened in TLR7 and TLR8 HEK293 cell line reporter assay and showed selective agonistic activity on TLR7. Compound **21a** was our most potent agonist TLR7 and had no cytotoxic effects on the HEK293 cell line. Importantly, it also induced the secretion of IL-1β, IL-12p70, IL-8, and TNF-α, suggesting that **21a** has the capability to regulate immune responses and trigger an immune cascade. **21a** shows significant potential for further development and structural optimization.



SAR exploration
45 compounds

21a
EC_{50} = 7.8 μM

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DESIGN, SYNTHESIS AND EVALUATION OF NOVEL INDAZOLE BChE/p38α MAPK DUAL INHIBITORS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder and represents a major cause of dementia [(1)]. Six drugs to treat AD are currently on the market, four of which are small-molecule cholinesterase inhibitors. Due to lack of convincing results and side effect such as vasogenic edema very few new biological drugs reach final stages of clinical trials. Design of novel small-molecule drugs targeting proteins responsible for early patophysiology of AD therefore presents a major challenge in medicinal chemistry[(2)]. Although a multitude of hypothesis tries to explain complex events in AD patophysiology, they all coalesce in the nowadays most extensively studied neuroinflammation hypothesis[(3)]. It states tha Aβ plaques together with phosphorylated tau overstimulate microglia, which stimulates expression of many enzymes that further contribute to an imbalance between anti- and proinflammatory cytokines. Among many enzymes that are overexpressed p38α MAP kinase (p38α MAPK) recently gained more attention. This ubiquitous enzyme further up-regulates proinflammatory cytokines such as TNF-α and IL-1β, promotes Aβ accumulation and drives the neurotoxic tau protein hyperphosphorylation[(4)]. This in turn makes it an interesting pharmacological target to combat AD.

Our aim is to design a dual inhibitor covering targets from both the neuroinflammation as well as the older cholinergic hypothesis. The latter states that forebrain cholinergic neuron loss is characteristic of AD. By inhibiting the hydrolytic action of cholinesterases, especially butyrylcholinesterase (BChE) we may augment the activity of surviving cholinergic neurons[(5)]. First, a library of small molecules with confirmed activity against p38α MAPK was prepared using ChEMBL and PDB (in ChEMBL activity threshold of $K_d/IC_{50} < 50$ μM was accounted for). Compounds were further divided into 30 clusters according to molecular fingerprint and docked into the BChE active site gorge. According to docking results 8 best small molecules were purchased and evaluated *in vitro* against BChE by the method of Ellman. Of the eight compounds, very promising activity against BChE was exhibited by ARRY-371797 (**Figure 1**) a p38α MAPK inhibitor of Pfizer Inc. This molecule was then subjected to further optimization.

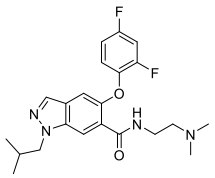

Figure 1. Structure of p38α MAPK inhibitor ARRY-371797

We started the synthesis of analogues by substituting the indazole core with various azines, bicyclic nitrogen heterocycles and 4-phenyl azines. The analogues with switched positions of the amide and ether were also synthesized. All synthesized compounds were initially evaluated for their *in vitro* activity against p38α MAPK and BChE with ADP-Glo kinase assay and Ellman's method respectively.

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DEVELOPMENT OF NEW HSP90B-SELECTIVE INHIBITORS BASED ON TOPOISOMERASE II LIGANDS

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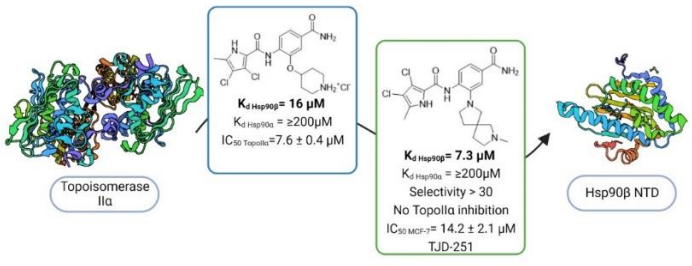
Ever since the the first N-terminal ATP-competitive inhibitor of Hsp90, geldanamycin, was discovered the Hsp90 family of proteins has been investigated as a potential target for the treatment of cancer. However, most of the clinically evaluated Hsp90 inhibitors had equal effect on all four members of the Hsp90 family (the mitochondrial TRAP-1, the endoplasmic reticulum localised Grp94, and the cytoplasmic Hsp90α and Hsp90β) that can lead to an induction of the heat shock response (HSR). This in turn may attenuate the anticancer effect of pan-Hsp90 inhibitors. To circumvent the HSR induction of pan-Hsp90 inhibitors, a switch was made to the design of inhibitors selective for only one of the four paralogues. However, the structure of the entire Hsp90 family is highly conserved. In particular, the cytoplasmic isoforms Hsp90α and Hsp90β are approximately 85% homologous and show an astonishing 95% identity when considering only the N-terminal ATP-binding site. Therefore, it is not surprising that only two structural classes of Hsp90β and one structural class of Hsp90α have been described so far and consequently new approaches to develop these inhibitors are in great demand. Looking at the binding sites of the GHKL (**G**yrase, **H**sp90, Histidin **K**inase and Mut**L**) protein family, a very distinct Bergerat fold makes the pockets susceptible to the binding of similar inhibitors.[1]

For this reason, the aim of our research was to repurpose known inhibitors of topoisomerase IIα prepared by our group[2] and redesign them into Hsp90β selective compounds. Indeed, our hit compound (IC_{50} (TopoIIα) = 7.6 ± 0.4 μM) from the "in-house" inhibitor library was shown to bind Hsp90β with a K_d value of 16 μM (CI:[11 μM;24 μM]). Simultaneously, the binding affinity to Hsp90α was significantly weaker ($K_d \geq 200$ μM). Therefore, we prepared a focused library of analogues to explore the structure-activity relationship and attempt to improve the affinity and the selectivity of the hit compound. Our efforts resulted in an inhibitor TJD-251 with no binding affinity for topoisomerase IIα, with an increased affinity for Hsp90β (K_d = 7.3 μM – CI:[4.8 μM;11 μM]) and with more than 30-fold selectivity for this isoform when compared to Hsp90α. The binding position of TJD-291 to Hsp90β was further explored by STD NMR. Additionally the compound induces apoptosis in MCF-7 breast cancer cell line in which it can reduce the concentration of oncogenic Hsp90 client proteins CDK-4 and ERα.



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NOVEL POTENTIAL ANTICANCER COMPOUNDS TARGETING THE HUMAN H_V1 PROTON CHANNEL

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Voltage-gated proton channels (H_V1) are proton-selective voltage-dependent channels that have been found in various mammalian and cancer cells. They regulate the intracellular pH, preventing intracellular acidification [1]. In physiological conditions, at the resting membrane potential, the channels are closed; however, when various pathological conditions occur, these channels can open even at the resting membrane potential. In this acidic microenvironment, tumor cells can adapt extremely well, while immune cells functions are impaired.

The aim of our work is to discover and evaluate a series of new H_V1 inhibitors. At present, there are no selective inhibitors specific for H_V1 proton channels. A selective H_V1 inhibitor would allow us to modulate the acidic tumor microenvironment.

An open structure of the human H_V1 channel was used to perform virtual screening (VS) of an in-house library of compounds and selected known H_V1 inhibitors [2, 3]. Compounds were docked to the binding site of guanidine derivatives, on the voltage-sensing domain [4]. A series of molecules was selected to be tested by manual patch-clamp on CHO (Chinese hamster ovary) cells expressing hH_V1 and other channels. A small series of analogues was prepared by organic synthetic procedures.

Virtual screening results were evaluated, and a series of most promising hits were selected for biological evaluation on H_V1 channels. Seven hits were found to have an effect on proton currents (more than 50% block at 50 μM) and six of them had the same scaffold in their structure (Fig.1 left). Results obtained using the patch-clamp technique show that NZ-58, one of the hit molecules, blocks dose-dependently the channels, it binds when the VSDs are resting or deactivated and that the binding rate is state independent; it is likely that it binds from the extracellular side. Most of the hit molecules turned out to have low selectivity: they also act on voltage-gated sodium and potassium channels. However, NZ-13 the scaffold itself and one of the hit molecules, had lower affinity for the other channels than for H_V1 and the smallest effect on T cell proliferation (Fig.1 right).

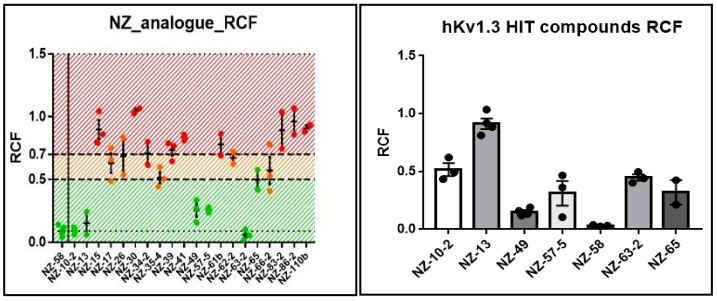

Figure 2. (left) Effects of NZ analogues - remaining H_V1 current fraction measured at +100 mV in the presence of 50 μM of the compounds. (right) Effects of NZ analogues - remaining $K_V1.3$ current fraction in the presence of 50 μM of the compounds.

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SYNTHESIS OF MITOCHONDRIA-TARGETING ION CHANNEL INHIBITOR IN THE FINAL ROUND OF SYNTHETIC MASTERCHEF OF THE FACULTY OF PHARMACY

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Calcium and potassium ion channels have an important role in regulating cell cycle and proliferation. Cancerous cells have a higher expression rate of voltage-gated potassium channels Kv1.3 than the healthy ones. Kv1.3 channels are also embedded in the mitochondrial inner membrane and their inhibition causes apoptosis.[1] PAP1 is a small-molecular selective inhibitor of Kv1.3 channels, which has previously been combined with a Mitochondria Targeting Moiety (MTM), a triphenylphosphonium cation, to promote selective apoptosis of cancer cells in vivo.[2]

Synthetic Masterchef of the Faculty of pharmacy was a pilot project aimed at implementation of the principles of research-based curriculum, and was supported by the Development Fund of the University of Ljubljana (RSF). In the first round, the 3rd year MPharm students were given 23 weekly theoretical synthetic challenges prepared by researchers at the Department of Pharmaceutical Chemistry in connection to their current scientific projects. 6 best students were selected for the final round, where they were paired and challenged with a "mystery box molecule". The aim of all teams within the final round was to prepare a conjugate between PAP1 and any non-phosphonium MTM, supervised by experienced researchers, during a two-week laboratory pressure test.

We have improved the literature synthetic procedure towards the modular PAP1–MTM alkyl iodide precursor,[3] and successfully used it for quaternization of a precursor of F16, a fluorescent molecule known to accumulate in mitochondria and induce cell apoptosis.[4] The PAP1–F16 conjugate thus prepared will be evaluated for its potential in anticancer therapy and fluorescence-based imaging.

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EXPANDING THE CHEMICAL SPACE OF *N*-PHENYLPYRROLAMIDES AS DNA GYRASE B INHIBITORS

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The growing threat of antibacterial resistance is intensifying the need for antibiotics with novel mechanisms of action (1). DNA gyrase and topoisomerase IV are enzymes essential for DNA double helix replication in bacteria (2). Both are constructed as heterotetrametric complexes consisting of two subunits responsible for the cleavage and transport of DNA (DNA gyrase, 2x GyrA; topoisomerase IV, 2x ParC) and two subunits responsible for the hydrolysis of ATP (DNA gyrase, 2x GyrB; topoisomerase IV, 2x ParE) (1).

In this work, we focused on expanding the knowledge of *N*-phenylpyrrolamides as DNA gyrase B inhibitors. They primarily exhibit high affinity for the GyrB subunit, while they often also show good inhibitory values toward the ParE subunit (3). Our goal is to advance the development of an *in vitro* effective antibacterial agent against "ESKAPE" pathogens.

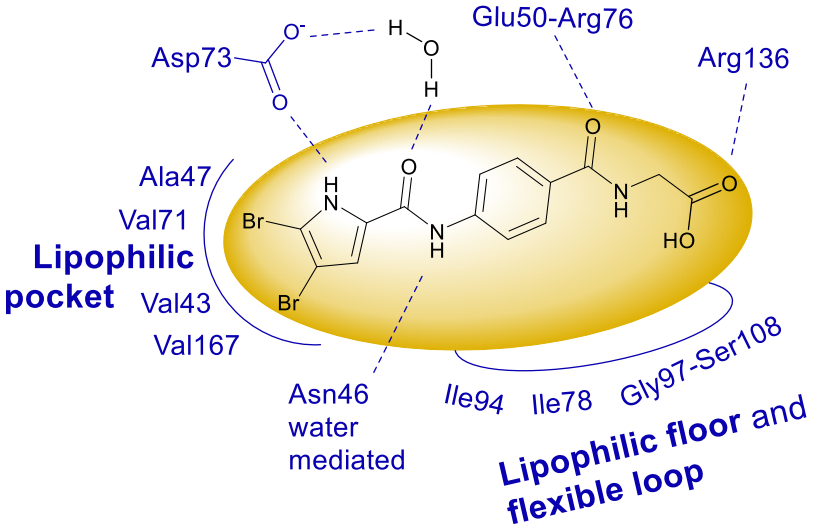

Figure 1. Representative substrate-enzyme interactions

By preparing different analogs, we investigated the influence of two key structures. When targeting the lipophilic floor (analogs bearing alkoxide substituents in ortho-position to the amide bond on the central benzene ring), our goal was to enhance the hydrophobic and H-bond interactions while improving the physiochemical properties of our compounds. In addition, we optimized the eastern part of the molecule by introducing groups capable of forming ionic or hydrogen bonds with the positively charged Arg136 residue and/or cation-π interactions with the Glu50-Arg76 salt bridge.

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IMMUNOPROTEASOME INHIBITION SHAPES THE CHEMOKINE MICROENVIRONMENT IN CO-CULTURES OF BREAST CANCER CELL LINES WITH PLATELETS

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Proteasomes are responsible for maintaining intracellular protein homeostasis[1]. Hematopoietic cells assemble a specialized form of proteasomes, known as the immunoproteasomes, in which the constitutive catalytic sites are replaced by the cytokine-inducible homologs ß1i, ß2i and ß5i.[1,2] As proteasome is a well-known target in haematological malignancies, we sought to investigate whether the level of catalytic subunits of proteasomes affects the susceptibility of solid tumours to the cytotoxic effects of proteasome inhibitors. Additionally, we explored whether the levels of proteasomes in breast cancer cells affects the interaction with platelets. Namely, it has been shown that platelets enable the formation of metastases by binding to tumour cells, providing immunosuppressive microenvironment and protecting them from cytotoxic immune cells.[3]

Venous blood was obtained from informed healthy donors and platelet isolation was prepared as described by Schwarz et al.[4] Platelets as well as breast cancer cell lines MCF-7, MDA-MB-231, and SKBR-3 were treated in a time- and dose-dependent manner in the presence or absence of IFN-γ and/or proteasome inhibitors (e.g., LMP7-IN-1, KZR-504). Next, inhibitor pre-treated isolated platelets and IFN-γ or inhibitor pre-treated breast cancer cells were co-cultured and levels of selected chemokines (IL-8, IP-10, CCL5, MIG, MCP-1) were assessed by flow cytometry.

In our research we used co-cultures of platelets and breast cancer cells, as such *in vitro* models better mimic the *in vivo* conditions in terms of the immunosuppressive microenvironment that enables the formation of metastases. Our preliminary findings demonstrate that co-culturing platelets with IFN-γ-stimulated MDA-MB-231 cells enhance the release of chemokines, which can be influenced by the addition of selective inhibitors. Inhibiting the β1i subunit resulted in reduced levels of IP-10 and MIG, while inhibiting β5i specifically decreased MIG levels. Notably, inhibiting this subunit led to an increase in IP-10 secretion. Collectively, these observations indicate that the degranulation process can be influenced by treating cells with selective immunoproteasome inhibitors. However, comprehensive studies are required to ascertain whether these alterations have a positive impact on cancer cell-platelet interactions or can serve as an effective therapeutic intervention against metastasis formation.

Our study revealed that the presence of IFN-γ can upregulate immunoproteasome subunits in breast cancer cells. Surprisingly, this increase in subunits did not influence the sensitivity of the cells to proteasome inhibitors. However, when breast cancer cells were co-cultured with platelets, with or without the presence of proteasome inhibitors, significant alterations were observed in the levels of secreted IP-10 and MIG. These findings suggest that the proteasome may play a crucial role in shaping the tumour microenvironment.

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SYNTHESIS OF MITOCHONDRIA-TARGETING ION CHANNEL INHIBITOR IN THE FINAL ROUND OF SYNTHETIC MASTERCHEF OF THE FACULTY OF PHARMACY

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The development of small-molecule anti-cancer inhibitors featuring a covalently linked mitochondria-targeting moiety (MTM) is a trending approach in modern cancer research initiatives. MTMs are small fragments containing a chemical functionality which exhibits a (permanent) positive charge; this property can be used to selectively target the mitochondria (which are abundantly expressed in carcinogenic tissues), owing to the highly negative potential of mitochondrial membranes (the latter arises as a result of oncogenic mutations). One of the possible targets of inhibitors containing an MTM warhead are voltage gated potassium channels Kv1.3, which are highly expressed in cancerous cells (in comparison with healthy cells). From a structural perspective, all MTM-containing inhibitors are designed to take at least two most important characteristics into account: (i) sufficient lipophilicity, and (ii) a presence of a (permanent) positive charge. The most frequently explored examples of such structures in the field are delocalized lipophilic cations; e.g. TPP (most commonly used), DQA, F16, (bi)guanido derivatives, rhodamine-containing fragments, pyridines, as well as mitochondria-penetrating peptides. The key remaining limitations of MTMs currently explored for this purpose are their low target selectivity, resulting in high levels of toxicity towards healthy cells, and insufficient solubility.

Our research project has been conducted as a part of 'Synthetic Masterchef UL FFA' initiative, the goal of which was to synthesise a potentially novel MTM-bearing small-molecule candidate that could be adjoined to anticancer drug PAP-1 in order to improve its functionality and selectivity in targeting malignant cells (in comparison with existing PAP-1–MTM derivatives). The idea behind our selected molecule was to incorporate the natural amino acid arginine, which contains a guanidino moiety. The use of the latter has been heavily explored for its cationic properties, hence we postulated that it could potentially exert MTM effects when combined with PAP-1 fragment. Our synthetic rationale therefore combines cationic, lipophilic, and peptide-like properties, found in many researched MTMs. Care was taken to also prepare more lipophilic analogues of the final target molecule, to be able to assess the suitability of a terminal guanido moiety on permeability.

Starting from an alkyl iodide precursor, we began the synthetic sequence with the nucleophilic substitution with an azide function. Our next step was the one-pot conversion of the azide group to the corresponding primary amine, which was accomplished in a near-quantitative yield using standard conditions for a Staudinger azide reduction. We next proceeded to convert the commercial carboxylic acid Boc-Arg(Z)2-OH to its corresponding NHS ester, which was then directly coupled to the prepared primary amine. From this point onward, our synthesis diverged into combinatory Cbz and Boc deprotection steps, ultimately yielding four potential MTM inhibitors, all of which will be tested in vitro.

EATRIS – EUROPEAN INFRASTRUCTURE FOR TRANSLATIONAL MEDICINE

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Translational medicine serves as a bridge between scientific discoveries and their practical application in healthcare. It enables the implementation of novel preventive, diagnostic and therapeutic solutions into clinics to overcome serious diseases. However, for a new product to enter clinical practice, a long development timeline is required, demanding joint efforts from authorities, governments and industry to address this issue. Recognizing the challenge, one of the national and European priorities has been the creation of infrastructures that provide high-quality services for translational medicine. One such initiative is the European Infrastructure for Translational Medicine (EATRIS), which provides access to an extensive range of preclinical and clinical expertise and resources from more than 144 academic institutions in 15 European countries. EATRIS enhances and streamlines the early stages of drug, vaccine and diagnostic development through five scientific platforms: therapy medicinal products, and tracing, molecules, Vaccine, inflammation and immune monitoring, and Biomarkers. EATRIS not only promotes research, but also actively collaborates with public funders, nonprofit organizations, and policymakers to develop tailored initiatives that improve the translational research ecosystem. The host and coordinator of the Slovenian EATRIS node is the University of Ljubljana, Faculty of Pharmacy (UL FFA). Access to a large research infrastructure enables Slovenian scientists to participate in international projects and network globally to achieve tangible results in pharmaceutical sciences, clinical biomedicine, medicine and biotechnology to improve patient outcomes.