WP1.1. Targeting enzymes involved in extracellular matrix degradation (Mareš)

Research aims

- To develop a new class of specific inhibitors of ECM-degrading peptidases as potential anticancer drugs.
- To characterize small-protein scaffolds with versatile loops as a tool for specific binding to the surface of ECM-degrading peptidases.
- To design specific inhibitors of ECM-degrading peptidases based on small-protein scaffolds and their synthetic polycyclic mimetics by using structure-based rational and combinatorial approaches

Research plan and methodology

Structural analysis of small-protein scaffolds: Proteins from the I3A inhibitor family (β-trefoil scaffold) and the I31 inhibitor family (IGFBP scaffold) will be analysed at the structural level (Fig. 1). The 3D structures of five members of these families will be determined by X-ray crystallography and NMR in the form of their complexes with model peptidases with a focus on cysteine peptidases[1]. The structures of the reactive sites and the inhibition mode will be identified. Based on this analysis, we will select the critical binding loops and their key residues that control the inhibitory interaction. This part of the project will be performed in collaboration with P. Rezacova's group (IOCB).

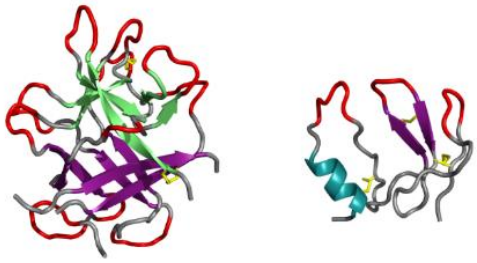

Fig. 1. Small-protein scaffolds employed by the project to design specific ligands. Left: the β-trefoil scaffold; right: the IGFBP scaffold. 3D structures are shown in cartoon representation. Candidate loops that will be engineered for binding are highlighted in red. Secondary structure elements are in magenta/green (β-strands) and blue (α-helix); disulfide bridges are in yellow.

Protein engineering and functional assays: The reactive site (typically two or three loops) on the protein scaffold will be modified for specific binding to the peptidase CTSK (a pilot target investigated). For this purpose, two approaches will be combined: phage display selection technology and in silico docking simulations[2]. The designed inhibitors will be produced as recombinant proteins and functionally characterized in vitro and in vivo. Here we will extensively use new FPLC and preparative LC apparatuses, which are proposed capital investments of this project. Firstly, the inhibitors will be screened in vitro against CTSK (and related peptidases) to determine their potency and selectivity[3]. Various ECM protein substrates (and glycosaminoglycan modulators) will be used in the activity assay[4,5], including e.g. collagens, elastins and IDP proteins. Secondly, the inhibitors will be tested in several cell-based assay systems with relevant types of cancer cells, including 3D assays with ECM protein matrices (in collaboration with J. Reseland's laboratory, University of Oslo, Norway, and D. Bromme's laboratory, University of British Columbia, Canada). The most effective inhibitors will be structurally analysed in complex with CTSK as a base for further development of inhibitors with improved binding properties.

In silico analysis of inhibitors: This part of the project will be performed in collaboration with J. Vondrasek's group and P. Hobza's group (IOCB) and O. Schueler-Furman's laboratory (Hebrew University, Israel). Computational methods will be used to analyze protein-protein interactions in the 3D structures of complexes of the protein inhibitors, and based on this, to design polycyclic peptide inhibitors as mimetics of the conformation of the binding loops in the protein inhibitors. Such polycyclic peptides are able to cover several non-contiguous binding subsites involved in the interaction[2]. The binding interface will be evaluated by the interaction energy map method[6] and the predicted structural substitutions by the Eris protein stability estimator and FoldX docking algorithm. Further, affinity of the

designed mimetics will be assessed using quantum mechanics-based scoring to obtain a detail quantitative picture of noncovalent interactions in the complexes and select the best mimetic structures (for methods, see the project by P. Hobza, WP2.3.).

Synthesis of mimetic inhibitors: This part of the project will be performed in collaboration with P. Majer's group (IOCB) and with J. Cvačka mass spectrometry department (IOCB) using new high-resolution mass spectrometer, which is a part of the proposed capital investment of this project. The mimetic inhibitors with the structure of polycyclic (bicyclic or tricyclic) peptides will be prepared and optimized by chemical randomization (Fig. 2). For this purpose, head to tail cyclization or tethering of individual side chains can be utilized. Besides cycles tethered by amide bond we plan to use thiol alkylation and/or olefin metathesis as alternative ring closing reactions. As extension of technology pioneered by Heinis[2] we plan to introduce three cysteine residues into cyclic peptide to form a tricyclic structure with three distinct binding domains. Residue side chains at selected positions will optimized by combinatorial chemistry approach[7].

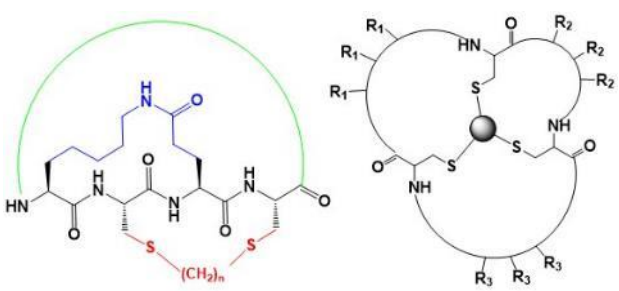

Fig. 2. Synthesis of polycyclic mimetic inhibitors. Left: three cyclization strategies: head to tail (green), side chain tethering by amide (blue) and hydrophobic side chain tethering by S-alkylation (red). Right: example of tricyclic peptidomimetics based on alkylation of three Cys redidues by trifunctional alkylating agent.

[1] Jilkova, A.; Rezacova, P.; Lepsik, M.; Horn, M.; Vachova, J.; Fanfrlik, J.; Brynda, J.; McKerrow, J. H.; Caffrey, C. R.; Mares, M., *J. Biol. Chem.* **2011**, 286 (41), 35770-35781.

[2] Baeriswyl, V.; Heinis, C., *Chem. Med. Chem.* **2013**, 8 (3), 377-384.

[3] Salat, J.; Paesen, G. C.; Rezacova, P.; Kotsyfakis, M.; Kovarova, Z.; Sanda, M.; Majtan, J.; Grunclova, L.; Horka, H.; Andersen, J. F.; Brynda, J.; Horn, M.; Nunn, M. A.; Kopacek, P.; Kopecky, J.; Mares, M., *Biochem. J.* **2010**, 429 (1), 103-112.

[4] Aguda, A. H.; Panwar, P.; Du, X.; Nguyen, N. T.; Brayer, G. D.; Brömme, D., *Proc. Natl. Acad. Sci. USA* **2014**, 111 (49), 17474-17479.

[5] Sharma, V.; Panwar, P.; O'Donoghue, A. J.; Cui, H.; Guido, R. V.; Craik, C. S.; Brömme, D., *Biochem. J.* **2015**, 465 (1), 163-173

[6] Bendova-Biedermannova, L.; Hobza, P.; Vondrasek, J., *Proteins* **2008**, 72 (1):402-413.

[7] Doleckova-Maresova, L.; Pavlik, M.; Horn, M.; Mares, M., *Chem. Biol.* **2005**, 12 (12), 1349-1357.

Research schedule

2017-2020

- Preparation of small-protein scaffolds: recombinant expression or isolation of protein inhibitors.
- Engineering of small-protein scaffolds using rational and combinatorial methods.
- Structural analysis of small-protein scaffolds: crystallization screening, crystallographic or NMR analysis of the inhibitor complexes.
- In silico analysis: computational analysis of 3D structures of the protein inhibitor complexes, prediction of mimetic inhibitors.
- Functional analysis of protein inhibitors: in vitro screening against peptidases and various substrates, in vivo screening in cell-based assay systems.

2020-2022

- Preparation and engineering of protein inhibitors and preparation of their complexes.
- In silico computational analysis of the complexes of the protein inhibitors and mimetic inhibitors.
- Synthesis of mimetic inhibitors.

- Functional analysis of protein inhibitors and mimetic inhibitors: in vitro screening against peptidases and various substrates, in vivo screening in cell-based assay systems.
- Optimization of functional properties of protein inhibitors and mimetic inhibitors: from designing to testing.
- Structural analysis of the complexes of optimized protein and mimetic inhibitors.

Publications and patents

Publications (Jimp)

	Jimp
2017	0
2018	1
2019	2
2020	2
2021	2
2022	2
Total	9

Journal of Biological Chemistry
Journal of Molecular Biology
Protein Science
Biochemistry
Journal of Structural Biology
ACS Chemical Biology
FEBS Journal

Patents and patent applications

	Patents (granted)	International patent applications (filed)
2017	0	0
2018	0	0
2019	0	0
2020	0	0
2021	0	1
2022	1	0
Total	1	1

We expect IP protection in the following areas:
Small protein derived scaffolds as peptidase inhibitors.

Cooperation with foreign institutions

- Dr. J. Reseland's laboratory at the University of Oslo (Norway),
- Dr. D. Bromme's laboratory at the University of British Columbia (Canada)
- Dr. O. Schueler-Furman's laboratory at the Hadassah Medical School, Hebrew University (Israel)