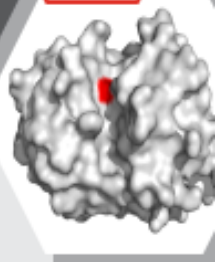
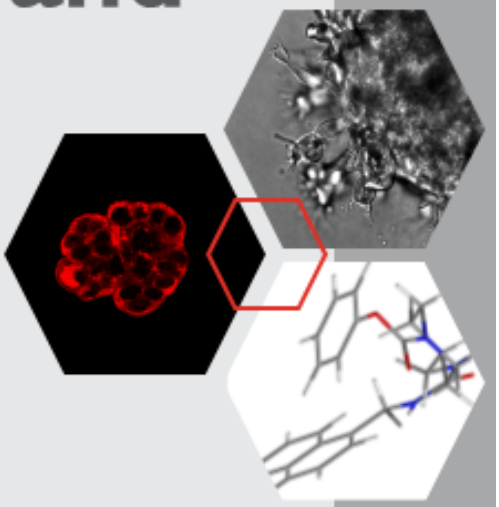
Minisymposium

UNIVERSITY OF LJUBLJANA | FFA Faculty of Pharmacy

15th - 17th April 2024



Design and application of peptidase inhibitors and active probes



Faculty of Pharmacy
University of Ljubljana

Minisymposium

Design and application of peptidase inhibitors and active probes

Organizer
Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia

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Overview

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Date: 15th April – 17th April 2024

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Anja Pišlar, Faculty of Pharmacy, University of Ljubljana, Slovenia
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Izidor Sosič, Faculty of Pharmacy, University of Ljubljana, Slovenia
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Foreword

Degradation and modulation of proteins represent one of the main physiological processes in our cells. Proteolytic enzymes, which catalyze the breakdown of peptide bonds in proteins, are regulated at various levels. The most important for regulating the activity itself are peptide inhibitors, which can reversibly or irreversibly inhibit enzymatic activity. The action of endogenous inhibitors is insufficient in various diseases and uncontrolled proteolytic activity can lead to tissue damage, increased cell proliferation and invasiveness, or accumulation of certain proteins that cells cannot degrade quickly enough. The application of endogenous or other natural inhibitors for the treatment of diseases associated with uncontrolled proteolytic activity has not been successful, primarily due to their non-specificity, poor biological availability, and instability. Small synthetic inhibitors have proven to be more useful, specifically binding to a particular target and not causing side effects. In addition to peptide inhibitors, active probes are important tools for research and clinical use, enabling differentiation between individual peptidases and precise definition of their sites of action.

Modern methods in drug design and chemical synthesis have led to the preclinical and clinical use of a variety of anti-peptidase agents suitable for the treatment of cancer, osteoporosis, pancreatitis, immune, neurodegenerative, inflammatory, parasitic, bacterial, and viral diseases. At the symposium, we aim to present some new approaches in the development of peptidase inhibitors and active probes and demonstrate successful examples of their use in inhibiting various pathological processes. With our research, we aim to foster the transfer of this knowledge to clinical investigations and contribute to better patients' treatment.

Programme

Monday, 15th April

Workshop 1 — **Old Computer classroom (1st floor)**

13.00 – 14.00	**In silico design of reversible and irreversible cathepsin B inhibitors** *Anže Meden, Faculty of Pharmacy, University of Ljubljana*

Tuesday, 16th April

Symposium — **PD lecture hall**

12.00 – 12.05	**Introduction** *Janko Kos, Faculty of Pharmacy, University of Ljubljana; Jožef Stefan Institute*
12.05 – 12.45	**Two Tags in One Probe and More: Activity-based Probes for Cysteine and Serine Proteases** *Michael Gütschow, University of Bonn, Germany*
12.45 – 13.05	**Tackling immunoproteasome with different medicinal chemistry strategies** *Izidor Sosič, Faculty of Pharmacy, University of Ljubljana*
13.05 – 13.25	**Cathepsins B and X: Relationship status – it is complicated** *Ana Mitrović, Faculty of Pharmacy, University of Ljubljana; Jožef Stefan Institute*
13.25 – 13.55	Coffee break
13.55 – 14.15	**Unraveling the Role of Proteasomes in Platelets** *Martina Gobec, Faculty of Pharmacy, University of Ljubljana*
14.15 – 14.35	**Metallodrugs targeting cysteine proteases** *Jakob Kljun, Faculty of Chemistry and Chemical Technology, University of Ljubljana*
14.35 – 14.55	**Exploring the Potential of Cathepsin V Inhibitors in Regulation of Cytotoxicity of Immune Cells** *Emanuela Senjor, Jožef Stefan Institute; Faculty of Pharmacy, University of Ljubljana*
14.55 – 15.15	**Tunable nucleofugality in carbamate inhibitors and activity-based probes for serine hydrolases – lessons learned from cholinesterases and beyond** *Anže Meden, Faculty of Pharmacy, University of Ljubljana*
15.15 – 15.25	**Concluding remarks** *Stanislav Gobec, Faculty of Pharmacy, University of Ljubljana*

Programme

Wednesday, 17th April

Workshop 2 — **Laboratory of Chair of Pharmaceutical biology**

13.30 – 15.30 **Methods of biological evaluation of peptidase inhibitors**

1. **Cultivation and observation of mammalian cell lines**
   Anja Pišlar, Faculty of Pharmacy, University of Ljubljana
2. **Preparation of tumor spheroids and use for inhibitor evaluation**
   Ana Mitrović, Faculty of Pharmacy, University of Ljubljana; Jožef Stefan Institute
3. **Effects of peptidase inhibitor on cell proliferation**
   Emanuela Senjor, Jožef Stefan Institute; Faculty of Pharmacy, University of Ljubljana
4. **Effects of peptidase inhibitors on lymphocyte agglutination**
   Milica Perišić Nanut, Jožef Stefan Institute

Abstracts

Two Tags in One Probe and More: Activity-based Probes for Cysteine and Serine Proteases

Michael Gütschow[1]

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Activity-based protein profiling has emerged as a valuable strategy for deciphering the functions of enzymes. We have developed specific activity-based probes for cysteine and serine proteases of therapeutic importance. Our probes addressed *e.g.* cathepsin K,[1,2] a bone matrix-degrading enzyme that proved to be a therapeutic target for osteoporosis, or matriptase-2,[3,4] a serine protease that plays a key role in the iron homeostasis in humans. Other probes detect rhodesain,[5] a cysteine protease of *Trypanosoma brucei,* recognized as a target enzyme to combat Human African trypanosomiasis.

The design and chemical synthesis of the activity-based probes is reported, together with the biochemical determination of parameters of protease inactivation and the utilization of the probes for the target detection by in-gel fluorescence. X-ray crystal structure analyses were conducted to demonstrate the binding mode of the probes. Several fluorophores have been employed, for example for the chemical introduction of the green fluorescence.[1] Michael acceptor[2,5] and phosphonate warheads, available through Kabachnik-Fields chemistry,[3,4] were utilized. Bimodal rhodesain probes combined in-gel or on-blot detection with affinity chromatography and size exclusion chromatography with fluorescence detection.[5]

The design of an activity-based probe for human leukocyte elastase relied on a coumarin fluorophore, linker-connected to a warhead. An anthranilic acid function was utilized to include the protein's tryptophan residues in a sequential two-step Förster resonance energy transfer system. This function was generated in the course of a Lossen rearrangement, initiated by the enzymatic ring cleavage of a sulfonyloxyphthalimide motif. The probe-elastase interactions were studied by means of fluorescence kinetics.[6]

[1] M. Frizler et al. *Org. Biomol. Chem.* **2013**, *11*, 5913.
[2] C. Lemke et al. *J. Med. Chem.* **2021**, *64*, 13793.
[3] D. Häußler et al. *Chem. Eur. J.* **2016**, *22*, 8524.
[4] D. Häußler et al. *Chem. Eur. J.* **2017**, *23*, 5205.
[5] C. Lemke et al. *Chem. Eur. J.* **2022**, 62, e202201636.
[6] A.C. Schulz-Fincke et al. *Biochemistry* **2018**, 57, 742.

Tackling immunoproteasome with different medicinal chemistry strategies

Nika Strašek Benedik[1], Andrej Šterman[1], Lara Smrdel[1], Martina Gobec[1], Aleš Obreza[1], Zdenko Časar[1], Damijan Knez[1], Stanislav Gobec[1], Izidor Sosič[1]

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The proteasome is an intracellular protease that represents a vital part of the ubiquitin-proteasome system. It degrades many proteins and has critical functions in several biological processes. The constitutive isoform (cCP) of the proteasome is expressed in all eukaryotic cells while its immunomodulatory isoform, the immunoproteasome (iCP) is mainly expressed in cells associated with the immune system. Notably, the expression of the iCP can be induced in non-immune tissues by pro-inflammatory cytokines. The research shows that selective inhibition of the iCP has great potential as a novel approach for the treatment of a wide range of autoimmune disorders.[1] So far, the majority of iCP inhibitors encompass compounds of peptidic type that are prone to poor metabolic stability and low bioavailability.[2]
In our research, we are focusing on the identification and development of non-peptidic compounds of both non-covalent and covalent nature that selectively inhibit catalytically active subunits of the iCP. With the aim to widen the chemical space of the iCP inhibitors, three distinct medicinal chemistry strategies are being pursued. In the first one, we are utilizing scaffold morphing and hopping to further improve previously described non-peptidic inhibitors of the iCP.[3] As a second strategy, we are using fragment-based ligand finding coupled with optimization of initial hit compounds.[4,5,6] The last approach is based on a new synthetic methodology to prepare diverse enantiomerically pure aminoboronic acids that was developed in our lab.[7] This enables us to develop a variety of novel bortezomib analogs as proteasome modulators.

[1] Muchamuel, T. et al. *Nat. Med.* **2009**, *15*, 781–787
[2] Huber, E. M. et al. *Cells* **2021**, *10*, 1029
[3] Gobec, M. et al. *Acta Pharm.* **2023**, *73*, 441–456
[4] Kollar, L. et al. *Eur. J. Med. Chem.* **2021**, *219*, 113455
[5] Kollar, L. et al. *Cells* **2021**, *10*, 3431
[6] Proj, M. et al. *Bioconjug. Chem.* **2023**, *34*, 1271–1281
[7] Šterman, A. et al. *Chem. Sci.* **2022**, *13*, 2946–2953

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Cathepsins B and X: Relationship status – it is complicated

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Cathepsins B and X are lysosomal cysteine peptidases that are involved in multiple stages of tumor progression and have been identified as promising targets in cancer therapy. Among the lysosomal cysteine peptidases, cathepsin B and X are unique due to their carboxypeptidase activity. In addition, cathepsin B can also act as an endopeptidase, its activity being determined by the position of the occluding loop, extra structural element that is pH-dependent and regulates access of substrates to the active site. A compensatory role between two cathepsins is known, as the loss of one cathepsin is replaced by the other. In cancer progression, we have shown that cathepsins B and X promote epithelial-mesenchymal transition,[1] with their higher expression and activity being associated with an invasive mesenchymal cell phenotype. Furthermore, we demonstrated that the expression and activity of cathepsin B and X is increased in cancer stem cells (CSC). These are small cell populations in tumors that are resistant to most conventional antitumor therapies and are responsible for cancer recurrence. Due to their important role in many stages of cancer progression, cathepsin B and X have been recognized as promising targets in cancer therapy. Using selective reversible small molecule inhibitors of cathepsin B and X, we demonstrated that inhibition of cathepsin B and X is also effective against CSCs, where cathepsins inhibition increased differentiation of CSCs and thus improve the efficacy of conventional chemotherapy. Furthermore, simultaneous inhibition of cathepsin B and X resulted in a synergistic effect and additionally reduced tumor invasion and migration in cell-based in vitro assays of tumor cell migration and spheroid growth [2]. Taken together, the use of cathepsin B and X inhibitors, alone or in combination, represents a promising approach that could overcome the limitations of existing antitumor therapy and increase the efficacy of cancer treatment.

[1] Mitrović, A. et al. *Eur J Cell Biol.* **2017**, *96, 622-631*
[2] Mitrović, A. et al. *Cell. Mol. Life Sci.* **2022**, *76, 1-14*

Unraveling the Role of Proteasomes in Platelets

Lara Smrdel[1], Martina Gobec[1]

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Platelets are non-nucleated, disc-shaped cell particles, which play a critical role in haemostasis. Evidence in the past decades, however, shows that they have a significant role in several other processes, including inflammation, metastasis, and growth of tumour cells. These diverse facets of platelet functions are enabled by numerous molecules in them, which have immune-associated functions.[1,2] Recently, the presence of active constitutive proteasome and immunoproteasome subunits in platelets was reported.[3] Proteasomes are large protein complexes responsible for intracellular proteolysis. They have three specific subunits responsible for the catalytic activity (ß1, ß2, ß5). Hematopoietic cells assemble a specialized form of proteasomes, known as the immunoproteasome, in which the constitutive catalytic sites are replaced by the cytokine-inducible homologs ß1i, ß2i, and ß5i. Proteasomes play a key role in MHC class I antigen processing and in forming the innate and adaptive immune response through regulation of cytokine production, immune cell differentiation, survival, and proliferation.[4] However, the role of (immuno)proteasomes in platelets and their potential role in shaping the microenvironment remains unclear.

In our research, we identified differences in the expression and activity of catalytic proteasome subunits. For instance, β1c, β1i, and β5i were more prominently expressed in human individuals. Preliminary results also showed that selective inhibitors of β1i, β5i and/or β5c subunit (LMP7-IN-1, KZR-504, and carfilzomib) can modulate the signaling pathways involved in platelet activation (e.g. PI3K/Akt and MAPK/Erk axis). However, the observed effects did not manifest into modulation of platelet activation. The latter is marked with increased ATP release, degranulation, and activation. Under the investigated conditions, ATP release and secretion of selected chemokines (IL-8, RANTES, MIG, MCP-1, and IP-10) were not affected. Given the context-dependency of platelet activation, further investigations are warranted under agonist-stimulated conditions, such as ADP, TRAP-6, or collagen activation. Our findings suggest a potential role for proteasome subunits in modulating platelet function, with implications for various physiological and pathological processes. Further research is needed to elucidate the precise mechanisms underlying the interplay between proteasomes and platelet activation, offering potential avenues for therapeutic interventions in platelet-related disorders.

[1] Ali, R. A. et al. *Curr. Trends Immunol.* **2015**, *16, 65-78*
[2] Andrade, S. S. et al. *Oncotarget.* **2017**, *8, 16851-16874*
[3] El-Kadiry, A. E.-H. et al. *Int. J. Mol. Sci.* **2021**, *22, 3999*
[4] Murata, S. et al. *Nat. Immunol.* **2018**, *19, 923-931*

Metallodrugs targeting cysteine proteases

Jakob Kljun[1], Ana Mitrović[2,3], Jerneja Kladnik[1], Ana Dolinar[1], Izidor Sosič[2], Marko Novinec[1], Janko Kos[2,3], Stanislav Gobec[2], Iztok Turel[1]

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The lysosomal cysteine peptidase cathepsin B (catB) is an important tumor-promoting factor involved in tumor progression and metastasis and represents an important target for the development of new antitumor agents. The first part of the presentation will describe the synthesis of eleven ruthenium compounds with the clinical agent nitroxoline, previously identified as a potent selective reversible inhibitor of catB activity, or its derivatives. We have shown that organoruthenation is a viable strategy to obtain highly potent and specific inhibitors of catB endo- and exopeptidase activity, as demonstrated by enzyme kinetics and microscale thermophoresis. Furthermore, we were able to show that the novel metallodrugs significantly impair processes of tumor progression in *in vitro* cell based functional assays by catB inhibition at low non-cytotoxic concentrations. In general, using metallodrugs we observed an improvement in catB inhibition, a reduction in extracellular matrix degradation and tumor cell invasion compared to free ligands and a correlation with the reactivity of the monodentate halide leaving ligand.[1]

In the second part of the talk I will present the synthesis of zinc pyrithione and seven zinc pyrithione analogs as well as a ruthenium pyrithione complex. At the height of the COVID pandemic these compounds were evaluated for the stability in biologically relevant media and anti-SARS-CoV-2 activity. Zinc pyrithione showed strong *in vitro* inhibition of cathepsin L (IC_{50}=1.88 ± 0.49 μM) and PL^{Pro} (IC_{50}=0.50 ± 0.07 μM), enzymes involved in SARS-CoV-2 entry and replication, respectively, as well as antiviral entry and replication properties in an ex vivo system derived from primary human lung tissue. All zinc complexes expressed high *in vitro* inhibition. On the contrary, the ruthenium complex and the ligand pyrithione itself expressed poor inhibition of both enzymes, indicating the importance of the selection of metal core and structure of metal complex for antiviral activity.[2]

[1] Mitrović, A. et al. *Inorg Chem.* **2019**, 58*, 12334-12347*
[2] Kladnik, J. et al. *J. Enzyme Inhib. Med. Chem.* **2022**, 37*, 2158-2168*

Exploring the Potential of Cathepsin V Inhibitors in Regulation of Cytotoxicity of Immune Cells

Emanuela Senjor[1,2], Ana Mitrović[1,2], Marko Jukić[2], Matic Proj[2], Anja Dremelj[1,2], Stanislav Gobec[2], Janko Kos[1,2], Milica Perišić Nanut[1]

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Cathepsin V (CTSL2), a lysosomal cysteine peptidase closely related to cathepsin L, exhibits distinct tissue distribution, substrate specificity, and functions. While predominantly expressed in the thymus, testis, and corneal epithelium under physiological conditions, elevated levels of cathepsin V have been linked to various pathological processes, including cancer. Notably, its expression correlates with the dysregulation of cell cycle and growth regulatory genes in cancer, suggesting its involvement in tumor progression. Recent evidence also implicates cathepsin V in tumor microenvironment remodeling and immune cell infiltration alterations, with high cathepsin V expression correlating with poorer prognosis across multiple cancer types[1].
Natural Killer (NK) cells are innate immune cells that are crucial in cancer immune surveillance. They employ the granzyme perforin pathway as a major mechanism to eliminate cancer stem cells. It is regulated by proteolytic activation of granzymes and perforin by cathepsins C, H, and L. However, their cytotoxic function is susceptible to immunosuppressive factors within the TME, including cystatin F, an inhibitor of cathepsins C, H, and L. Unlike other type II cystatins typically downregulated in tumors, cystatin F is upregulated in some cancers, correlating with reduced survival. This upregulation compromises NK cell cytotoxicity. Cystatin F's activity is regulated by various factors- expression levels, N-glycosylation, and proteolytic activation[2,3]. In the lysosomes, cystatin F is activated from inactive dimeric form to active monomer and cathepsin V was shown to be the key peptidase involved in the activation of cystatin F. To counteract cystatin F-mediated immunosuppression, we developed a novel small molecular inhibitor targeting cathepsin V, the activating peptidase of cystatin F. Compound 7 was identified as a reversible, selective, and potent inhibitor of cathepsin V, through molecular docking of small molecular compounds from commercial libraries with cathepsin V and enzyme kinetics for the enzyme inhibition, selectivity, and reversibility of binding[4]. Treatment with Compound 7 reduced proliferation in cancer cell lines and reduced the conversion of cystatin F to its active monomeric form in monocyte cell line and NK cells. Compound 7 also increased cytotoxic potential of cytotoxic cell lines NK-92 and TALL-104 against K-562 cells and increased the cytotoxicity of primary NK cells against glioblastoma stem cells.
Targeting cathepsin V presents a promising strategy to mitigate the detrimental effects of cystatin F on NK cell function and enhance their cytotoxicity against cancer cells. Our findings underscore the therapeutic potential of selective cathepsin V inhibitors in cancer treatment, offering novel avenues for therapeutic intervention.

[1] Lecaille, F. et al. *Mol. Aspects Med.* **2022**, *88, 101086*
[2] Senjor, E. et al. *Cell Oncol (Dordr).* **2021**, *44, 1051-1063*
[3] Senjor, E. et al. *Cell. Mol. Life Sci.* **2024**, *81, 1-19*
[4] Mitrović, A. et al. *Comput. Struct. Biotechnol J.* **2022**, *20, 4667-4687*

Abstracts

Tunable nucleofugality in carbamate inhibitors and activity-based probes for serine hydrolases – lessons learned from cholinesterases and beyond

Anže Meden[1], Damijan Knez[1], Xi Chen[2], Xavier Brazzolotto[3], Maša Zorman[1], Anja Pišlar[4], Chang-Guo Zhan[5] and Stanislav Gobec[1]

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In the last three decades, activity-based protein profiling (ABPP) has proven to be a versatile tool in the field of serine hydrolases (SHs) and beyond.[1,2] However, only a narrow variety of carbamate warheads are utilised in chemical biology to target SHs.[3] We present an in-depth profiling of diverse halogen, chalcogen, and nitrogen-based leaving groups to identify the optimal leaving groups (nucleofuges) to tune the reactivity of the electrophilic carbamate group. Although originating from a case study on cholinesterases (ChEs), this is a first comprehensive structure-*reactivity* exploration of the carbamate warhead, rather than one-target-oriented structure-*activity* study. With computational tools we correlated the experimentally observed reactivities with steric and electronic factors of the warheads. Intrinsic chemical reactivity and spatial complementarity could be inferred from the "rough and cheap" DFT calculations combined with molecular docking and dynamics. However, only multiscale QM/MM studies in the context of topology and electrostatics of the enzymatic environment were able to explain how small changes in the nucleofuge (e.g., carbon-nitrogen substitution) allowed covalent bond to form. Additionally, by enlarging the *N*-carbamoyl moiety, we were able to slow down decarbamoylation to a point where inhibition by an otherwise pseudo-irreversible carbamate electrophile became *de facto* irreversible. Looking at the bigger picture beyond ChEs, the proteome-wide selectivity of a handful of the developed carbamate warheads was determined. A long-neglected electrophilic warhead and an archetypal carbamate with high reactivity, carbamoyl fluoride, for which we had also devised a new, practical synthesis route, showed a surprising selectivity, differing significantly from the pan-SH fluorophosphonate warhead. Additionally, 3-*O*-isoxazolyl carbamate warhead demonstrated fair selectivity for ChEs. Slight differences in the carbamate warhead were reflected in markedly different proteome labelling patterns, which would enable further development of subclass-selective ABPP probes or small molecule covalent inhibitors for certain niche proteins.

[1] Fang, H. et al. *Chem. Sci.* **2021**, *12, 8288-8310*
[2] Faucher, F. et al. *Cell Chem. Biol.* **2020**, *27, 937-952*
[3] Chang, J. W. et al. *ACS Chem. Biol.* **2013**, *8, 1590-1599*

Workshops

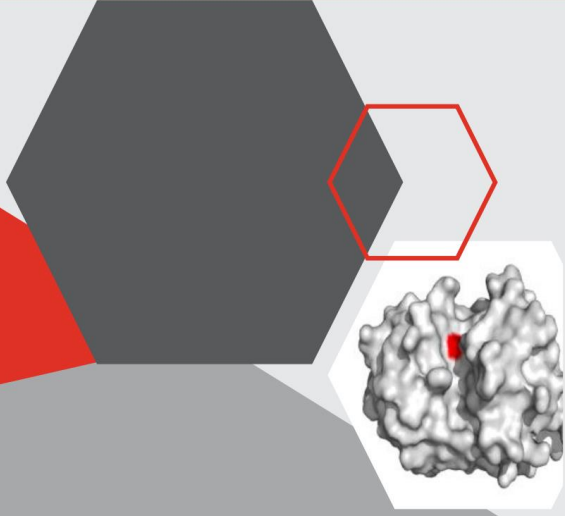
Workshop 1:
In silico design of reversible and irreversible cathepsin B inhibitors

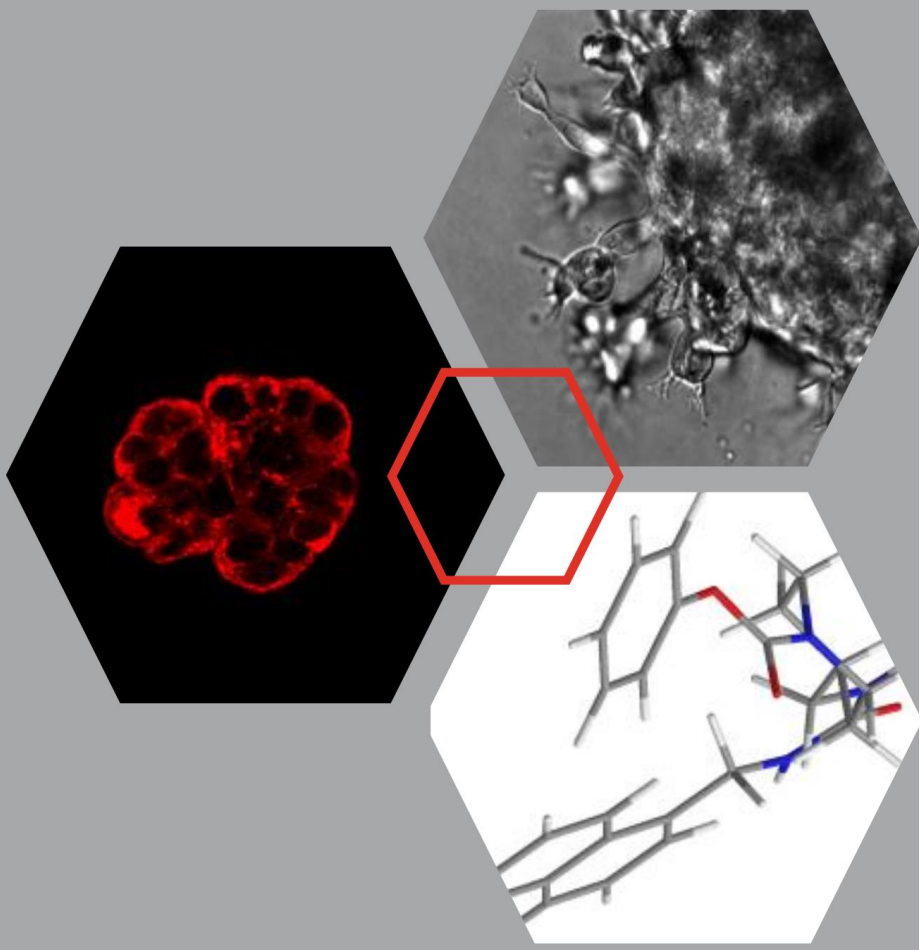
Participants will be introduced to computer tools that are used for docking and design of reversible and irreversible enzyme inhibitors, working on the case of cathepsin B.
Starting just from the structures of the protein target and prospective ligands, participants will learn the necessary steps for protein and ligand preparation, docking, covalent docking, evaluation of results, and the iterative process of enzyme inhibitors design.

Workshop 2:
Methods of biological evaluation of peptidase inhibitors

Attendees will be introduced to cell culture techniques, which are used as a tool for the biological evaluation of peptidase inhibitors, and some techniques for evaluating the impact of inhibitors in vitro.

- **Cultivation and observation of mammalian cell lines**
  The workshop will demonstrate the principle of cell culture subcultivation and the morphological observation of some mammalian cell lines (e.g. SH-SY5Y, BV2, U87, MCF7, MDA-MB-231, Jurkat).
- **Preparation of tumor spheroids and use for inhibitor evaluation**
  Effect of peptidase inhibitors on the spheroid growth – brightfield microscopy
  The workshop will demonstrate the preparation and manipulation of 3D cell- based models and show the effect of peptidase inhibitor on the growth of the tumor spheroid.
- **Effects of peptidase inhibitors cell proliferation**
  The workshop will demonstrate the evaluation of cell proliferation using flow cytometry.
- **Effects of inhibitors on lymphocyte aggregation/agglutination**
  The workshop will demonstrate the effects inhibitors on of lectin-induced lymphocyte aggregation with brightfield and fluorescence microscopy.





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