Summary

Proteases may be secreted or released into the extracellular environment through exocytosis of secretory vesicles. In addition to these soluble enzymes, a distinct subgroup of membrane-anchored proteases also exists. Three members of this subgroup (matriptase-1 (MT1), matriptase-2 (MT2), and furin) are the focus of my dissertation.

MT1 and MT2 are serine proteases anchored in a cell membrane. MT1 was first described as a novel matrix-degrading enzyme found in human breast cancer cells. It is ubiquitous in number of epithelial tissues where it plays crucial role in the maintenance of epithelial integrity. In healthy tissues, activity of MT1 is kept under tight control by two independent mechanisms: autoactivation and rapid inhibition by the Kunitz type hepatocyte growth factor activator inhibitors HAI-1 and HAI-2. Imbalanced ratio of MT1 and its inhibitors leads to MT1 dysregulation and, in consequence, to pathology. An increased expression of MT1 was revealed in variety of epithelial cancers, such as prostate, breast, ovarian, cervical and stomach cancers. What is more, an overexpression of MT1 in breast and prostate cancers is linked with tumor grade and stage, and it is considered as the predictive factor of poor disease prognosis. Therefore, MT1 is thought to be the attractive target for cancer treatment and its inhibitors are prospective anti-cancer agents. The oncogenic properties of MT1 still focus scientists' attention. MT2 is almost exclusively produced by hepatocytes. Numerous studies have underlined the role of MT2 in human iron homeostasis, but its exact function in that process is elusive. Notwithstanding, a very interesting observation is a reverse correlation between MT2 and cancers development, as compare to MT1. In contrast to MT1, presence of MT2 in breast cancer cells is considered as a good prognostic factor. This underscores the need to develop highly specific inhibitors that selectively target only one of MTs, i.e. the cancer-associated MT1 while sparing MT2.

Furin is a prototypical and the best-characterized member of the proprotein convertase subtilisin/kexin type (PCSK) family. This enzyme is also termed Paired Basic Amino Acid Cleaving Enzyme, PACE. It is a 794-amino acid type I transmembrane protein that is found in all vertebrates and many invertebrates. High levels of furin are found in salivary glands, liver and bone marrow. Furin might be found mainly in two subcellular localizations: the trans-Golgi network and on the cell surface. In the trans-Golgi network, furin cleaves and activates mostly substrates delivered by host cells. Among them are cellular precursor proteins, such as: growth factors, neuropeptides, hormones, adhesion molecules, blood coagulation factors and receptors. On the cell surface it cleaves many external pathogenic substrates. Apart from its physiological relevance, furin is also involved in the development of various inflammatory diseases, cancers, pathogen infections (both viral and bacterial), atherosclerosis and neurodegenerative disorders. Furin takes part in growth and progression of various malignancies,

including colon carcinoma, rhabdomyosarcoma, head and neck cancers, lung, skin and brain tumors. Also many pathogen molecules, like bacterial toxins (for example diphtheria toxin or *Pseudomonas* exotoxin), can be activated by furin. Host furin is also exploited by viruses to promote infection through the processing of viral surface glycoproteins. Furin-mediated cleavage has been described for glycoproteins produced by numerous, evolutionarily diverse viruses, e.g. HIV, influenza, Dengue fever, Ebola, Marburg virus, to name a few.

The aim of my work was to design and obtain highly potent, selective, non-covalent inhibitors of MT1 and furin that remain stable in proteolytic and reducing environments. The structural scaffold for the new MT1 inhibitors was a truncated analogue of *Huia versabilis* Bowman-Birk inhibitor (HV-BBI), which exhibits exceptionally strong inhibitory activity against MT1 (K_i = 8 nM) and almost 1000 fold selectivity over MT2. The development of inhibitors that selectively target only one of the matriptases is extremely challenging due to the high structural homology between MT1 and MT2. Furin inhibitors developed in this study were designed on the basis of a potent furin inhibitor that is an analogue of the Sunflower trypsin inhibitor-1 (SFTI-1) that also belongs to Bowman-Birk family of inhibitors. The structures of these reference inhibitors are shown below (cyclization indicated by "&").

HV-BBI: Ser-Val-Ile-Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-Val-Lys-NH_2

SFTI-1: $\&^1$Gly-Arg-Cys($\&^2$)-Thr-Lys-Ser-Ile-Pro-Pro-Ile-Cys($\&^2$)-Phe-Pro-Asp$\&^1$

My research initially focused on the development of MT1-specific inhibitor. I synthesized two 5(6)-carboxyfluorescein-labeled analogues of a potent MT1 inhibitor derived from an SFTI-1 scaffold. One of these peptides was additionally *N*-methylated to improve its proteolytic stability. However, with the identification of a more potent MT1 inhibitor, an analogue of HV-BBI (inhibitor **3**), this scaffold became the focus of subsequent optimization.

Molecular dynamics studies indicated that replacing Phe13 in inhibitor **3** could strengthen its binding interactions and thereby enhance its inhibitory potency. Consequently, this position was substituted with various Phe derivatives containing more bulky side chains, as well as amino acids differing in size and physicochemical properties, such as Lys. Unfortunately, this modification did not improve inhibitory activity. Therefore, I performed alanine scanning, to identify residues essential for inhibitory function. The results of this analysis enabled the selection of amino acids that could potentially be removed to generate inhibitors with reduced molecular weight. Size reduction is a modification that may enhance proteolytic stability and bioavailability. I designed and synthesized 9 truncated analogues of inhibitor **3**. I also synthesized an analogue in which the disulfide bridge was replaced with a triazole bridge to increase its stability in reducing conditions. **Two of the obtained shortened analogues revealed inhibitory activity comparable to that of the starting inhibitor. Notably, the lower-molecular-weight analogue displayed the highest stability in human serum among all MT1 inhibitors examined.**

The second group of compounds which I synthesized were furin inhibitors. Using a potent furin inhibitor, an SFTI-1 analogue previously developed by the Fittler group, as a template, we created a peptide library employing combinatorial chemistry. Subsequent iterative deconvolution allowed us to determine the optimal amino acids at the P1, P2, P4, and P5 positions. After selecting the most promising furin inhibitor, I introduced additional basic amino acid at the *N*-terminus, following literature reports indicating a positive effect of such modification on inhibitory potency towards furin. I also applied an additional cyclization to enhance the proteolytic stability of the peptides. **Enzymatic evaluation led to the identification of three potent inhibitors (40, 42, 46) with nanomolar K_i values against furin**. I subsequently obtained 5(6)-carobxyfluorescein labeled analogues of these inhibitors and an additional analogue of one of them in which the disulfide bridge was replaced with a triazole bridge.

One of furin inhibitors (**42**) and its analogues with replaced disulfide bridge were evaluated for anticancer activity. The anticancer studies did not yield clear conclusions. The analogues containing the triazole (peptide **44**) and diselenide (peptide **45**) bridges demonstrated improved anticancer activity toward U251 and A549 cell lines compared with the parent furin inhibitor (containing disulfide bridge). However, the intended positive control (commercial furin inhibitor, Dec-RVKR-CMK) showed no anticancer activity under the tested conditions. This finding suggests that the anticancer properties observed for the synthesized furin inhibitors cannot be attributed exclusively to furin inhibition.

In contrast, antiviral tests against the Zika virus (ZIKV) revealed that all of the three potent furin inhibitors (**40, 42, 46**) reduced ZIKV titers, as measured using the plaque assay. Notably, the most potent furin inhibitor also exhibited exceptionally strong antiviral activity against the ZIKV replication cycle, surpassing the activity of the commercially available, non-selective furin inhibitor Dec-RVKR-CMK. **Importantly, our inhibitor was less cytotoxic and demonstrated an approximately five-fold higher therapeutic index (SI). These results indicate the potential therapeutic applicability of this inhibitor in Flaviviruses infections.**

Moreover, since I applied *N*-methylation to selected peptides to improved their stability in human serum, I decided to optimize this method. Until recently, the method commonly used in our group was a three-step on-resin *N*-methylation protocol requiring 4 h to complete. Given the substantial acceleration of the peptide synthesis achieved through ultrasonic agitation, I decided to investigate whether this method could be applied in place of standard shaking during *N*-methylation. For the optimization studies, the tripeptide Arg-Trp-Gly-NH_2 was selected as a model compound. Each step of the procedure was progressively shortened. **As a result, I optimized and significantly reduced total duration of the on resin peptide *N*-methylation process (from 4 h to 40 min).** After the optimal conditions were established, the method was subsequently applied to the *N*-methylation of various *N*-terminal amino acid in the model tripeptide in place of Arg, to assess its versatility. The residues tested were: His, Ser, Trp, Tyr, Asp, Glu, Cys, Phe, and Ala. Furthermore, the efficiency of my, time-reduced

N-methylation method was compared using a microwave-assisted peptide synthesizer and standard laboratory shaker. Finally, after confirming the applicability and efficiency of the optimized method, I synthesized a peptide methylated at three positions, further demonstrating the method's utility.