



University of Gdańsk, Faculty of Chemistry

Department of Molecular Biochemistry

Design, chemical synthesis and enzymatic studies of transmembrane serine proteases inhibitors: matriptase-1, matriptase-2 and furin

Projektowanie, chemiczna synteza oraz ocena aktywności inhibitorów transmembranowych proteinaz serynowych: matryptazy-1, matryptazy-2 oraz furyny

Doctoral dissertation

mgr Aleksandra Dorota Helbik-Maciejewska

Supervisor: prof. dr hab. Krzysztof Rolka

Assistant Supervisor: dr Agata Gitlin-Domagalska

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Table of contents

Summary.....	6
Streszczenie.....	10
Abbreviations	14
Introduction.....	17
1. Proteases.....	17
1.1. Serine proteases.....	18
1.1.1. Transmembrane serine proteases	21
1.1.2. Type I transmembrane serine proteases	22
1.1.3. Type II transmembrane serine proteases	22
1.1.3.1. Matriptase-1	25
1.1.3.2. Matriptase-2.....	29
1.1.3.3. Matriptase-1 and matriptase-2 catalytic domains - differences and similarities	32
1.1.4. Proprotein convertases	33
1.1.4.1. Furin	35
1.1.4.1.1. Furin in tumor progression.....	39
1.1.4.1.2. Role of furin in viral pathogenicity	40
1.1.4.1.3. Furin and ZIKV relationship.....	41
2. Serine proteases inhibitors	44
2.1. Classification of serine protease inhibitors by inhibition mechanism.....	44
2.2. Classification of serine protease inhibitors by structural diversity	46
2.2.1. Bowman-Birk inhibitors	48
2.2.1.1. Sunflower Trypsin Inhibitor-1 (SFTI-1).....	49
2.2.1.2. Huia versabilis Bowman-Birk inhibitor (HV-BBI).....	51
2.3. Matriptase-1 inhibitors	52
2.4. Furin inhibitors.....	53
3. Molecular imaging - peptide-based molecular probes	55
3.1. Activity-based probes.....	56
3.2. Affinity based probes	57
Scientific goals of the study	58
Experimental section.....	62
4. Materials used in the synthesis and purification of inhibitors.....	62
4.1. Reagents and solvents	62
4.2. Amino acids and derivatives	63

4.3.	Resins	64
5.	Analytical methods.....	64
6.	Methodology of peptide synthesis	64
6.1.	Attachment of the <i>C</i> -terminal Fmoc-amino acid derivative to the 2-chlorotrityl resin	65
6.2.	Solid phase peptide synthesis – manual synthesis	66
6.3.	Solid phase peptide synthesis using automatic synthesizer.....	67
6.4.	Solid phase peptide synthesis using semi-automatic synthesizer.....	67
6.5.	Monitoring of peptide coupling and deprotection reactions	67
6.6.	Cleavage of peptide from solid support	68
6.7.	Synthesis of cyclic peptides	68
6.7.1.	Formation of disulfide bridge	68
6.7.2.	Formation of the triazole bridge applying “click” chemistry.....	69
6.7.2.1.	Confirmation of the presence of triazole bridge.....	73
6.7.2.1.1.	Test with DTT	73
6.7.2.1.2.	Incubation with trypsin	76
6.7.3.	Formation of the diselenide bridge	78
6.7.4.	Backbone cyclization	79
6.8.	Synthesis of peptomer	79
6.9.	Labeling peptides – introduction of fluorescent tag.....	80
6.9.1.	Labeling of monocyclic peptides – introduction of fluorescent tag in the <i>N</i> -termini	81
6.9.2.	Labeling of bicyclic peptides – introduction of fluorescent tag in the peptide side chain.....	82
6.9.2.1.	Attachment of 5(6)-carboxyfluorescein to Fmoc-Lys	82
6.9.2.2.	Attachment of 5(6)-carboxyfluorescein to Fmoc-Phe(4-NH ₂).....	85
6.10.	<i>N</i> -methylation of peptides	86
6.11.	Purification of peptides	88
7.	Enzymatic studies	88
7.1.	Materials and methods used in enzymology studies	88
7.1.1.	Enzymes.....	88
7.1.2.	Substrates	88
7.1.3.	Others.....	88
7.1.4.	Procedure for measuring the inhibitory activity of MT1 and MT2 inhibitors ..	89
7.1.5.	Procedure for measuring the inhibitory activity of of furin inhibitors	89
7.1.6.	Procedure of a IC ₅₀ determination.....	90
7.1.7.	Procedure of K _m determination	90

7.1.8.	Procedure of inhibition constant (K_i) determination.....	90
8.	Study of inhibitors' proteolytic stability.....	90
Results and discussion		91
9.	Design and enzymatic evaluation of MT1 inhibitors.....	91
9.1.	Analogues of SFTI-1.....	91
9.2.	Analogues of HV-BBI.....	92
9.2.1.	Replacement of Phe13 in peptide 3	96
9.2.2.	Alanine Scanning in peptide 3	100
9.2.3.	Reduction and optimization of the molecular size of inhibitor 3	103
9.2.3.1.	Truncation of peptide 3	103
9.2.3.2.	Deletion of Trp and Pro residues in peptide 3	104
9.2.4.	Analogue of peptide 3 with triazole bridge.....	106
9.2.5.	Study of MT1 inhibitors' stability in serum.....	108
10.	Synthesis of MT2 inhibitor	113
11.	Design and enzymatic evaluation of furin inhibitors	115
11.1.	Antiviral properties of furin inhibitors 40 , 42 , and 46	120
11.2.	Inhibitor 42 analogues with disulfide bridge mimetics.....	125
11.2.1.	Anticancer properties of furin inhibitors 42 , 44 , and 45	127
11.3.	Study of furin inhibitors' stability in serum	129
11.4.	Conformational analysis and structural organization of inhibitors 42 and 46	131
11.5.	Design of furin inhibitors for albumin-binding assessment.....	132
12.	Optimization of the peptide <i>N</i> -methylation method.....	135
Conclusions		141
References		144
List of Figures.....		166
List of Tables.....		169
Academic achievements.....		170

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-NH₂

SFTI-1: &¹Gly-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)-carboxyfluorescein 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-NH₂ 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.

Streszczenie

Proteazy są hydrolazami katalizującymi rozcinanie wiązań peptydowych. Znaczną część z nich stanowią enzymy wydzielane do płynów ustrojowych. Oprócz tych enzymów występujących w formie rozpuszczalnej, istnieje także podgrupa proteaz zakotwiczonych w błonie komórkowej. Trzej przedstawiciele tej grupy (matryptaza-1 (MT1), matryptaza-2 (MT2) oraz furyna) są obiektem badań przedstawionych w niniejszej pracy.

MT1 i MT2 są serynowymi proteazami zakotwiczonymi w błonie komórkowej. MT1 została odkryta w komórkach raka piersi jako nowy enzym degradujący macierz zewnątrzkomórkową. Enzym ten jest szeroko rozpowszechniony w licznych tkankach nabłonkowych, gdzie odgrywa kluczową rolę w utrzymaniu integralności nabłonka. W zdrowych tkankach aktywność MT1 jest ściśle regulowana przez dwa niezależne mechanizmy: autoaktywację oraz inhibicję przez inhibitory aktywatora czynnika wzrostu hepatocytów HAI-1 i HAI-2. Zaburzona równowaga pomiędzy MT1 a jej inhibitorami prowadzi do deregulacji aktywności enzymu, a w konsekwencji do licznych stanów patologicznych. Podwyższoną ekspresję MT1 wykazano w różnych nowotworach nabłonkowych, takich jak rak prostaty, piersi, jajnika, szyjki macicy czy żołądka. Co więcej, nadekspresja MT1 w nowotworach piersi i prostaty koreluje ze stopniem zaawansowania nowotworu oraz może stanowić negatywny czynnik prognostyczny dla pacjenta. Z tego względu MT1 jest uważana za atrakcyjny cel terapeutyczny w leczeniu nowotworów, a jej inhibitory stanowią obiecującą grupę potencjalnych leków przeciwnowotworowych. Z kolei ekspresję MT2 obserwuje się głównie w hepatocytach. Liczne badania podkreślają rolę MT2 w homeostazie żelaza u człowieka, choć jej dokładna funkcja w tym procesie nie jest wciąż w pełni poznana. Co istotne, w przeciwieństwie do MT1, odnotowano odwrotną zależność pomiędzy ekspresją MT2, a rozwojem nowotworów. Obecność MT2 w komórkach raka piersi jest uważana za dobry prognostyk dla pacjenta. Podkreśla to konieczność opracowania wysoce specyficznych inhibitorów, które selektywnie blokują jedynie MT1, związaną z rozwojem nowotworów, a nie wpływają na aktywność MT2.

Furyna jest najlepiej scharakteryzowaną subtylizyno-podobną konwertazą proproteinową. Enzym ten jest również określany jako PACE (ang. Paired Basic Amino Acid Cleaving Enzyme). Furyna składa się z 794 reszt aminokwasowych i jest białkiem zakotwiczonym w błonie komórkowej typu I. Jest szeroko rozpowszechniona w ludzkim organizmie. Wysoki poziom ekspresji furyny obserwuje się w śliniankach, wątrobie oraz szpiku kostnym. W komórce jest zlokalizowana w dwóch miejscach: na powierzchni lub w sieci trans-Golgiego. W sieci trans-Golgiego furyna odpowiada za przekształcanie i aktywację głównie białek gospodarza, takich jak czynniki wzrostu, hormony, cząsteczki adhezyjne, czynniki

krzepnięcia krwi czy receptory. Z kolei na powierzchni komórki furyna przetwarza liczne substraty patogenne. Furyna bierze udział w rozwoju wielu chorób zapalnych, neurodegeneracyjnych oraz infekcji bakteryjnych i wirusowych. Jej aktywność jest powiązana również z rozwojem licznych nowotworów, m.in. jelita grubego, płuc, skóry, mózgu, głowy i szyi. Ponadto, furyna aktywuje toksyny bakteryjne (np. toksynę błoniczą czy egzotoksynę *Pseudomonas*). Z kolei wirusy (m.in. HIV, wirus grypy, dengi, Ebola, czy Marburg) wykorzystują furynę gospodarza do przetwarzania glikoprotein znajdujących się na ich powierzchni, proces ten skutkuje rozpoczęciem infekcji.

Celem mojej pracy było zaprojektowanie oraz otrzymanie silnych, selektywnych, niekowalencyjnych inhibitorów MT1 i furyny. Strukturą wyjściową dla nowych inhibitorów MT1 był skrócony analog inhibitora wyizolowanego ze skóry płaza *Huia versabilis*, należący do rodziny inhibitorów Bowmana-Birki, (ang. *Huia versabilis* Bowman-Birk inhibitor, HV-BBI), który wykazywał niezwykle silną aktywność wobec MT1 ($K_i = 8$ nM) oraz niemal tysiącrotnie wyższą selektywność względem MT2. Otrzymanie selektywnych inhibitorów zdolnych do hamowania aktywności tylko jednej z matryptaz jest zadaniem niezwykle wymagającym ze względu na podobną budowę MT1 i MT2. Z kolei inhibitory furyny zostały zaprojektowane w oparciu o strukturę silnego inhibitora furyny, będącego analogiem inhibitora trypsyny wyizolowanego z nasion słonecznika (ang. Sunflower trypsin inhibitor-1, SFTI-1). Struktury wspomnianych inhibitorów zostały przedstawione poniżej (cyklizacja została oznaczona symbolem „&”).

HV-BBI: Ser-Val-Ile-Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-Val-Lys-NH₂

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

Moje badania początkowo skupiały się na opracowaniu inhibitora specyficznego wobec MT1. Zsyntezowałam dwa znakowane 5(6)-karboksyluoresceiną analogi silnego inhibitora MT1, (zaprojektowanego na podstawie struktury SFTI-1). Jeden z tych peptydów został dodatkowo poddany *N*-metylowaniu w celu zwiększenia jego stabilności proteolitycznej. Jednak po zidentyfikowaniu bardziej skutecznego inhibitora MT1, będącego analogiem HV-BBI (inhibitor **3**), to właśnie ta struktura stała się związkiem wyjściowym w dalszych pracach.

Badania z wykorzystaniem symulacji dynamiki molekularnej wskazały, że zastąpienie reszty Phe13 w inhibitorze **3** mogłoby wzmocnić jego oddziaływanie z enzymem, a tym samym zwiększyć jego aktywność inhibitorową. W związku z tym Phe13 została zastąpiona różnymi pochodnymi Phe, zawierającymi bardziej rozbudowane łańcuchy boczne, a także aminokwasami różniącymi się rozmiarem i właściwościami fizykochemicznymi, takimi jak Lys. Niestety, modyfikacja ta nie poprawiła aktywności inhibitorowej. Dlatego, przeprowadziłam skaninę

alaninowy w celu zidentyfikowania reszt aminokwasowych kluczowych dla oddziaływań z enzymem. Wyniki tych badań pozwoliły mi wytypować aminokwasy, które potencjalnie można usunąć, aby uzyskać inhibitory o obniżonej masie cząsteczkowej. Celem zmniejszenia rozmiaru cząsteczki było zwiększenie stabilności proteolitycznej oraz ewentualnej biodostępności inhibitorów. Zaprojektowałam i zsyntetyzowałam dziewięć skróconych analogów inhibitora **3**. Ponadto otrzymałam analog, w którym mostek disulfidowy zastąpiłam mostkiem triazolowym, aby zwiększyć stabilność inhibitora w warunkach redukujących. **Dwa skrócone analogi wykazały aktywność inhibitorową porównywalną z inhibitorem wyjściowym. Co istotne, analog o niższej masie cząsteczkowej wykazywał najwyższą stabilność w surowicy ludzkiej spośród wszystkich badanych inhibitorów MT1.**

Drugą grupą związków, które zsyntezowałam stanowiły inhibitory furyny. Wykorzystując strukturę silnego inhibitora furyny (analogu SFTI-1) opracowanego wcześniej przez grupę Fittlera stworzyliśmy bibliotekę peptydów z użyciem chemii kombinatorycznej. Kolejne etapy iteracyjnej dekonwolucji biblioteki peptydowej pozwoliły określić optymalne aminokwasy w pozycjach P1, P2, P4 oraz P5. Po wybraniu najbardziej obiecującego inhibitora furyny, wprowadziłam dodatkowy aminokwas zasadowy na *N*-końcu, zgodnie z doniesieniami literaturowymi wskazującymi na pozytywny wpływ takiej modyfikacji na siłę hamowania furyny. Zastosowałam również dodatkową cyklizację w celu zwiększenia stabilności proteolitycznej peptydów. **Badania enzymatyczne umożliwiły zidentyfikowanie trzech silnych inhibitorów (40, 42, 46) wykazujących nanomolowe wartości K_i aktywność wobec furyny.** Następnie otrzymałam analogi tych inhibitorów znakowane 5(6)-karboksyluoresceiną oraz dodatkowy analog jednego z nich, w którym mostek disulfidowy zastąpiłam mostkiem triazolowym.

Jeden z inhibitorów furyny (**42**) oraz jego analogi z wymienionym mostkiem disulfidowym zostały poddane ocenie pod kątem aktywności przeciwnowotworowej. Badania te nie przyniosły jednak jednoznacznych wniosków. Analogi zawierające mostki triazolowe (peptyd **44**) oraz diselenowy (peptyd **45**) wykazały zwiększoną aktywność przeciwnowotworową wobec linii komórkowych U251 i A549 w porównaniu z inhibitorem wyjściowym (zawierającym mostek disulfidowy). Jednakże zastosowany w badaniach, jako kontrola pozytywna, komercyjny inhibitor furyny (Dec-RVKR-CMK) nie wykazał aktywności przeciwnowotworowej. Może to sugerować, że aktywność przeciwnowotworowa peptydów **44** oraz **45** niekoniecznie wynika z hamowania furyny.

Z kolei badania przeciwwirusowe przeprowadzone wobec wirusa Zika (ZIKV) wykazały, że wszystkie trzy silne inhibitory furyny (**40, 42, 46**) obniżały miano ZIKV. Co istotne, najsilniejszy inhibitor furyny wykazał również najsilniejszą aktywność przeciwwirusową wobec ZIKV, przewyższając działanie komercyjnego, nieselektywnego inhibitora furyny

Dec-RVCR-CMK. Ważnym aspektem jest również niższa cytotoksyczność naszego inhibitora oraz około pięciokrotnie wyższy indeks terapeutyczny (SI). Wyniki te wskazują na potencjalne zastosowanie terapeutyczne tego inhibitora w leczeniu zakażeń Flawiwirusami.

Ponadto w związku z *N*-metylowaniem wybranych peptydów w celu zwiększenia ich stabilności w surowicy ludzkiej, zdecydowałam się zoptymalizować tę metodę. Do niedawna powszechnie stosowaną procedurą w naszej grupie była czterogodzinna, trzyetapowa metoda *N*-metylowania na nośniku stałym. Biorąc pod uwagę doniesienia wskazujące na znaczące przyspieszenie syntezy peptydów osiągane dzięki zastosowaniu ultradźwięków, postanowiłam zbadać, czy technika ta mogłaby zastąpić standardowe wytrząsanie podczas procesu *N*-metylowania. Jako związek modelowy do badań wybrałam tripeptyd Arg-Trp-Gly-NH₂. Każdy etap procedury był przeze mnie stopniowo skracany. **W rezultacie zoptymalizowałam i istotnie skróciłam całkowity czas trwania procesu *N*-metylowania peptydu na nośniku stałym, z 4 godzin do 40 minut.** Po ustaleniu optymalnych warunków metoda została następnie zastosowana do *N*-metylowania różnych aminokwasów znajdujących się na *N*-końcu w modelowym tripeptydzie. Testowanymi resztami były: His, Ser, Trp, Tyr, Asp, Glu, Cys, Phe i Ala. Ponadto oceniłam wydajność skróconej metody wykorzystując syntezytor peptydów wspomagany mikrofalami oraz standardową wytrząsarkę laboratoryjną. W końcowym etapie, po potwierdzeniu efektywności zoptymalizowanej metody, zsyntetyzowałam peptyd metylowany w trzech pozycjach, co dodatkowo potwierdziło użyteczność tej metody.

Abbreviations

The abbreviations of amino acids and their derivatives are in accordance with recommendations of IUPAC - IUB Join Commission on Biochemical Nomenclature, that are included in *Nomenclature and symbolism for amino acids and peptides*, Eur. J. Biochem., 138, 9-37 (1984), as well as in *A Short Guide to abbreviations and Their Use In Peptide Science*, J. Pept. Sci., 5, 465-471 (1999) and in *Revised Guide to Abbreviations In Peptide Science and Plea for Conformity*, J. Pept. Sci., 12, 1-12, (2006). In the cases that are not included in the mentioned guidelines, abbreviations commonly used in peptide chemistry were applied.

α1-PDX	α 1-antitrypsin Portland
A1AT	Alpha 1 antitrypsin
ACN	Acetonitrile
AcOH	Acetic acid
Agb	2-amino-3-guanidinopropionic acid
Aha	Azidohomoalanine
AMC	7-amino-4-methylcoumarin group
BBB	Blood brain barrier
BBLTIs	Bowman-Birk-like trypsin inhibitors
BMPs	Bone morphogenic proteins
Bpa	Benzoyl-L-phenylalanine
BSA	Bovine serum albumin
C	Core
CC₅₀	Cytotoxic concentration 50%
Cf	5(6)-carboxyfluorescein
Cit	Citrulline
CMK	Chloromethylketone
CPPs	Cell-penetrating peptides
CUB	Cls/Clr, urchin embryonic growth factor and bone morphogenetic protein-1
DAPI	4',6-diaminidino-2-phenylindole
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DENV	Dengue virus
DHB	2,5-dihydroxybenzoic acid matrix
DIC	<i>N,N'</i> -diisopropylcarbodiimide
DIPEA	<i>N,N</i> -diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMBA	7,12-dimethylbenzanthracene
DMF	<i>N,N</i> -dimethylformamide
Dpa	3,3-diphenyl-L-alanine
DTNP	2,2'-dithiobis(5-nitropyridine)
DTT	Dithiothreitol
E	Envelope
EC	Enzyme Commission
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
Fmoc	9-fluorenylmethoxycarbonyl
fXa	Factor Xa
Gnf	4-guanidinophenylalanine

GPI	Glycosylphosphatidylinositol
HA	Hemagglutinin
HAI	Hepatocyte growth factor activator inhibitors
HArg	Homoarginine
HAT/DESC	Human airway trypsin-like protease/differentially expressed in squamous cell carcinoma
HATU	O-(7-azabenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HBeAg	hepatitis B e antigen
HBTU	<i>N,N,N',N'</i> -tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HBV	Hepatitis B virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HIF-1	hypoxia-inducible factor-1
HIV-1	Human immunodeficiency virus-1
HJV	Hemojuvelin
HNSCC	Head and neck squamous cell carcinoma
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HSA	Human serum albumine
HV-BBI	Huia versabilis Bowman-Birk inhibitor
IC₅₀	Half-maximal inhibitory concentration
IGFs	Insulin-like growth factors
IR	Insulin receptor
IRIDA	Iron refractory iron deficiency anemia
IsOH	Isopropanol
ITC	Isothermal titration calorimetry
JEV	Japanese encephalitis virus
KD	Kunitz-type serine proteinase inhibitor domain
K_i	Inhibitory constant
K_m	Michaelis-Menten constant
LDL	Low-density lipoprotein
LDLRA	Low-density lipoprotein receptor class A domain
MAM	Meprin, A5 antigen, and receptor protein phosphatase μ domain
MCs	Mast cells
MeOH	Methanol
Mob	<i>p</i> -methoxybenzyl
MOI	Multiplicity of infection
MT1	Matriptase-1
MT2	Matriptase-2
MT3	Matriptase-3
MW	Microwave-assisted peptide synthesizer
N	Stoichiometry
NC-IUBMB	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
NMM	<i>N</i> -methylmorpholine
NMP	1-metylo-2-pyrrolidone
NS	Non-structural
NSCLC	Non-small cell lung cancer
<i>o</i>-NBS-Cl	<i>o</i> -nitrobenzenesulfonyl chloride
Orn	Ornithine
PACE	Paired basic amino acid cleaving enzyme
PAI-1	Plasminogen activator inhibitor-1
P_{app}	Apparent permeability coefficients

PBMPs	Peptide-based molecular probes
PCs	Proprotein convertases
PCSK	Proprotein convertase subtilisin/kexin type
PDB	Protein Data Bank
PDGFs	Platelet-derived growth factors
PepT1	Peptide transporter 1
Phe(4-F)	4-fluoro-L-pheynylalanine
Phe(4-NH₂)	4-amino-L-phenylalanine
Phe(4-NO₂)	4-nitro-L-phenylalanine
Phe(4-Ph)	4-pheynyl-L-phenylalanine
Phe(4-tBu)	4-tert-butyl-L-phenylalanine
Pin1	Potato type I serine proteinase inhibitor
Pin2	Potato type II serine proteinase inhibitor
Pra	Propargylglycine
prM	Precursor membrane
pro-ANP	Pro-atrial natriuretic peptide
proIR	Precursor of insulin receptor
PRSS31	Protease serine member S31
PTFE	Polytetrafluoroethylene
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
Pyr	Pyroglutamic acid
RCL	Reactive center loop
RP-HPLC	Reverse-phase high performance liquid chromatography
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCC	Squamous cell carcinoma
SEA	Sea urchin sperm protein, enteropeptidase, agrin domain
Sec	Selenocysteine
SERPINC1	Antithrombin III
SI	Selectivity index
SKTI	Soybean Kunitz trypsin inhibitor
SMAD	Suppressor of mother against decapentaplegic
SMI	Small-molecule inhibitors
SPPS	Solid phase peptide synthesis
SR	Scavenger receptor domain
T2DM	Type 2 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBEV	Tick-borne encephalitis virus
TEER	Transepithelial/transendothelial electrical resistance
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TGN	<i>trans</i> -Golgi network
TIPS	Triisopropylsilane
TMPRSS	Transmembrane protease/serine S1
t_R	Retention time
Tris-Cl	Trizma® chydrochloride
TTSPs	Type II transmembrane serine proteases
UA	Ultrasonic agitation
VEGF-C	Vascular endothelial growth factors C
VEGF-D	Vascular endothelial growth factors D
WNT	Wingless-related integration site
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

Introduction

1. Proteases

The vast diversity of enzymes necessitated the establishment of a standardized nomenclature system by scientific community. Each enzyme is assigned to a unique four-digit code preceded by the prefix 'EC', which stands for Enzyme Commission. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) classifies enzymes into seven major classes, each designated by an EC number:

- EC 1 Oxidoreductases,
- EC 2 Transferases,
- EC 3 Hydrolases,
- EC 4 Lyases,
- EC 5 Isomerases,
- EC 6 Ligases,
- EC 7 Translocases.¹

Proteases, also known as peptidases, constitute a subclass of hydrolases. They are responsible for hydrolysis of peptide bonds and are essential for all living organisms. They play critical roles in protein and peptide biosynthesis (e.g. hormones) and in post-translational modifications. Furthermore, they are responsible for the degradation of proteins, determining their ultimate fate through hydrolysis.²

A comprehensive classification of proteases was introduced in 1993 by Rawlings and Barrett, who organized peptidases into clans and families. In 1996, they established the MEROPS database, which has since been continuously updated and expanded.^{3,4} This database is a source of information about peptidases, and also their substrates and inhibitors. This strictly hierarchical division categorizes proteins into species, families and clans.⁵ According to this system a family comprises enzymes with similar amino acid sequences in regions responsible for catalytic activity, while a clan includes families that are evolutionally related, even if they do not share sequence similarity. Some families are further divided into subfamilies. Each clan and family is associated with a specific catalytic type.⁶ The first letter in the classification denotes the catalytic type: A (aspartic peptidases), C (cysteine peptidases), G (glutamic peptidases), M (metallopeptidases), N (asparagine peptide lyases), S (serine peptidases), T (threonine peptidases), and U (peptidases of unknown catalytic type).⁵ However, some families and clans comprise enzymes with multiple catalytic types. In such cases, their names begin with the letter P. For example, family P1 includes enzymes with cysteine, serine, and threonine catalytic types, while clan PA comprises both serine and cysteine proteases.^{4,7}

The specificity of a protease is determined by the composition of its active site, which dictates the substrate that will form a non-covalent Michaelis complex and undergo proteolytic cleavage. The nomenclature used to describe protease binding sites was introduced by Schechter and Berger in 1967⁸ and is illustrated schematically in Figure 1. Briefly, the enzyme's binding pockets are designated as non-primed sites (S1, S2, S3, etc.) and primed sites (S1', S2', S3', etc.), depending on their position relative to the active site. The corresponding substrate/inhibitor residues are labeled P1, P2, P3, etc. (toward the *N*-terminus) and P1', P2', P3', etc. (toward the *C*-terminus). The P1 position is a location directly prior the cleavage site.⁸

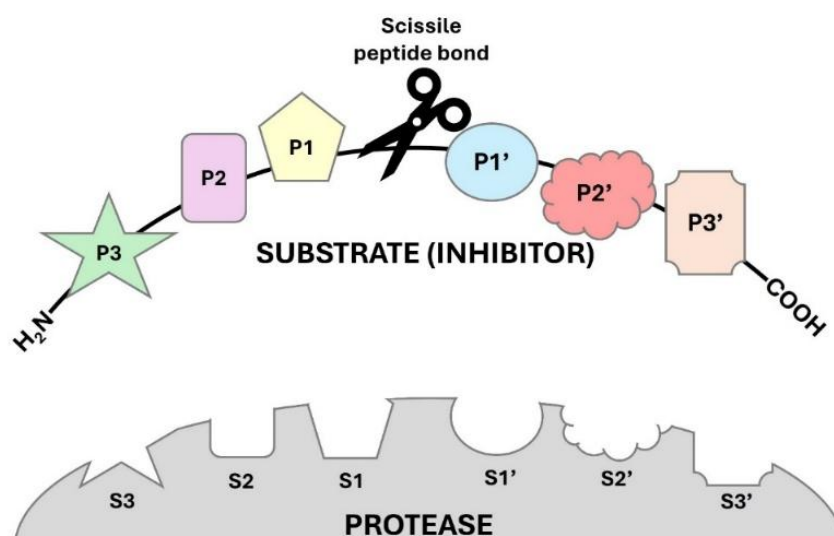


Figure 1. The scheme of substrate (inhibitor) - protease interaction according to Schechter-Berger⁸ model.

1.1. Serine proteases

According to data published in the MEROPS database, serine proteases constitute the largest group of proteolytic enzymes. Approximately one-third of all proteases are classified as serine proteases, which are characterized by a nucleophilic serine residue in their active sites.⁹ The database currently lists 1,728 serine proteases with MEROPS identifiers, including 188 enzymes with assigned EC number.¹⁰

This group of enzymes is the most extensively characterized, which is a result of decades of intensive scientific investigation. Among the best-studied serine proteases are chymotrypsin, trypsin, and elastase. The first protease structure determined at atomic resolution was bovine chymotrypsin, described by Matthews *et al.* in 1967.¹¹

The serine proteases' class got its name from the presence of a Ser residue in the active site, whose hydroxyl group acts as a nucleophile during peptide bond hydrolysis. The catalytic triad of serine proteases consists of three key residues: His57, Asp102, and Ser195 (numbering based on chymotrypsin structure). The mechanism of peptide bond cleavage, illustrated in the Figure 2, relies on the synergistic action of these residues. Ser195 serves as the nucleophile, His57 acts as a general base that abstracts a proton from the hydroxyl group of Ser195, and Asp102 stabilizes the positive charge on the imidazole ring of His57. This arrangement facilitates a nucleophilic attack on the carbonyl carbon of the scissile peptide bond, leading to the formation of a tetrahedral intermediate.

This intermediate is stabilized by the so-called oxyanion hole, which is formed by hydrogen bonds between the backbone amide groups of Ser195 and Gly193 and the negatively charged oxygen atom of the substrate. Subsequently, a proton is transferred from His57 to the amine group of the substrate, resulting in the cleavage of the peptide bond and formation of an acyl-enzyme intermediate. The cleaved peptide fragment with a free amine group is then released. In the next step, a water molecule attacks the acyl-enzyme intermediate, forming a second tetrahedral intermediate. The final step yields the release of the second peptide fragment with a free carboxyl group and regeneration of the hydroxyl group of Ser195.¹²

The largest group of serine proteases is classified as S1 family, also known as chymotrypsin family. Their members exhibit three main activity types: trypsin-, chymotrypsin-, and elastase-like. This group does not contain any exopeptidases, however there are some proteins which does not exhibit any peptidase activity, for example: azurocidin, procarboxypeptidase A complex component III or protein Z.⁶

As mentioned in the Section 1., Barrett and Rawlings developed a revolutionary classification system for proteases. Nevertheless, serine proteases can also be broadly categorized based on their substrate specificity into trypsin-like, chymotrypsin-like, subtilisin-like, elastase-like, kallikrein, or cathepsin. However, the three main groups are:^{12,13,14}

- Trypsin-like proteases, which cleave peptide bonds after basic amino acids such as Arg and Lys, for example urokinase, plasmin, thrombin, and VIIa, IXa, Xa, XIIa factors;¹⁵
- Chymotrypsin-like proteases, which cleave after bulky, hydrophobic residues such as Phe, Trp, Tyr, for example chymotrypsin-C, chymotrypsinogen B1, chymotrypsinogen B2, chymase;¹⁶
- Elastase-like proteases, which cleave after small, aliphatic residues such as Ala, Gly, Val, for example neutrophil elastase or proteinase 3.¹⁷

However there are exceptions to this classification, such as granzyme B, which cleaves substrates after Asp residues.^{12,13}

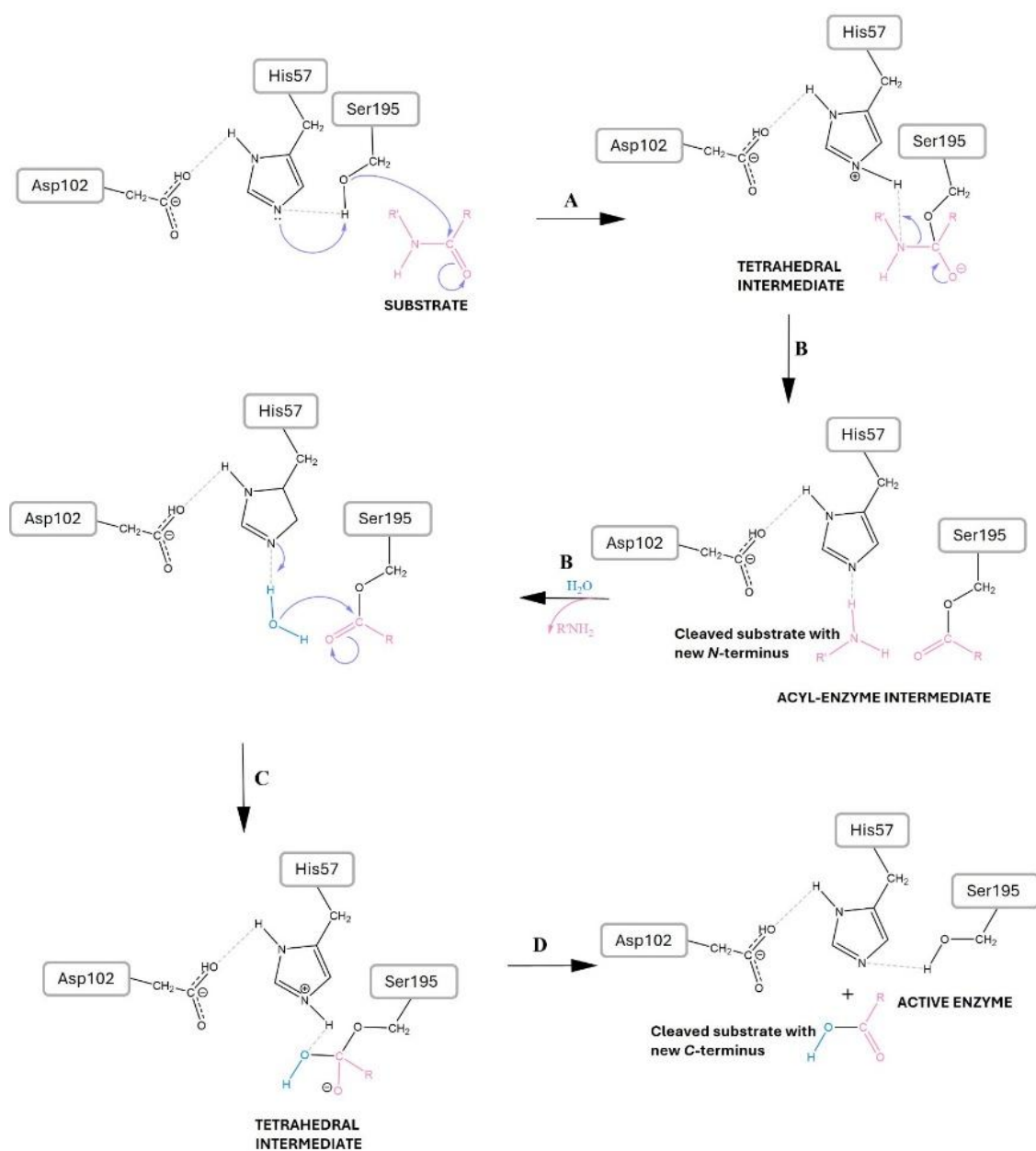


Figure 2. Catalytic mechanism of serine proteases on the example of chymotrypsin.

- A)** Nucleophilic attack by the hydroxyl group of Ser195 on the carbonyl carbon atom of the substrate;
- B)** Breakdown of the tetrahedral intermediate and release of the substrate fragment with a free N-terminus; formation of the acyl-enzyme intermediate, followed by nucleophilic attack of a water molecule;
- C)** Formation of a second tetrahedral intermediate;
- D)** Cleavage of the substrate, release of the fragment with a free C-terminus, and regeneration of the hydroxyl group of Ser195.

1.1.1. Transmembrane serine proteases

Proteases could be secreted or exocytosed from secretory vesicles into the extracellular environment, such as already mentioned trypsin or chymotrypsin. These enzymes are also present in the cytosol and within intracellular organelles, such as lysosomes.

As it was mentioned in Section 1.1., most of serine proteases are classified as S1 family. Among members of this group significant majority are secreted enzymes. However, in addition to these soluble proteases, there is a distinct subgroup of membrane-anchored proteases, being members of S1 family.¹⁸

Based on the orientation in the membrane, there are three types of these enzymes distinguished, that are schematically shown in the Figure 3. The type I is anchored *via* carboxy-terminal transmembrane domain, type II (type II transmembrane serine proteases, TTSPs) is anchored by an amino-terminal transmembrane domain with a cytoplasmic extension and GPI-anchored proteases contain glycosylphosphatidylinositol (GPI) linkage.¹⁸ More than 20 human and murine membrane-anchored SP were identified to date.^{18,19}

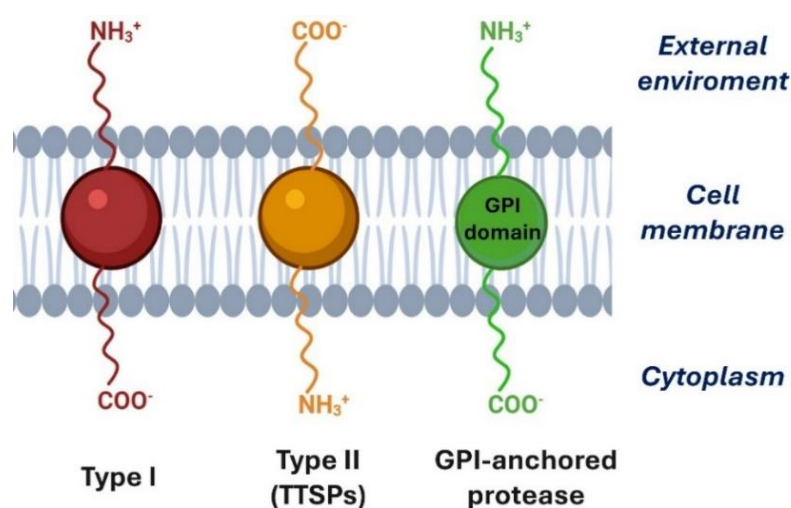


Figure 3. Types of membrane anchored serine proteases.

Membrane anchored serine proteases play crucial role in the activation of numerous precursors of biologically important molecules within the pericellular environment. These include peptide hormones, enzymes, receptors, adhesion molecules, as well as growth and differentiation factors. Moreover, these proteases have been implicated in the pathogenesis of cancer progression, as well as in viral and inflammatory diseases.¹⁸

Transmembrane serine proteases are synthesized as single-chain precursors, or zymogens, which subsequently undergo activation. This process results in the formation of a two-chain structure, in which the chains are linked *via* a disulfide bond, and the active enzyme remains

anchored in the cell membrane. Zymogen activation occurs through proteolytic cleavage at conserved Arg or Lys residues located within a highly conserved activation motif that separates the pro-domain from the catalytic domain.¹⁸ A detailed activation process of one representative of the transmembrane serine proteases, matriptase-1, is described in Section 1.1.3.1.

1.1.2. Type I transmembrane serine proteases

Both type I transmembrane SP and GPI-anchored proteases possess a hydrophobic C-terminal domain located intracellularly. Overall, these two groups contain fewer members compared to the TTSPs. The only known representative of type I serine proteases is transmembrane tryptase, also known as tryptase γ or protease serine member S31 (PRSS31). This small enzyme consists of 321 amino acids, which are grouped into two domains: transmembrane and catalytic. Tryptase γ has been identified in the secretory granules of mouse and human mast cells (MCs).^{19,20} Although the precise physiological function of PRSS31 remains unclear, studies involving genetically modified mice lacking this protease have not revealed any aberrances and number of MCs in their tissues remained unchanged. Nevertheless, compared to wild-type mice from the same litter, modified animals exhibited increased resistance to the development of chronic obstructive pulmonary disease and colitis.²¹

1.1.3. Type II transmembrane serine proteases

TTSPs represent the largest group among membrane-anchored serine proteases. They are divided into four subfamilies: HAT/DESC (human airway trypsin-like protease/differentially expressed in squamous cell carcinoma); hepsin/TMPRSS (transmembrane protease/serine S1); corin and matriptases family.¹⁸ A schematic representation of the structural organization of TTSPs is presented in Figure 4A. Figure 4B¹⁹ shows all members of the serine proteases anchored in the cell membrane, along with their respective domain compositions.

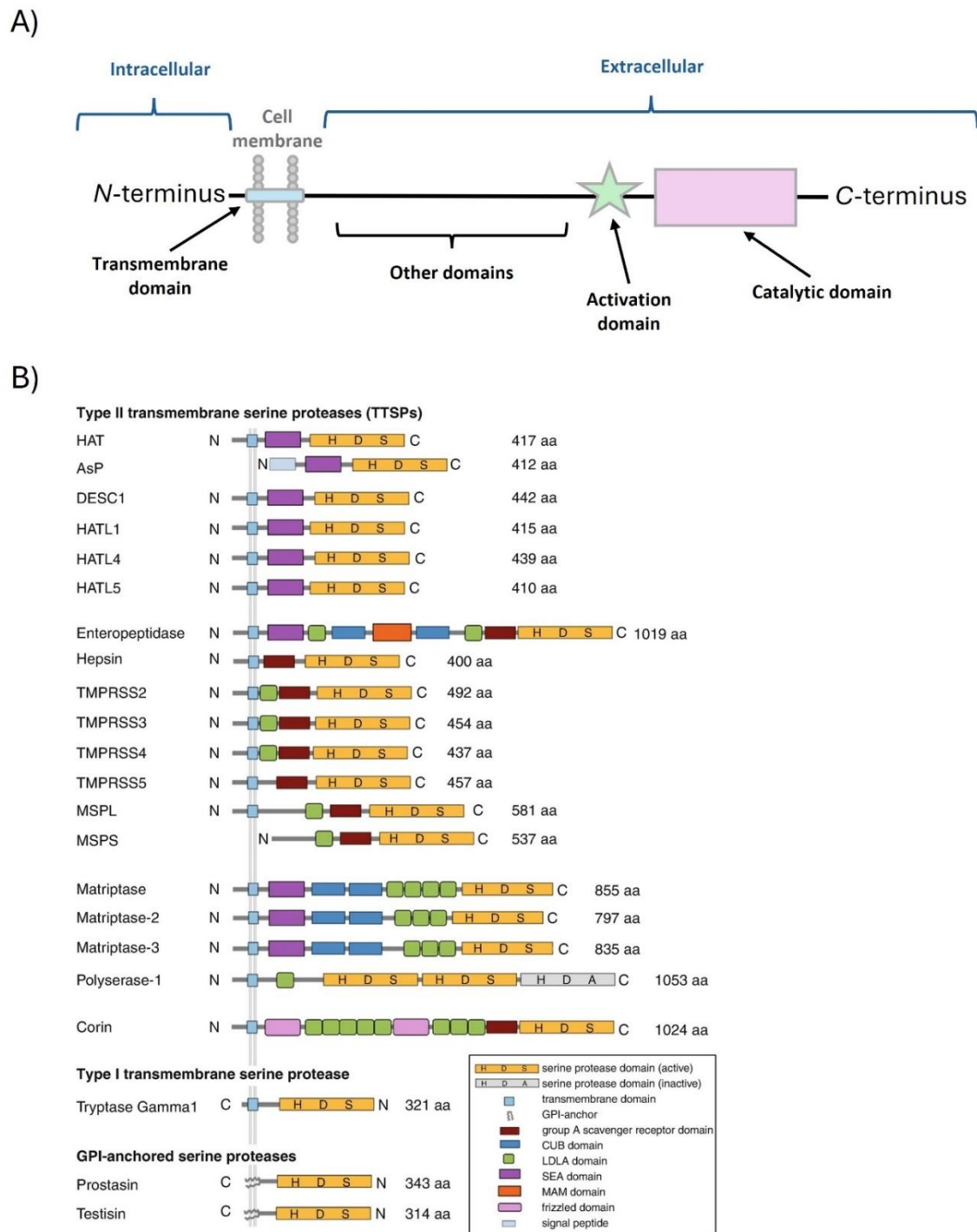


Figure 4. Schematic representation of the structural composition of TTSPs (A) and overview of all known transmembrane serine proteases and their domain organization (B)¹⁹.

GPI- glycosylphosphatidylinositol; CUB- CIs/Clr, urchin embryonic growth factor and bone morphogenetic protein-1; LDLA- low-density lipoprotein; SEA- sea urchin sperm protein, enteropeptidase, agrin domain; MAM- meprin, A5 antigen, and receptor protein phosphatase μ domain;

HAT/DESC subfamily is characterized by the least complex structure among all TTSPs. Its representatives possess only one domain in area between activation and cell domains, which is sea urchin sperm protein, enteropeptidase, agrin domain (SEA). HAT is one of few TTSPs which cleave hemagglutinin (HA)- glycoprotein of the influenza virus. HAT cleaves precursors proteins of HA after Arg residue which initiates membrane fusion of the virus. This process contributes to the virus activation and spread.¹⁸

Members of hepsin/TMPRSS family include Group A scavenger receptor domain (SR) located before activation domain. Furthermore, TMPRSS2, TMPRSS3, TMPRSS4 and MSPL possess single low-density lipoprotein (LDL) receptor class A domain (LDLRA). Whereas, the most complex member of this subfamily is endopeptidase. This enzyme possesses a diverse array of domains, including Cls/Clr, urchin embryonic growth factor and bone morphogenetic protein-1 (CUB); meprin, A5 antigen, and receptor protein phosphatase μ domain (MAM); SEA and LDLRA,¹⁸ as is illustrated in Figure 4B.

Corin is a single member of corin subfamily. Its stem region consists of two frizzled domains, eight LDLRA domains and one Group A SR domain.¹⁸ It was identified in 1999 in human heart,²² where it is responsible for converting pro-atrial natriuretic peptide (pro-ANP) to its active form. ANP regulates blood pressure and cardiac function due to its crucial role in vasodilation, natriuresis, lipid metabolism, inflammatory responses and vascular remodeling.^{19,23} Corin possesses unique structure among all TTSPs. It is the only member containing frizzled-like cysteine rich domains, which were found mainly in proteins that interact with Wingless-related integration site (WNT) signaling molecules.²⁴ Among others, secreted frizzled-related proteins are involved in activation WNT signaling pathways, which are a part of innate immunity and development of inflammation.²⁵ Moreover, experiments which were comparing the soluble corin containing only extracellular fragment with the enzyme anchored in cell membrane revealed that both proteins exhibited similar activity. These results indicated that transmembrane domain is not crucial for its enzymatic activity.²⁴

Matriptase subfamily comprises four members: matriptase-1 (MT1), matriptase-2 (MT2), matriptase-3 (MT3) and polyserase-1. All matriptases share a similar domain architecture, typically consisting of one SEA, two CUB and three (MT2 and MT3) or four (MT1) LDLRA domains. In turn, polyserase-1 has single LDLRA domain and, notably, features one enzymatically inactive and two active serine protease domains.^{18,19} Despite similar structures, matriptases play completely different biological roles in human body. MT3 is a 90 kDa protein that was identified in 2005. Its mRNA has been detected, among others, in the brain, skin, and reproductive tissues. The physiological role has not yet been fully elucidated,¹⁹ however recent reports suggest that loss of the gene encoding MT3 may be associated with neurodevelopmental disorders.²⁶ The roles of MT1 and MT2 are discussed in greater detail in the following Sections 1.1.3.1 and 1.1.3.2.

1.1.3.1. Matriptase-1

In 1993 MT1 was first described as a novel matrix-degrading enzyme found in human breast cancer cells.²⁷ It is a 80-90 kDa cell surface glycoprotein, consisting of 855 amino acid residues and encoded by the *STI4* gene. In 2002 Friedrich *et al.*²⁸ solved crystal structure of MT1 with benzamidine and bovine pancreatic trypsin inhibitor.²⁸ Schematic domain structures of MT1 and its ribbon stereo plot are shown in Figure 5. MT1 is ubiquitous in number of epithelial tissues and its main role is participation in epithelial development and maintaining adult tissue homeostasis.

MT1 and prostatic serine protease (PRSS8/CAP-1), is a GPI-anchored membrane serine protease. It is synthesized as a ~ 40 kDa inactive zymogen, which is activated by a cleavage after Arg14, located in a fragment QPRITG. It is not able to undergo autoactivation.²⁹ In 2013 Friss *et al.*²⁹ proved that MT1 and prostatic serine protease form a bilateral zymogen activation complex and also that prostatic serine protease is able to non-enzymatically influence the activation of MT1. Prostatic serine protease acts as a critical co-factor during MT1 activation, however its zymogen conversion and also catalytic activity are not required. What is more, activation of prostatic serine protease is dependent on MT1 zymogen.²⁹ In turn, in 2016 Su *et al.*³⁰ published distinct findings, which indicated that prostatic serine protease is not required and involved in MT1 autoactivation, because MT1 is able to undergo activation in cells devoid of prostatic serine protease. Also, according to their research, MT1 is not required and involved in prostatic serine protease activation, due to the fact that active form of this GPI-anchored protease was observed in cells which do not express endogenous MT1. Moreover, zymogens of these membrane-anchored enzymes are activated under un-coupled mechanisms.³⁰ In 2018 Lee *et al.*³¹ conclude that relationship between mentioned enzymes is related to subcellular localization and cannot be generalized. Prostatic serine protease is activated mainly in high levels of skin, in granular layers. Whereas the activation of MT1 zymogen occurs in cells of the lower, basal layer.³¹

However, setting aside the controversies regarding the mutual influence of enzymes and their zymogens during activation, both proteins play a crucial role in epithelial function. Prostatic serine protease together with furin (enzyme described in detail in Section 1.1.4.1) cleaves and activates the epithelial sodium channel. This process leads to an increase in sodium ion concentration, which promotes membrane depolarization and facilitates calcium ions entry into the cell. Increased concentration of Ca^{2+} activates various proteases contributing, for example, to the processing of profilaggrin into filaggrin monomers, as well as to the formation of lamellar bodies and lamellae of the cornified layer.³²

Expression of MT1 was found in epidermis, cornea, salivary gland, oral and nasal cavities, thyroid, thymus, esophagus, trachea, bronchioles, alveoli, stomach, pancreas, gallbladder, duodenum, small intestine, colon, rectum, kidney, adrenals, urinary bladder, ureter, seminal vesicles, epididymis, prostate, ovaries, uterus and vagina.³³ MT1 plays crucial and global role in

maintenance of epithelial integrity across multiple organ systems. It has been shown to be essential for epidermal barrier function, hair follicle development, and thymic homeostasis. MT1 is involved in terminal epidermal differentiation processes, including lipid extrusion, profilaggrin processing and cornified envelope formation. MT1 is also expressed in simple, stratified, transitional and pseudostratified epithelia throughout the body that do not undergo cornification, secrete specialized lipids, or express profilaggrin.^{34,35}

MT1 is synthesized as inactive single-chain zymogen.³³ For this enzyme, both autoactivation and also prostaticin-mediated activation have been reported.^{29,36} The process of activation is complex and it requires two sequential cleavages. In the first step hydrolysis occurs after Gly149 located in the conserved fragment GSVIA in the *N*-terminal SEA domain. After this cleavage the enzyme remains attached to the cell due to noncovalent interactions. This process occurs in the secretory pathway. Afterwards, another cleavage appears after Arg614 in the highly conserved motif RRVGG. This activation in the active site requires proteolytic activity of MT1. It was shown that mutations in residues of the catalytic triad result in an inability of MT1 to undergo an autoactivation. The process of the activation is controlled by the stem region, posttranslational modification, and also by cellular localization of the enzyme. It was noticed that LDLRA domain plays a significant role in preventing uncontrolled activation of MT1. Complete deletion of all four LDLRA domains resulted in efficient activation. Moreover, also mutations in the Ca²⁺-binding motifs prevent activation.³³

In the human body there are also molecules that regulate MT1 activity, including hepatocyte growth factor activator inhibitors HAI-1 and HAI-2, also named Spint1 and Spint2. These proteins control MT1 activation and are type I transmembrane proteins belonging to the Kunitz type inhibitor family.^{37,38,39} HAI-1 and HAI-2 are expressed mostly by epithelial cells. They consist of two extracellular Kunitz-type serine proteinase inhibitor domains (KD1 and KD2), a single-pass transmembrane domain, and a short intracytoplasmic domain. The difference between HAI-1 and HAI-2 relies on presence additional domains in HAI-1, which are absent in HAI-2. These domains are: 8 cysteine (MANEC) domain and polycystic kidney disease (PKD)-like domain which are located in *N*-terminus. Also between KD1 and KD2 there is LDLRAp domain.⁴⁰ HAI-1 is the prominent inhibitor for MT1 and prostaticin. However it is also a chaperone-like protein, responsible for the intracellular trafficking- after MT1 autoactivation, or activation by other proteases, it deactivates the enzyme and promotes transport of MT1 to the plasma membrane.³² HAI-1 plays a crucial role in maintaining epithelial integrity. Experiments with mice with global *Spint1* knockout revealed that all individuals died during fetal life due to flawed formation of placental labyrinth. An attempt of rescue pups by introducing *HAI-1/Spint*^{+/+} blastocysts with *HAI-1/Spint*^{+/+} embryonic stem cells resulted in formation of chimeric embryos that developed normal placentas. However the neonates exhibited scaly skin, due to abnormal epidermal keratinization and also showed abnormal hair shafts. Mice died by 16 days.⁴¹ Also

Spint2^{-/-} mice were embryonic lethal.⁴² Activity of HAI-2 is the most significant in intestinal tissues and cultured colon carcinoma cells. It is also significant MT1 inhibitor in breast cancer cells. The inhibitory role of HAI-2 is limited in basal and spinous layer keratinocytes, due to its intracellular localization.^{31,40}

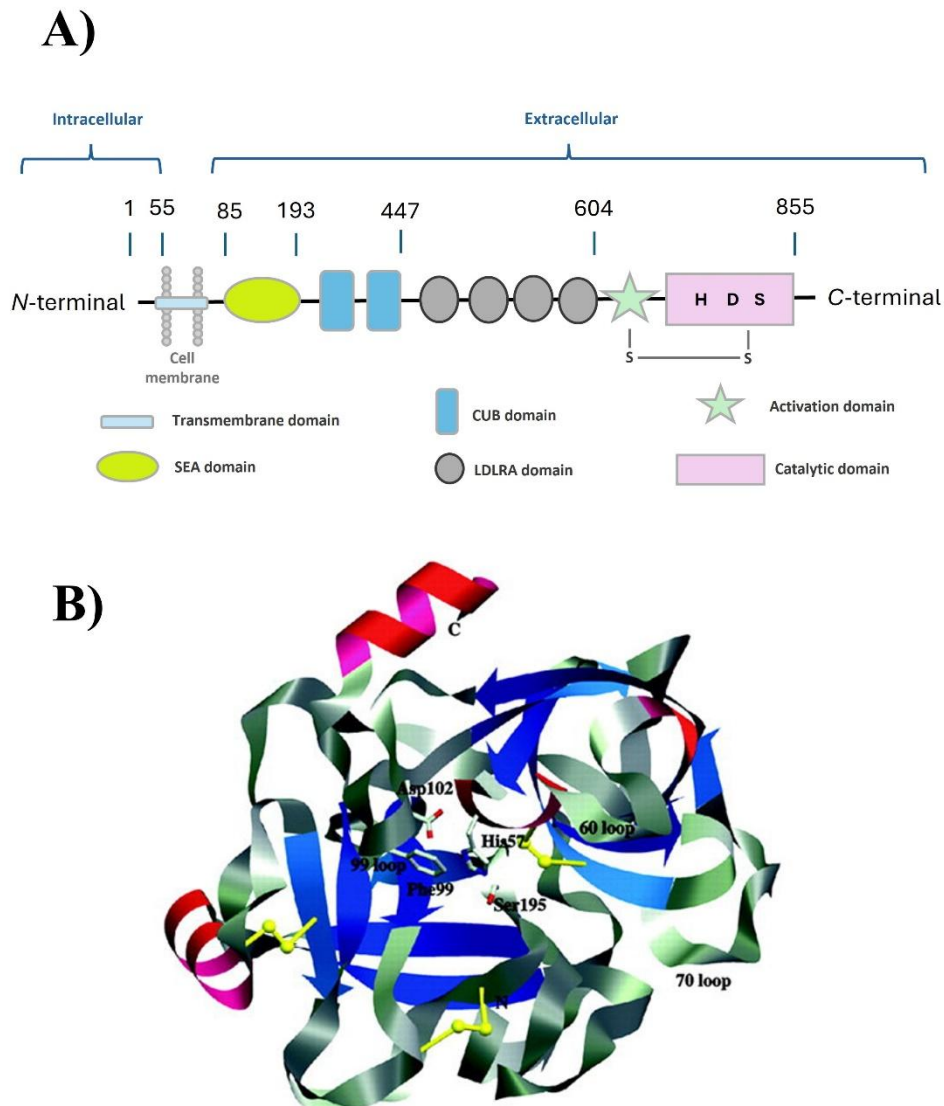


Figure 5. Schematic presentation of MT1 domains (A) and a ribbon stereo plot of MT1 (B).

A) Each domain is illustrated by a distinct shape and color. Arabic numeral above the domains represent the numbers of the subsequent amino acid residues. CUB- Cls/Clr, urchin embryonic growth factor and bone morphogenetic protein-1; SEA- Sea urchin sperm protein, enteropeptidase, agrin domain; LDLRA- Single low-density lipoprotein receptor class A domain;

B) Structure of MT1 obtained by Friedrich et al.²⁸

Skin and mucosa constitute the first line of defense against environmental microorganisms and toxins. As previously mentioned, MT1 plays a crucial role in maintaining epidermal integrity. Therefore its dysregulated activity, or inhibition, may lead to epithelial barrier disfunctions. In healthy tissues, activity of MT1 is kept under tight control maintained by mentioned already two independent, but closely associated mechanisms. These are autoactivation⁴³ and rapid inhibition by the endogenous inhibitors HAI-1 and HAI-2.^{37,38,39} Imbalanced ratio of MT1 and its inhibitors leads to MT1 dysregulation and, in consequence, to pathology including malignancies development. This was recently confirmed by Pawar *et al.*,⁴⁴ who demonstrated a new link between excessive MT1 activity and disruption of cell-cell adhesion, critical for metastasis in epithelial ovarian cancers. Nevertheless, there are other probable models of pathological and oncogenic MT1 hyperactivation, but, at least with respect to their *in vivo* relevance, they are unclear.^{37,39,45,46,47}

Several studies revealed increased expression of MT1 in variety of epithelial cancers, such as skin and head, prostate, breast, ovarian and cervical or stomach cancers.^{38,48,49,50} MT1 is both an initiator and a strong tumor-promoter in squamous cell carcinoma (SCC).⁴⁸ Studies on transgenic mice with MT1 overexpression revealed spontaneous epidermal neoplasia in 100% animals. Moreover, application of a single topical dose of the chemical carcinogen 7,12-dimethylbenzanthracene (DMBA) resulted in development of SCC within 40 weeks, in almost all transgenic mice. It is worth to notice that simultaneous overexpression of inhibitor HAI-1 with MT1 in epidermis abolished the development of SCC following DMBA exposure.^{48,51} MT1 is also highly expressed in head and neck SCC (HNSCC), such as carcinomas of the tongue, lip, larynx and gingiva. Moreover, in this case, MT1 is often co-expressed with the receptor tyrosine kinase c-Met.^{48,52}

What is more, an overexpression of MT1 in breast and prostate cancers is linked with tumor grade and stage, and high MT1 expression is predictive factor of poor disease prognosis.^{37,53,54,55} Importantly, in long-term survival study of node-negative (without nodal involvement) breast cancer patients, 30-years survival data demonstrated that high expression of both MT1 and c-Met (the receptor for hepatocyte growth factor, HGF) was significantly associated with worse disease-free survival.⁵⁴ Other research from 2017 reported by Ji *et al.*⁵⁶ confirmed overexpression of MT1 in tissue samples from patients diagnosed with ovarian serous adenocarcinoma. The results indicated that elevated levels of MT1 were frequently observed in early stages of this cancer, suggesting its potential utility as a marker for early diagnosis. Furthermore, research from 2014 described by Ha *et al.*⁵⁷ demonstrated that overall survival was significantly lower among patients with esophageal squamous cell carcinoma who exhibited high MT1 expression. Taking into consideration relation between MT1 and cancer progression, this enzyme is thought to be the attractive target for cancer treatment and its inhibitors are prospective anti-cancer agents.⁵⁸ The

oncogenic properties of MT1 continue to be a subject of active research.^{39,44,59,60,61} Known inhibitors of MT1 are described in Section 2.3.

It is worth to mention that besides its role in cancer progression, MT1 is one of the host proteases responsible for cleaving the major surface glycoprotein hemagglutinin of certain H1N1⁶² and H9N2⁶³ influenza viruses, which is a significant step for virus infectivity. It has been shown that potent MT1 ketobenzothiazole-based inhibitors exhibit antiviral activity against H1N1 virus.⁶⁴ Mentioned results, described by Colombo *et al.*⁶⁴, indicated on non-selective properties of these compounds and on influence on other transmembrane serine protease, i.e. TMPRSS2, which is involved in the entry mechanism of the virus. Furthermore, other TMPRSS2/ MT1 small molecule, 3-amidinophenylalanine inhibitors, have been tested *in vitro* toward H1N1 and H9N2.⁶⁵ Also their hepatotoxic properties and modulatory effects on plasma proteins have been tested.⁶⁶

Recently,⁶⁷ elevated levels of circulating MT1 were observed in patients suffering from type 2 diabetes mellitus (T2DM). Owing to the presence of glycosylation sites, MT1 is considered to be involved in glucose metabolism. Correlations between MT1 levels and various metabolic and inflammatory parameters were reported. It was noted that patients with T2DM exhibited increased MT1 levels compared with the control group. Moreover, a positive correlation was observed between MT1 levels and, among others, insulin concentration, fasting blood glucose, and postprandial blood glucose. These findings suggest that MT1 may be involved in glucose metabolism.

1.1.3.2. Matriptase-2

MT2 is a protein consisting of 797 amino acid residues¹⁸ with molecular mass of approximately 90 kDa⁶⁸ produced predominantly in the adult and fetal liver, and- in much lower concentrations- in kidney, uterus and nasal mucosa.⁶⁹ MT2 is encoded by *TMPRSS6* gene in humans and the *Tmprss6* gene in mice.⁶⁸

Crystal structure of MT2 is not available, however in 2010 Sisay *et al.*⁷⁰ published comparative three-dimensional model of its catalytic domain. Schematic domain structure and model obtained *in silico* by Sisay *et al.*⁷⁰ through homology modeling are shown in Figure 6. The model was obtained using crystal structures of closely related enzymes. In this procedure, a 3D protein structure was generated by aligning its sequence with that of a structurally known homolog and transferring atomic coordinates accordingly. The model was obtained using a semi-automated modeling server, complemented by extensive manual input, to ensure high accuracy in the active site region. After specific research of Protein Data Bank (PDB), using the sequence of the catalytic domain of MT2, the most suitable enzyme for this homology modeling turned out to be MT1 (45%, PDB: 1EAX). Plasma kallikrein, coagulation factor XIa, catalytic domain of

hepsin, plasminogen, R-tryptase and thrombin also exhibited similar level of homology (around 40%). The mentioned crystallographic structure of MT1 (PDB: 1EAX) was selected because it exhibited the highest resolution. Alignment of the sequences indicated on significant similarity between MT1 and MT2. The major difference was noticeable in fragment called “60 insertion loop”, where MT1 has insertion with three more amino acids (DDRGFRYSD) than MT2 (EDSMAS). Moreover, in MT1 two additional single residue deletions are located in the following loops: “37 loop” and “70-80 loop”.

In 2014, group of Prof. Boudreault⁷¹ also applied similar strategy and obtained homology model of MT2. Differences and similarities between MT1 and MT2 are described more detailed in Section 1.1.3.3.

Numerous studies have indicated on the role of MT2 in iron homeostasis. MT2 serves as the proteolytic regulator of iron level in human body, however its exact function is elusive. Maintenance of iron absorption and balance relies on complex process. In blood iron ions are carried by transferrin and then taken by cells during endocytosis. Inside the cell ions are released and stored in the form of ferritin. When level of this element in plasma is low, iron is released from the following cells: enterocytes, hepatocytes and macrophages. This export is possible due to ferroportin, a transmembrane protein. In turn, when the level is high, iron will remain stored. Hepcidin, peptide consisting of 25 amino acids, interacts with ferroportin by binding with this membrane-anchored protein. This process induces phosphorylation, causing internalization of ferroportin and its degradation in lysosomes. In consequence, hepcidin decreases ferroportin expression and export of iron ions from hepatocytes to plasma, it also reduces iron absorption in guts and prevents recycling of this element in macrophages. Hepcidin is encoded by gene *Hamp*, which is mainly expressed in the liver. The major activators of expression of gene *Hamp* are Bone morphogenic proteins (BMPs). The binding of BMP to its cell surface receptor activates the suppressor of mother against decapentaplegic (SMAD) signaling pathway and in consequences induces expression of *Hamp*. BMP has a co-receptor, hemojuvelin (HJV), which promotes expression of *Hamp*. Until recently, it was believed that the influence of MT2 on iron homeostasis relies on the degradation of HJV by MT2 and thus, that the enzyme is able to inhibit hepcidin expression.^{72,73,74}

It has been revealed that mutations in the MT2 encoding gene, *TMPRSS6*, lead to overexpression of hepcidin,⁷² whereas mice lacking MT2 encoding gene demonstrated, beside increased hepcidin transcription, an alopecia phenotype and iron deficiency anemia.⁶⁸ As already mentioned, hepcidin, a small peptide hormone secreted by hepatocytes, inhibits iron absorption from the intestine and controls iron recycling and release by regulating the cellular concentration of ferroportin.⁷⁵ Chronic excess of hepcidin stops the cellular iron export, resulting in a decreased supply of plasma iron, which in long-term may lead to iron-restricted anemia.⁷⁵ On the other hand, hepcidin deficiency may be also harmful causing iron overload.^{75,76} The role of MT2 in the

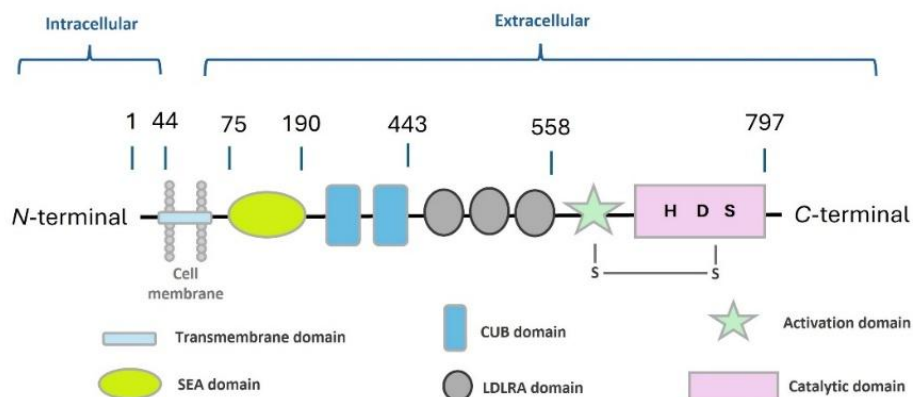
hepcidin regulatory pathway was further confirmed when the combined knockout of HJV and MT2⁷⁷ or BMP and MT2⁷⁸ led to hepcidin insufficiency and iron overload in mice. However, results of research on zebrafish embryogenesis indicated an alternative, ancillary, MT2-mediated pathways controlling hepcidin transcription.⁷⁹

Further studies on MT2 confirmed that iron concentration control system is even more complicated than it was initially assumed.^{80,81} In 2017 Wahedi *et al.*⁸² took an attempt to determine the mechanism of suppression of hepcidin expression driven by MT2 and suggested that also other proteins are involved in the process. It was indicated that the development of obesity-associated conditions, such as hepatic steatosis and insulin resistance, can be suppressed in deficiency of MT2.⁸³ Indeed, Zimiao *et al.*⁸⁴ demonstrated correlation between iron overload and visceral fat mass and other body composition parameters.⁸⁴ In humans, mutations in the *TMPRSS6* gene result in a high hepcidin expression and lead to iron refractory iron deficiency anemia (IRIDA).^{68,85,86}

Recent literature reports confirm that MT2 is a limiting factor in the suppression of hepcidin expression and it can suppress hepcidin expression independently of its proteolytic activity. This, in turn, shows that major function of MT2 is to set the basal levels of hepcidin expression. However, studies in mice models with global *Tmprss6* knockout (*Tmprss6*^{-/-}) suggest that lack of MT2 does not impact the iron induction of hepcidin.⁸⁷ Also other research^{88,89} support this finding and indicate that hepcidin expression is independent of MT2 proteolytic activity.

Notwithstanding, a very interesting issue is a reverse correlation between MT2 and cancers, as compare to MT1. Expression of MT2 was found for example in breast cancer cell lines of a general low/ noninvasive nature (MCF7 and ZR-751). Also, MT2 is plentifully produced by liver cancer cell line (PLC-PRF-5), but this results from the fact that liver is a main source of MT2 in the body. It is worth to notice that MT2 was not found in the highly invasive breast cancer cell line (MDA-MB-231), stromal fibroblasts (MRC-5), the endothelial cells (HEVC) or the prostate cancer cells (DU-145 and CA-HPV-10).⁹⁰ In contrast to MT1, expression of MT2 was reduced significantly with higher tumor grade. Notably, MT2 levels were markedly diminished in undifferentiated ductal or lobular tumors. Furthermore, overall poor prognosis for patients was correlated with reduced MT2 levels in breast cancer tissues.^{90,91} Moreover, it was reported that overexpression of MT2 dramatically reduced the invasive and migratory properties of the breast cancer cells from cell line MDA-MB-231.⁹⁰ The prognostic value of MT2 was also confirmed also by Erhan *et al.*⁹² in study of breast cancer patients, who confirmed that expression of MT2 was significantly lower in tumor tissues compared with normal tissues. All these reports, showing correlations between matriptases and cancer prognosis, emphasize the importance of finding highly specific and exclusive inhibitors of only one of them, i.e. cancer-associated MT1, but not MT2.

A)



B)

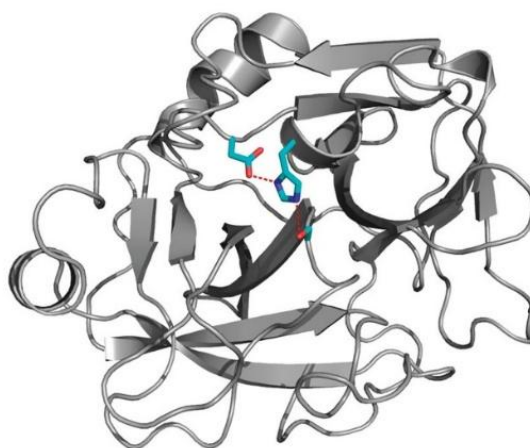


Figure 6. Schematic presentation of MT2 domains (A) and a ribbon stereo plot of MT2 (B).

A) Each domain is illustrated by a distinct shape and color. Arabic numeral above the domains represent the numbers of the subsequent amino acid residues.

CUB- Cls/Clr, urchin embryonic growth factor and bone morphogenetic protein-1; SEA- Sea urchin sperm protein, enteropeptidase, agrin domain; LDLRA- Single low-density lipoprotein receptor class A domain;

B) Structure of MT2 obtained by Sisay et al.⁷⁰

1.1.3.3. Matriptase-1 and matriptase-2 catalytic domains - differences and similarities

The catalytic domains of MT1 and MT2 adopt a chymotrypsin-like fold. Despite sharing approximately 44% similarity of their primary structures, subtle variations in their sequences confer distinct substrate specificity. These sequence divergences are critical for finding selective

inhibitors of both proteases.^{71,93} It is worth to recall that distinct substrate preferences of enzymes result mostly from different construction of their substrate binding pockets.^{70,71}

The first, critical difference between MT1 and MT2 is observed in S1 binding pocket, which is known to be crucial for enzyme specificity. In both enzymes, Asp residue is present at the bottom of this pocket, but in case of MT2, a subsequent position is occupied by Ala, whereas Ser is found in corresponding position in MT1. This makes the S1 pocket of MT2 a little more hydrophobic in compare to MT1. Thus, MT2 prefers binding with substrates having Arg in P1 position, as it is stabilized by a direct salt bridge between the guanidinium moiety of Arg and the carboxyl group of Asp. Binding of Lys is more preferred by MT1, as the presence of Ser enables the formation of a hydrogen bond with its hydroxyl group.^{70,94}

The presence of His in the S2 pocket of MT2, in place of Phe in MT1, also may be essential for the design of selective inhibitors. It was noticed that MT2 prefers small aliphatic residues, like Ala or Val in P2 position.⁹⁵

A further significant difference is observed in S3/S4 pockets. In case of MT2 the bottom part of this subsite extends to Leu which makes it more hydrophobic than in MT1, where Asp is located in this position. The upper part of S3/S4 in MT2 forms a negatively charged environment, as it is composed of Glu, Asp and Ser. In case of MT1, the corresponding positions are occupied by Asp, Phe and Thr. Thus, the S3/S4 site of MT2 prefers positively charged residues. Important distinction is observed also in the S2' pocket, which is smaller in MT2 compared to MT1 due to the substitution of Gly in MT1 by Ile in MT2.^{70,94}

Although the differences in the binding pockets of MT1 and MT2 are subtle, they are sufficient to permit the rational design of highly selective inhibitors for each protease, as has been proved by Gitlin-Domagalska *et al.*^{96,97} Furthermore, MT1 inhibitors are detailed described in Section 2.3.

1.1.4. Proprotein convertases

Proprotein convertases (PCs) are serine proteases that trigger the activation, inactivation or functional changes of wide range of protein substrates.⁹⁸ They share structural homology with bacterial subtilisin and yeast kexin proteases⁹⁹ and thus the joint abbreviation, PCSK, for proprotein convertase subtilisin/kexin type enzymes was proposed. Humans encode nine members of this protease family (PCSK1-9).¹⁰⁰ Seven of which, namely furin, PACE4, PC1/3, PC2, PC5/6, PC7 and PC4, catalyze the hydrolysis of peptide and protein substrates at paired basic residues. They recognize the consensus recognition motif in the primary structure of a protein substrate: Arg-X_{aa}-Lys/Arg-Arg↓ (RX_{aa}K/RR↓, the arrow indicates the cleavage site, whereas X_{aa} corresponds to any amino acid) and hydrolyze peptide bond after Arg residue.^{101,102}

The other two members of this family are S1P/SKI-1 and PCSK9, which cleave precursor proteins at the C-terminal end of a nonbasic amino acid.^{103,104}

These calcium (Ca²⁺)-dependent endoproteases are widely present in humans and take part in variety of physiological processes.¹⁰³ Since their discovery in the early 90's, many studies have focused on understanding how these enzymes are implicated in health and disease.^{100,105,106,107} The individual biological roles of all convertases are shortly presented in the Table 1.^{108,109}

Table 1. Functional overview of PCs members.

Proprotein convertase	Biological role
PC1/3	It maintains body weight homeostasis by processing proinsulin into insulin and proglucagon into glucagon-like peptides. Its activity is related to the activity of PC2.
PC2	It maintains body weight homeostasis by converting proinsulin into insulin and proglucagon into glucagon. Its activity is related to the activity of PC1/3.
Furin	It converts diverse human's substrates, e.g. hormones, growth factors, receptors. Besides its physiological role it also activates bacterial toxins and viral glycoproteins, thus contributing to the development of diseases.
PC4	It is mainly expressed in the testis and is involved in reproductive functions, particularly in sperm maturation and fertilization, although its broader physiological roles are less well characterized.
PC5/6	It plays role during embryogenesis and activates growth differentiating factor.
PACE4	It converts matrix metalloproteases (ADAMTS-4 and ADAMTS-5) in cartilage. Also it participates with furin in early embryonic patterning,
PC7	It plays a role in iron metabolism by shedding transferrin receptor-1. Also it participates in mood regulation by regulating central dopaminergic circuits.
SKI-1/S1P	It plays a role in regulation of cholesterol and fatty acids synthesis by cleaving their regulators and lysosomal targeting of proteins. It also activates somatostatin, and envelope glycoproteins of hemorrhagic fever viruses.
PCSK9	It binds LDL receptors and targets them for lysosomal degradation, thus controlling plasma LDL levels.

1.1.4.1. Furin

Furin is a prototypical and the best-characterized member of the PCSK family. This enzyme is also termed Paired Basic Amino Acid Cleaving Enzyme (PACE).¹⁰⁰ It is a 794-amino acid single-pass type I transmembrane protein that is found in all vertebrates and many invertebrates.^{105,110} Furin belongs to the S8 family of subtilisin-like serine proteases, according to MEROPS database classification.¹¹¹

Although furin is a type I transmembrane protein, with its carboxyl-terminal domain located in the cytosol, and functions as a serine protease with catalytic triad composed of Ser368, His194 and Asp153,¹¹⁰ it is not classified as transmembrane serine protease. Furin belongs to S8 family and the S8B subfamily. Most members of this family preferentially cleave peptide bonds after hydrophobic residues, however a characteristic feature of the subfamily S8B is the cleavage of substrates after paired basic amino acids. Furthermore, most members of the S8 family possess two calcium-binding sites in their structure and, unlike enzymes from S1 family, the majority of these proteases are not multidomain proteins.¹¹² As mentioned in Section 1.1.1., all transmembrane serine proteases are classified within the S1 family, and this specific feature precludes the classification furin within this group.

Its catalytic triad is subtilisin-like. During peptide bond cleavage, the activity of the catalytic triad is supported by two hydrogen bonds: one formed by the backbone amino group of Ser368 and the other by the side chain amide group of Asn295. These interactions form the oxyanion hole, which stabilizes the transition state of the substrate.^{110,113} A schematic representation of furin's domain structure and its three-dimensional conformation are shown in Figure 7.¹¹⁴

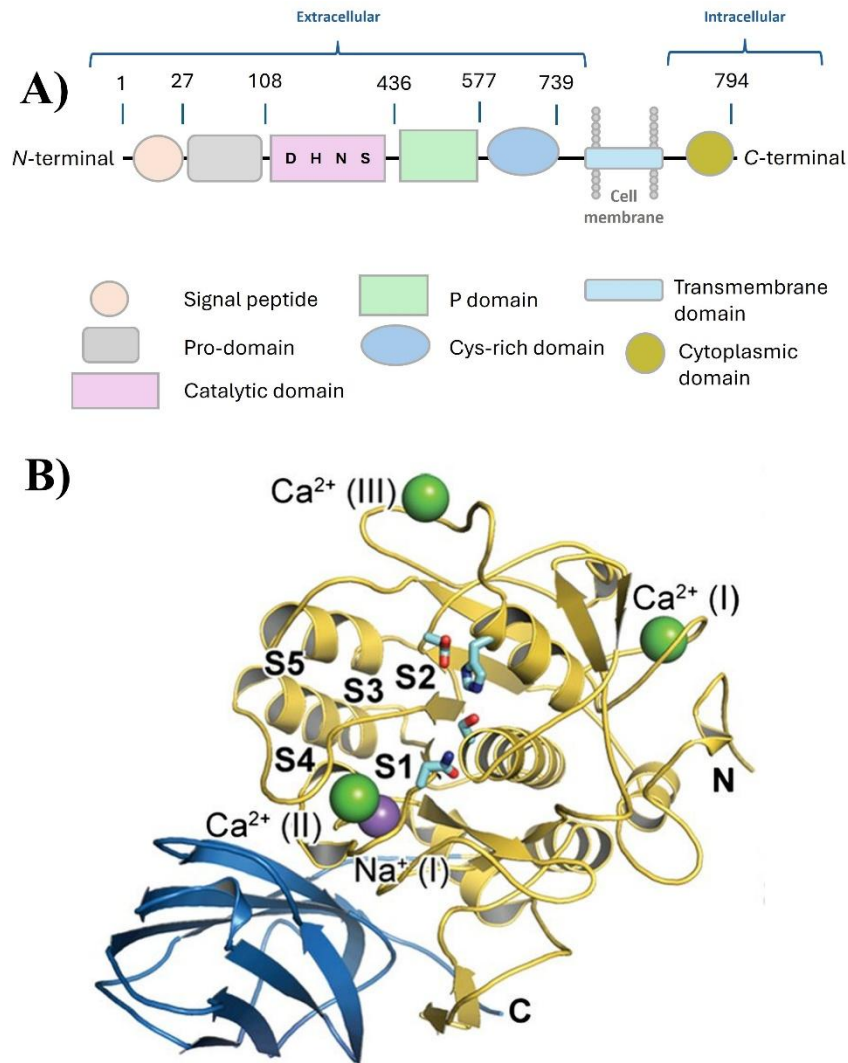


Figure 7. Schematic presentation of furin domains (A) and a crystal structure of a human furin (PDB: 5JXG) (B).

A) Each domain is illustrated by a distinct shape and color. Arabic numeral above the domains represent the numbers of the subsequent amino acid residues.

B) The P domain is marked by blue color, the catalytic domain is gold. The amino acid residues forming catalytic triad are demonstrated as cyan stick model.¹¹⁴

Furin is ubiquitously expressed across human tissues, to such an extent that no cases of complete furin deficiency have been documented in the literature. This underscores its essential role in physiological processes. Notably, high levels of furin have been observed in salivary glands, liver and bone marrow.¹¹⁵ This enzyme exhibits a broad pH activity range, with optimal functioning between pH 5 and 8.¹⁰⁵ Furin might be found mainly in two subcellular localizations: the *trans*-Golgi network (TGN) and on the cell surface.¹¹⁶

Expression of furin is driven by three different promoters: P1, P1A and P1B. Each of them harbours an alternative transcription start site. However the transcripts differ only in the first untranslated exon and express the same protein. Promotor P1 can be trans-activated upon cytokine stimulation, while P1A and P1B parallel promoters constitutively expressed housekeeping genes. During translation of the mRNA an inactive proenzyme of furin enters the secretory pathway.¹⁰⁰

Activation of furin is triggered by two sequential autocatalytic cleavages. In the first step, the proenzyme is integrated into the endoplasmic reticulum (ER), *via* its C-terminal transmembrane domain, and then it undergoes glycosylation. In the next step short, N-terminal signal peptide is cut off and autocatalytic cleavage occurs. As a result, the pro-domain (also named propeptide) becomes N-terminal. It acts as intramolecular chaperone. Pro-domain is required for regulation of the autoactivation mechanism, it also ensures proper folding of the catalytic domain. Trafficking of furin to the TGN, where pH is 6, triggers second cleavage and removal of pro-domain. Moreover, furin's glycans are trimmed and enzyme gains proteolytic activity. Furin accumulates in the TGN, however it can be further transported to the cell surface and back *via* endosomal pathway.¹⁰⁰ Process of furin's maturation is schematically presented in Figure 8A.¹⁰⁰

In the TGN, furin cleaves and activates mostly substrates delivered by host cell. While on the cell surface it cleaves many external pathogenic substrates.¹¹⁶ Furin is able to activate more than 100 proproteins. However, most of these substrates have not yet been verified as physiological. Moreover, proproteins can be cleaved in one tissue only by furin, and in another by one or more additional PCs.¹¹⁷ Among its host substrates are cellular precursor proteins, such as: growth factors, neuropeptides, hormones, adhesion molecules, blood coagulation factors and receptors.¹¹⁶

Apart from its physiological relevance, furin is also involved in the development of various inflammatory diseases, cancers, pathogen infections (both viral and bacterial),¹⁰⁰ atherosclerosis¹⁰⁶ and neurodegenerative disorders.^{107,118} Furin's involvement in cleavage and activation of mammalian, viral and bacterial proteins is schematically presented in Figure 8B.¹⁰⁰

Furin takes part in growth and progression of various malignancies, including colon carcinoma, rhabdomyosarcoma, head and neck cancers, lung, skin and brain tumors.¹¹⁹ Also many pathogen molecules, like bacterial toxins (for example diphtheria toxin or *Pseudomonas* exotoxin), can be activated by furin.¹²⁰ Furin's role in tumor development and progression is described in detail in Section 1.1.4.1.1.

In the case of viruses, it induces infection through processing of their surface glycoproteins (e.g. spike protein in Coronaviruses).¹⁰³ Furin-mediated cleavage has been described for glycoproteins of numerous evolutionarily diverse viruses, including HIV, influenza virus, dengue virus, Ebola virus, and Marburg virus.¹⁰³ An in-depth analysis of furin's role in viral infections is addressed in Sections 1.1.4.1.2 and 1.1.4.1.3.

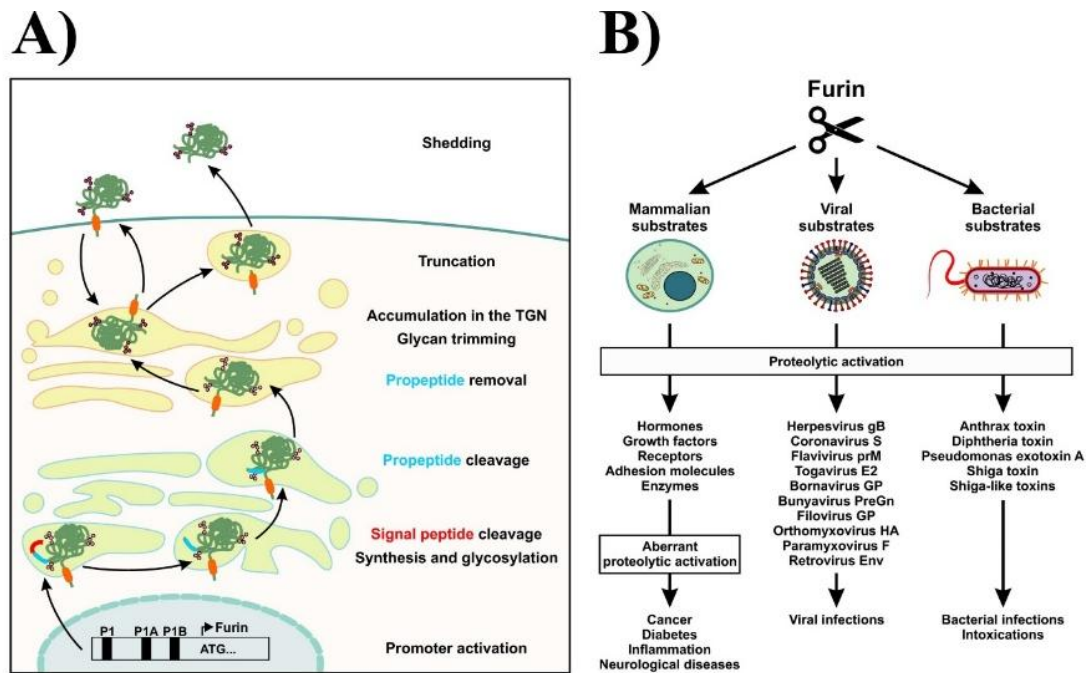


Figure 8. The maturation process of furin (B), and a schematic representation of the biological functions of furin in the body, including its role in the development of viral and bacterial infections (A).¹⁰⁰

A) Transmembrane domain is marked by orange color, signal peptide is marked by red color and propeptide (pro-domain) is blue. Endoplasmic reticulum is illustrated as light green structures, and *trans*-Golgi network (TGN) as light yellow structures.

B) E2- glycoprotein E2; Env- envelope; F- fusion protein; gB- glycoprotein B; GP- glycoprotein; HA- hemagglutinin; PreGn- precursor glycoprotein Gn; prM- premembrane protein; S- spike protein.

1.1.4.1.1. Furin in tumor progression

As previously mentioned, furin is widely expressed throughout the human body. Its oncogenic potential is associated with its ability to activate proteins involved in key processes, such as cell proliferation, angiogenesis, migration, and tissue invasion.

Among the proteins activated by furin are growth factors, including insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs), and nerve growth factor (NGF), which can promote cell proliferation and, consequently, tumor growth. Additionally, furin activates angiogenic and lymphangiogenic factors such as vascular endothelial growth factors C and D (VEGF-C and VEGF-D), which enhance vascularization and support tumor development.¹⁰⁰ Furthermore, furin promotes binding with hypoxia-inducible factor-1 (HIF-1), which is a transcription complex responsible for adaptation of cell for hypoxia. It should be noted that conditions where level of oxygen is decreased has an impact on tumorigenesis. Additionally hypoxia increases distribution of furin.^{121,122}

Proliferation is a crucial and fundamental mechanism for tumor cells. Overproduction or constitutive activation of growth factors results in sustained activation of this process.¹¹⁷ Examples of such factors are the mentioned above IGFs. IGF is a complex system comprising e.g. growth factor ligands or transmembrane growth factor receptors, like insulin receptor (IR) - A and -B. Hyper activation of this signaling pathway is related with the development, maintenance, progression, survival and response to chemotherapeutics in diverse types of cancers. IR-A and IR-B are matured in the TGN, where they undergo activation by furin.¹²³ The mature IR is a heterotetrametric transmembrane protein, which consists of two disulfide-linked dimers. Each dimer has ligand-binding α -subunit at *N*-terminus and a tyrosine kinase active β -subunit at *C*-terminus. These subunits are synthesized as a single precursor polypeptide (proIR), which is cleaved in TGN. The cleavage occurs after Arg735 located in the fragment RKRR735. This tetrabasic amino acid sequence is located at the junction between two subunits. There are two isoforms of IR, which differ by a presence of a 12-amino acid fragment located at *C*-terminus of subunit of IR-B (which is absent in IR-A).¹²⁴ It was noticed that cancer cells overexpress IGF and IR (mainly IR-A), and IR-A contributes to the amplification of IGFs signaling.¹²⁵

The first reports linking furin expression to tumor development appeared in 80s. In 1987 Schalken *et al.*¹²⁶ described increased expression of furin mRNA in non-small cell lung cancer (NSCLC),¹²⁶ later in 2001 Bassi *et al.*¹²⁷ reported the correlation of furin and its mRNA levels with the aggressiveness of lung, head and neck cancer cells.¹²⁷ Furin and other members of the proprotein convertases family (PC1, PACE4 and PC7) mRNAs were detected in 7 human breast cancer cell lines (MCF-7, T-47D, ZR-75-1, BT-20, MDA-MB-157, MDA-MB-231, MDA-MB468) and in 30 breast tumor tissues.¹²⁸ Among types of cancers related with furin activity are:

sarcomas, lung carcinomas, head and neck squamous cell carcinomas, endometrial, cervical, ovarian, breast, brain and central nervous system cancers.¹¹⁹

Consequently, furin has emerged as a promising tumor marker¹²⁹ and became a compelling target for anticancer therapeutic strategies. There are a few approaches that could be applied: inhibition of furin activity, silencing of furin's activity (through autologous cancer cell vaccine¹³⁰) or utilization of furin as a target receptor to deliver therapeutics.¹¹⁹ Moreover, scientific reports published over the past five years indicate that furin serves as a significant prognostic marker as its expression level correlate with cancer malignancy.^{131,132,133} Additionally, inhibition of furin has been shown to contribute to antitumor activity.^{132,134} Overview of furin inhibitors is presented in Section 2.4.

1.1.4.1.2. Role of furin in viral pathogenicity

Most of viral fusion proteins are synthesized as inactive precursors, which need to be processed by host cell proteases. Activation of viral glycoproteins allows viral entry into the host cell and promotes the spread of the infection. Usually viruses exploit host cell trypsin-like or subtilisin-like endoproteases, such as furin.¹⁰⁰ Furthermore, many glycoproteins require more than one proteolytic processing to form fully infectious virions.¹³⁵

In general, host proteases cleave the *N*-terminal extracellular region (which includes the receptor-binding domain) from *C*-terminal transmembrane domain. As a result, the fusion peptide is released, enabling membrane fusion and subsequent viral entry into the host cell. Additionally, such structural rearrangements can lead to exposing or masking of epitopes and thereby decreasing visibility of pathogen for antibodies. Furthermore, some viruses possess their own proteases. For example, human immunodeficiency virus-1 (HIV-1) utilizes its own protease to cleave Gag-Pol polyprotein and furin or related proprotein convertases to activate envelope protein. Also severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) employs both viral proteases (PL^{pro} and M^{pro}) and cellular enzymes, like furin, to enter the host cells. Afterwards, viral glycoprotein moves through the TGN and ER to the extracellular space. Different viruses are activated through diverse host proteases and in various subcellular compartments.¹³⁵

As it was mentioned previously, in Section 1.1.3., furin is involved in activation of viral proteins across a broad spectrum of viral families, including: Herpes-, Papillo-, Corona-, Flavi-, Toga-, Borna-, Bunya-, Filo-, Orthomyxo-, Paramyxo-, Pneumo-, Hepadna- and Retroviridae.¹⁰⁰

Flaviviruses are enveloped RNA viruses transmitted by arthropods. They enter host cells *via* receptor-mediated endocytosis and are subsequently transported to endosomes, where the acidic environment induces conformational changes in their structure. Then, genomic RNA is released and it can be translated to polyprotein precursor. This precursor gives rise to three structural proteins: core (C), precursor membrane (prM), envelope (E) and seven non-structural proteins

(NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. The structural proteins are essential for virion assembly, entry, and egress, whereas the NS partake in viral replication and in evasion of the host immune response. Immature virions migrate to endoplasmic reticulum where they congregate. Then, their maturation occurs in TGN, where furin recognizes characteristic sequence $RX_{aa}R/KR$, occurring in all flaviviruses. As a consequence, protein prM is cleaved to M protein and then mature, infectious particles are released outside the cell *via* exocytosis.¹³⁶

The Flaviviridae family includes several clinically significant pathogens, such as West Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV) which are responsible for severe encephalitis; dengue virus (DENV) and yellow fever virus (YFV), which lead to hemorrhagic fever and Zika virus (ZIKV), which causes relatively mild clinical symptoms, however it is associated with teratogenic effects on fetal development, posing a particular risk to pregnant women.¹³⁶ Detailed role of furin in ZIKV infection is described in next Section.

1.1.4.1.3. Furin and ZIKV relationship

ZIKV is an arbovirus transmitted to humans through the bite of infected arthropods, such as *Aedes aegypti* mosquitoes. The virus was first discovered in 1947 during studies on yellow fever in Uganda's Zika Forest, when it was isolated from a rhesus monkey. Following its detection, the first human cases were reported in the 1950s. The initial strain of ZIKV (ZIKV_{MR766}) was named after the identification number of the monkey from which the virus was isolated.¹³⁷

ZIKV can be transmitted *via* blood transfusion, sexual contact, and maternal-fetal routes. Most infections are asymptomatic (60–80%), while symptomatic cases typically present with fever, rash, arthralgia, and conjunctivitis. However, ZIKV infection may lead to neurological complications, such as Guillain-Barré syndrome, and poses a significant risk to pregnant women. In developing fetuses, infection can result in microcephaly, with the risk of this abnormality increasing up to 50-fold in infected mothers.¹³⁸

Since its discovery, ZIKV has spread globally. In 2007, outbreaks occurred in Micronesia, followed by further outbreaks in French Polynesia during 2013-2014. These events were associated with an increased incidence of Guillain-Barré syndrome and congenital abnormalities. In 2015, ZIKV reached the Americas, causing widespread outbreaks in Brazil, Central and South America, and the Caribbean. Although the spread of the virus has declined since 2017, it remains a significant public health concern. Furthermore, over nearly eight decades since its discovery, the ZIKV genome has undergone mutations, resulting in the emergence of more pathogenic strains.¹³⁷

The structure of ZIKV is icosahedral-like, with viral particles measuring between 45 and 75 nm in diameter. The mature particle contains 180 copies of the E and M proteins embedded in the viral membrane. Structurally, the E protein consists of four domains: a stem-transmembrane

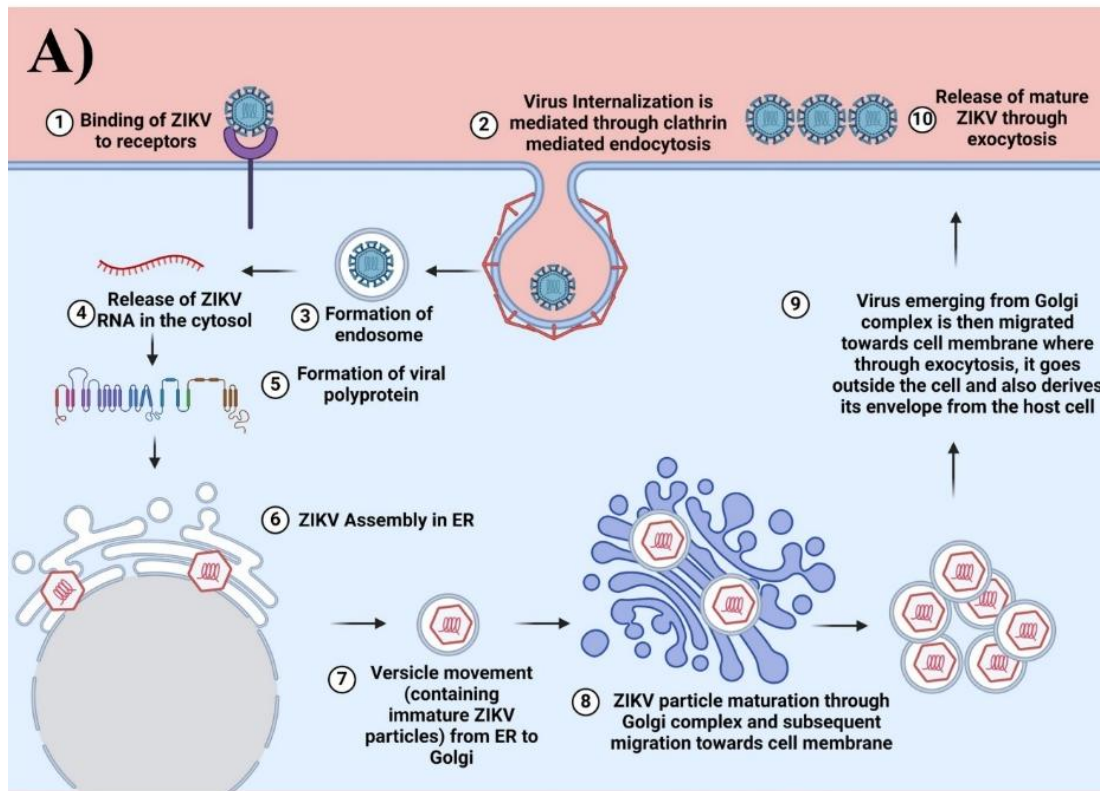
domain pair and three ectodomains (I, II and III), located outside of the membrane. The larger E protein predominates on the surface, while smaller M protein, with its short extracellular region and stem-transmembrane domains, is placed below. E proteins form dimers, with each monomer related to its neighbor by 2-fold symmetry. Three dimers lie parallel to each other, forming “raft” configuration. Each virion contains 30 rafts, resulting in 60 asymmetric units. Despite presence of 180 E and M proteins, the virion does not exhibit $T = 3$ quasi-equivalence, due to the unusual composition of E proteins.¹³⁹

As mentioned in the previous section, immature flaviviruses are composed of three structural proteins: C, prM (which is cleaved into pr and M), and E. Immature, spiky ZIKV particles contain three prM-E heterodimers located on the exterior of the viral membrane, with the fusion loop of the E protein exposed. The pr fragment is positioned at the apex of each spike, covering the fusion loop and thereby stabilizing the E protein by locking it into a rigid, immature conformation during transport through the secretory pathway. Thus, this mechanism protects against premature membrane fusion.^{137,139}

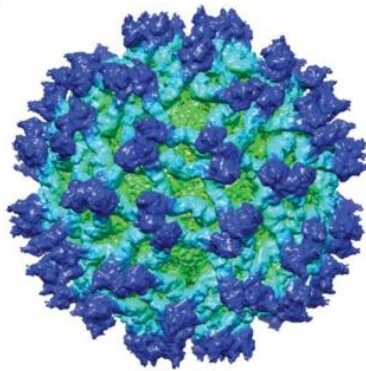
Viral E proteins bind to cellular receptors located on the surface of for example: fibroblasts, immature dendritic cells, epidermal keratinocytes, and stem-cell-derived human neural progenitor cells. After viral binding, internalization inside the cells occurs through clathrin-dependent endocytosis. Inside the endosome, viral glycoproteins undergo conformational changes and in consequences ZIKV nucleocapsid is released inside the host cell. The positive-sense RNA is translated into a polyprotein, which then enters the ER, where synthesis of structural and non-structural proteins ensue.¹⁴⁰

The spiky particles undergo glycosylation in the ER and are subsequently transported to the TGN, where glycan trimming occurs. Exposure to the locally acidic environment induces conformational rearrangement of the prM-E spikes, resulting in the alignment of E proteins parallel to the membrane. Consequently, the cleavage site within the prM protein becomes accessible to furin. Following maturation cleavage, the pr domain remains associated with the distal end of the E protein until the virion exits the host cell.^{137,139} Scheme of ZIKV entering the cell and its replication mechanism is presented in Figure 9A.¹⁴⁰ It is important to underline that furin is crucial for maturation process of ZIKV which occurs in human cells.

The remaining structural proteins serve distinct functions: the C protein is responsible for packaging the viral RNA genome and assembling new virions, while the E protein mediates receptor binding and facilitates membrane fusion during viral entry and subsequent endocytosis.¹³⁷ The structures of both immature and mature ZIKV particles are illustrated in Figure 9B and C.¹³⁹



B)



C)

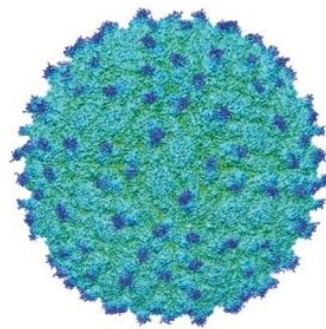


Figure 9. ZIKV replication cycle (A)¹⁴⁰ and structures of spiky, immature (B) and smooth, mature (C) forms of ZIKV.¹³⁹

ZIKV poses a particular threat to pregnant women due to its ability to replicate within Hofbauer cells of the placenta and fetal brain. These cells act as reservoirs for viral replication and dissemination.^{137,141}

Due to the global spread of ZIKV and the outbreaks described in previous sections, the development of effective therapeutic strategies is urgently needed. However, to date, no licensed

vaccines or monoclonal antibodies have been developed.¹⁴² This highlights the necessity of developing novel treatments against ZIKV.

Several therapeutic approaches are currently under investigation, including direct inhibitors targeting the E protein, inhibitors of viral fusion or attachment, and strategies aimed at modulating host factors.¹³⁷ One promising avenue involves the inhibition of furin activity, which could prevent the formation of mature ZIKV particles.

2. Serine proteases inhibitors

Protease inhibitors are present in diverse organisms, including plants, animals, and microorganisms, where they fulfill essential biological functions. Examples of their roles include protecting cells from unwanted proteolytic activity, regulating blood pressure, and facilitating hormone transport.¹⁴³

As it was discussed in previous sections of this thesis, inhibitors of serine proteases are of particular importance due to their diverse applications in medicine and pharmacy. Some of these inhibitors have been approved as therapeutic agents and have been successfully used for many years in clinical practice.

In addition to their established roles, serine protease inhibitors are increasingly recognized for their potential in treating viral infections, inflammatory diseases, and certain cancers. For instance, camostat mesylate, a serine protease inhibitor originally used for pancreatitis, has gained attention for its ability to block TMPRSS2- a host protease critical for the entry of coronaviruses into human cells.¹⁴⁴

Moreover, naturally sourced such as plant-derived protease inhibitors (e.g. Bowman-Birk inhibitors)^{145,146} and microbial peptides are being explored for their unique properties and potential synergistic effects when combined with conventional therapies.

Given their broad spectrum of activity and adaptability, serine protease inhibitors remain a highly promising class of therapeutic agents. Ongoing research aims to expand their therapeutic applications, particularly in areas where protease dysregulation plays a central role in disease pathogenesis.

2.1. Classification of serine protease inhibitors by inhibition mechanism

Serine protease inhibitors can be classified into four distinct groups based on their mechanisms of action: serpins, inhibitors targeting the protease autolysis loop, canonical inhibitors, and non-canonical inhibitors.¹⁴⁷

Serine protease inhibitors, termed serpins are irreversible covalent inhibitors composed of approximately 350-500 amino acids. Their mechanism of action involves the formation of a suicide complex with the target protease and is often compared to the function of a mousetrap.

They exhibit a highly conserved architecture consisting of eight-nine α -helices, three β -sheets, and a reactive center loop (RCL). Stereoviews of an example of typical serpin, α_1 -proteinase inhibitor, is presented in Figure 10.¹⁴⁸

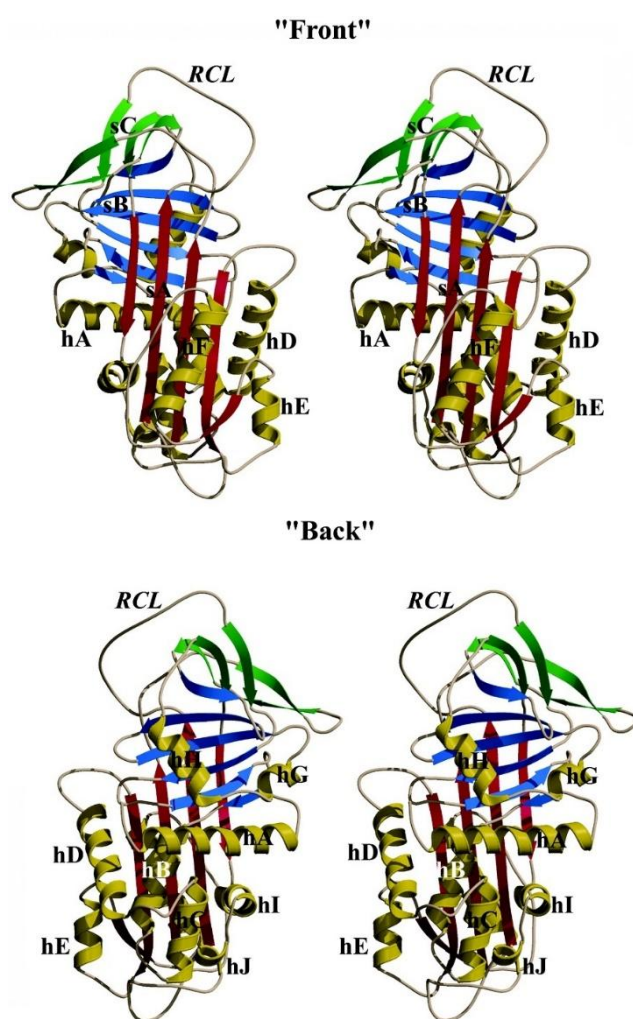


Figure 10. Front and back stereoviews of α_1 -proteinase inhibitor.¹⁴⁸

Eight α -helices are designated by the letters A–H, each preceded by a lowercase h (e.g., hA).

Three β -sheets (A, B, C) are shown in red, blue and green respectively.

RCL- reactive center loop.

RCL consists of roughly 17 amino acids, which acts as a bait for the enzyme. The RCL can adopt different conformations: in the active form of the inhibitor, it protrudes from the protein scaffold, whereas in the more stable, latent conformation, the loop is inserted into the central β -sheet A. Upon binding, the protease cleaves the RCL at the P1-P1' position and forms a covalent

bond with it. This complex is then translocated to the opposite end of the serpin molecule, resulting in the formation of an inactive complex.

Examples of serpins include alpha-1 antitrypsin (A1AT), antithrombin III (SERPINC1), and plasminogen activator inhibitor-1 (PAI-1).^{147,149}

Inhibitors that target the autolysis loop of serine proteases bind allosterically. The autolysis loop is part of the activation domain, and its inhibition induces significant conformational changes that disrupt the activation process and other functional regions of the enzyme, thereby abolishing protease activity. Three mechanisms of action have been proposed. The first one relies on the deformation of the oxyanion hole due to displacement of the oxyanion-stabilizing loop. The second one leads to disruption of the S1 specificity pocket. The third one leans on the interference with the salt bridge between the Asp of the catalytic triad and the *N*-terminal residue, resulting in the formation of an inactive, zymogen-like protease.¹⁵⁰ Examples of autolysis loop-targeted inhibitors are monoclonal antibodies (e.g. mAb-112 and mAb-12E6B10).¹⁵¹

Canonical serine protease inhibitors bind to the active site of the protease in a substrate-like manner but are not cleaved (or only partially cleaved). They present an exposed loop (like serpins) with a defined backbone conformation, referred to as the “canonical” structure. Their mechanism of action, known as the Laskowski or standard mechanism, involves substrate-like binding kinetics. The inhibitor is hydrolyzed, forming an acyl-enzyme complex, but the hydrolysis products remain bound within the active site. The peptide bond within the inhibitor can be reformed, leading to a significant reduction or complete inhibition of enzymatic activity.¹⁵²

Non-canonical inhibitors, in contrast, sterically block the active site of the protease. They interact with the enzyme via their *N*-terminal region and occupy the active site without forming new peptide bonds. As a result, the S1 pocket becomes inaccessible, preventing substrate entry and subsequent catalysis.¹⁵²

2.2. Classification of serine protease inhibitors by structural diversity

Taking into consideration their structure, serine protease inhibitors can be generally categorized into two groups: small-molecule compounds and biomacromolecules, including peptides and proteins.

The first group to be distinguished consists of small-molecule inhibitors (SMI). These compounds can interact with the catalytic site of the enzyme through covalent or non-covalent binding. Their advantages include low production cost, lack of immunogenicity, and long shelf life. However, they often exhibit broad specificity.^{153,152} Several SMIs are currently used in clinical practice. For example, the thrombin inhibitor Dabigatran, the active component of

Pradaxa®, is used in stroke prevention. Other examples include inhibitors of activated coagulation factor X (fXa), such as Apixaban, Betrixaban, and Rivaroxaban, which are employed in the treatment of thromboembolic disorders. Another clinically relevant compound is Nafamostat, a broad-spectrum serine protease inhibitor used in the management of acute pancreatitis and disseminated intravascular coagulation.¹⁵² The structures of selected SMIs are presented in Figure 11.¹⁵²

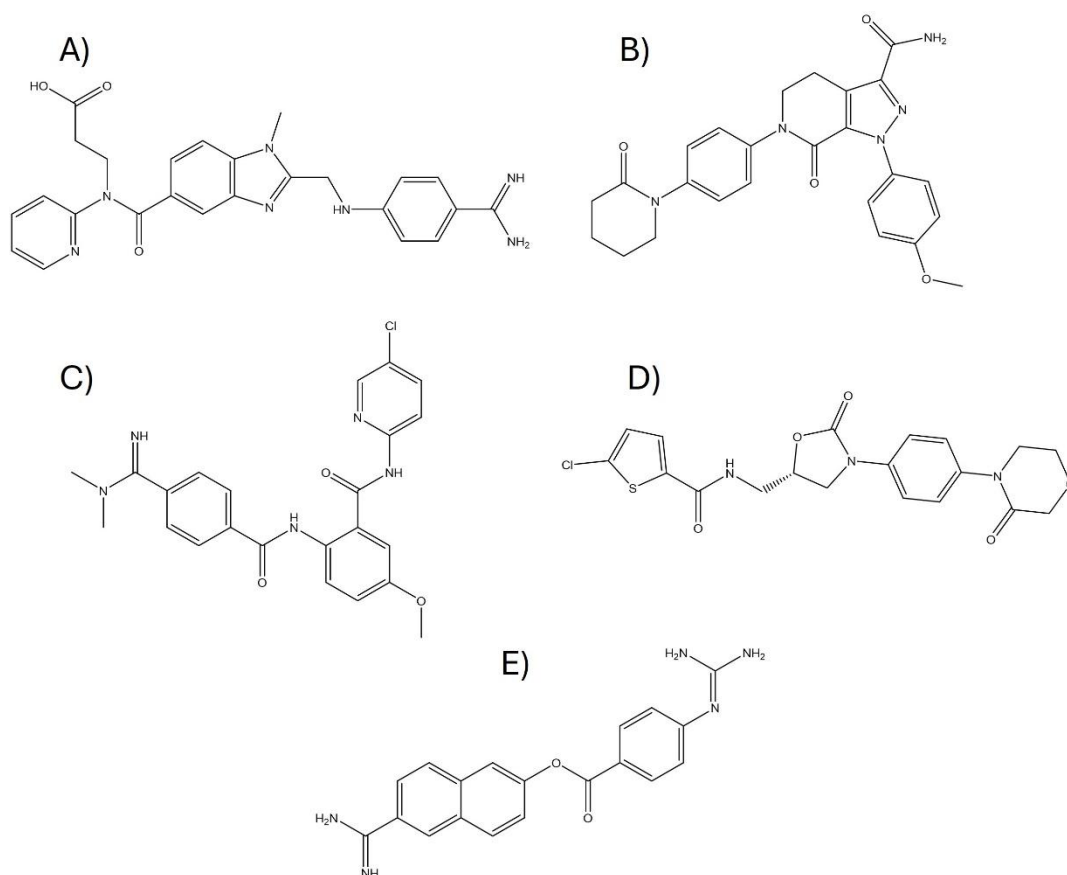


Figure 11. Structures of small-molecule inhibitors: Dabigatran (A), Apixaban (B), Betrixaban (C), Rivaroxaban (D), Nafamostat (E).¹⁵²

The second group comprises biomacromolecular inhibitors, which include proteins, peptides, and peptide mimetics. Proteinaceous protease inhibitors represent a ubiquitous class of compounds that have been studied for over 80 years. These inhibitors can be categorized into families such as Serpins, Kunitz, Kazal, Potato type I (Pin1), Potato type II (Pin2), and Bowman-Birk inhibitors.^{152,154} Generally, they are of natural origin and have been identified in a wide range of organisms, including microorganisms, animals, and plants. In plants, they can constitute 1-10% of the total protein content in storage organs such as seeds and tubers.¹⁵⁴

Serpins are the largest and most widely distributed family of protease inhibitors. They have been identified in Eukarya, Bacteria, Archaea, and even in some viruses. Serpins form a distinct group based on both their mechanism of action and structural features. In plants, serpins play a protective role against pathogens.¹⁵⁵ They were described in details in Section 2.1.

Kunitz-type inhibitors are particularly abundant in the plant family *Fabaceae* (*Leguminosae*), which includes subfamilies such as *Mimosoideae*, *Caesalpinioideae*, and *Faboideae/Papilionoideae*. The first Kunitz inhibitor, Soybean Kunitz trypsin inhibitor (SKTI), was described in 1945 by M. Kunitz.¹⁵⁶ These inhibitors primarily protect plants against bacteria, fungi, and insects. In addition, they have demonstrated anti-inflammatory, anticoagulant, antithrombotic, and anticancer properties in humans. Kunitz inhibitors typically have a molecular weight of 20-22 kDa and exist as single- or double-polypeptide-chain proteins, with variations in cysteine residues and disulfide bridges. They generally possess highly conserved primary structures. Their reactive sites commonly contain Arg and Lys residues, although some variants include Glu, Ala, or Val.¹⁵⁴

Kazal-type inhibitors are proteins composed of one or more Kazal inhibitory domains, each characterized by six highly conserved Cys residues forming three intradomain disulfide bridges. These inhibitors regulate proteases such as trypsin, chymotrypsin, subtilisin, thrombin, and elastase. They have been identified in vertebrates (particularly mammals and birds), parasites, insects, leeches, crustaceans, and fungi. Although Kazal inhibitors have also been found in plants, their specific role in these organisms remains unclear.¹⁵⁷

Potato type I (Pin1) and Potato type II (Pin2) serine protease inhibitors accumulate extensively in plant reproductive and storage organs and are major components of *Solanum tuberosum* (potato) tubers.¹⁵⁸ The first member of the Pin1 group, Potato Inhibitor I, was described in 1962 by Ryan and Balls.¹⁵⁹ These inhibitors primarily protect plants from pathogens and insect proteases, such as those from the Colorado potato beetle. Notably, Pin1 accumulation has been observed in both damaged and undamaged distal leaves of tomato and potato plants in response to pest attack. Members of the Pin1 group vary in the molecular weight and amino acid composition. Pin2 inhibitors contain between two and eight tandem repeated domains, each consisting of approximately 50 amino acids, including eight highly conserved Cys residues involved in disulfide bridges formation.¹⁵⁸

The Bowman-Birk inhibitors are described in detail in Section 2.2.1.

2.2.1. Bowman-Birk inhibitors

Bowman-Birk Inhibitors (BBIs) are stable, small (~ 8-10 kDa) peptide- and protein-based inhibitors. They usually possess two inhibitory loops active against two identical or non-identical proteases and fourteen Cys residues which form seven disulfide bridges. An exposed binding loop

consists of nine amino acids and it is flanked with two cysteines connected with disulfide bridge.^{160,161} First member of BBI family was isolated from soybean (*Glycine max*) by Donald E. Bowman in 1946,¹⁶² and then in 1963 it was characterized by Yehudith Birk *et al.*¹⁶³ Many BBIs are of plant origin, they occur in seeds of legumes and in cereal grains, e.g. SFTI-1 (from sunflower, *Sunflower trypsin inhibitor*), BTCI (from black-eyed pea, *Black-eyed pea trypsin and chymotrypsin inhibitor*), TBPI (from tepary bean, *Tepary bean protease inhibitor*) or LCTI (from lentil, *Lentil trypsin-chymotrypsin inhibitor*). Their most likely role is to control plants' endogenous proteases or protect them against exogenous proteases present in microbials and insects.¹⁶¹ Moreover, it was shown that BBIs also have anti-inflammatory,¹⁶⁴ anticancer,¹⁶⁵ antibacterial¹⁶⁶ and antifungal¹⁶⁷ properties. Interesting BBI's feature is an extreme stability in high temperatures (even at 100°C) and wide pH range (2-12). They own it to the presence of several disulfide bonds in their structure. Classical BBIs exhibit high resistance even in acidic environment in gastrointestinal tract.¹⁶¹

Not only plants produce substances which protect them against pathogens and predators. In addition to BBIs isolated from plants, there are also peptides which contain the canonical Bowman-Birk protease inhibitory motif and has been detected in the skin secretions of amphibians, mainly from *Ranidae* family members. These peptides, named Bowman-Birk-like trypsin inhibitors (BBLTIs), are composed, in general, from 17-18 amino acids residues, and feature a single disulfide bridge in their structure. However, there are some exceptions, such as ORB peptide, which is composed of 20 amino acids and PPF-BBI, composed of 16 residues.^{161,168,169} This group of inhibitors is continuously expanding, and new members are regularly being reported: *Odorrana schmackeri* Trypsin Inhibitor (OSTI) described in 2017,¹⁷⁰ *Pelophylax esculentus* Bowman-Birk proteinase inhibitor (PE-BBI) described in 2018,¹⁷¹ *Pelophylax plancyi fukienensis* Bowman-Birk proteinase inhibitor (PPF-BBI) described in 2019¹⁶⁷ or livisin found in the skin secretion of the *Odorrana livida* frog, described in 2022.¹⁶⁸

One of BBLTIs, described in 2008, is a peptide isolated from frog *Huia versabilis* skin, named HV-BBI (*Huia versabilis* Bowman-Birk inhibitor)¹⁷² described in details in Section 2.2.1.2.

2.2.1.1. Sunflower Trypsin Inhibitor-1 (SFTI-1)

Sunflower trypsin inhibitor-1 (SFTI-1) is one of the smallest natural-derived inhibitors, which acts according to Laskowski mechanism. This well-known inhibitor, derived from sunflower seeds, is a 14 amino acid (~ 1,5 kDa) bicyclic peptide with a single disulfide bond (Figure 12). Its sequence and structure were determined through X-ray crystallography of its complex with bovine β -trypsin. SFTI-1 comprises two anti-parallel β -strands that are connected by a type VIb

β -turn at one end and a type I β -turn at the other end. Its structure is rigid due to the presence of disulfide bridge, three Pro residues and intramolecular hydrogen bonds network.¹⁷³



Figure 12. Structure of SFTI-1.

The P1 position is marked in bold and the conserved binding loop is highlighted in blue.

Several studies confirm sub-nanomolar K_i values for SFTI-1 against bovine β -trypsin.^{174,175,176} Relatively small size, compared to other members of BBI and strong inhibitory potency, make the SFTI-1 interesting and valuable scaffold for designing inhibitors of other serine proteases. For example, replacing the Lys residue at the P1 position with one that is more preferred by a specific enzyme, such as Phe in the case of chymotrypsin, can significantly increase the inhibitory potency against that enzyme, while simultaneously decreasing activity against trypsin.¹⁷⁷ Similarly, placing Val in that position yields potent elastase inhibitor, but compound inactive toward trypsin. However, substitution in this position is rare, because Lys is crucial residue for protease selectivity.¹⁷³ In fact, every residue in SFTI-1 was examined using several approaches, like alanine scanning or substitutional analysis performed on cellulose, where all positions were substituted by remaining 19 proteinogenic amino acids.^{173,178}

Other modifications have focused on designing analogs with substituted amino acids to enhance activity against specific proteases. For example, the replacement of Ile10 with Arg resulted in a potent MT1 inhibitor.¹⁷⁹ Similarly, substitution of Arg2 with D-Arg yielded a potent MT2 inhibitor, as described in the paper by Gitlin-Domagalska *et al.*⁹⁶ mentioned in Section 1.1.3.3. Another approach is iterative optimization, which involves sequential screening of point mutations at multiple positions. This strategy has been employed to develop a series of inhibitors targeting, for example, MT1,¹⁸⁰ kallikrein-related peptidase 5,¹⁸¹ furin,¹⁸² cathepsin G,¹⁸³ proteinase 3,¹⁸⁴ or individual subunits of the 20S proteasome.¹⁸⁵

SFTI-1 is widely recognized as an excellent scaffold for designing protease inhibitors due to its compact, cyclic structure and high stability, which confer resistance to enzymatic degradation. Its canonical binding loop allows precise interactions with protease active sites, and targeted substitutions in positions crucial for tuning specificity toward various enzymes. The scaffold tolerates extensive modifications, making it highly versatile for engineering. Additionally, its structure has been well-characterized through crystallographic studies, facilitating rational design of novel inhibitors. What is more, it is a great starting structure for engineering potential therapeutics and it does not undergo oxidative folding during introducing non-native sequences.¹⁷³

2.2.1.2. *Huia versabilis* Bowman-Birk inhibitor (HV-BBI)

In 2008 Song *et al.*¹⁷² described novel amphibian skin Bowman-Birk-like trypsin inhibitor extracted from Chinese bamboo odorous frog *Huia versabilis*. Its inhibitory potency against trypsin was evaluated multiple times, with reported K_i values of 18,8 nM;¹⁷² 3,3 nM;¹⁸⁶ 120 nM.¹⁸⁷ HV-BBI is an 18 amino acid peptide with amide group on the C-terminus (Figure 13A). The most crucial residue for interaction with an enzyme is Lys8, which determinates both inhibitory activity and specificity against trypsin-like proteases.¹⁸⁸

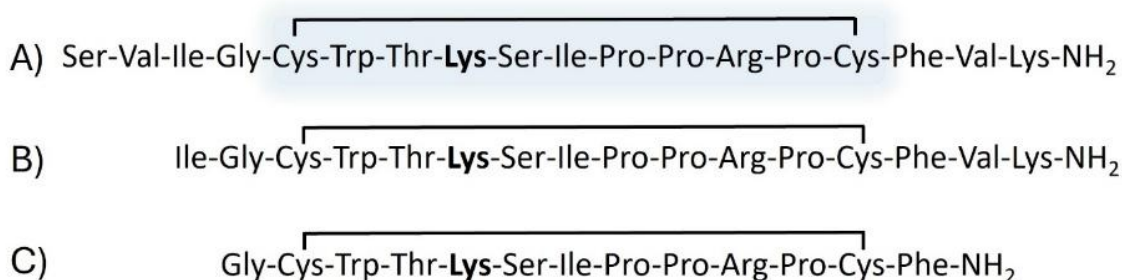


Figure 13. Structures of HV-BBI (A); HV-BBI(3-18) (B); HV-BBI(4-16) (C).

The P1 position is marked in bold and the conserved binding loop is highlighted in blue.

Crystal structure for HV-BBI was obtained actually for its truncated analog- peptide HV-BBI(3-18) (Figure 13B) with bovine β -trypsin (PDB: 4U2W) and it proved interaction between S1 pocket in the enzyme and Lys residue in inhibitor.¹⁸⁸

HV-BBI analog with Arg instead of Lys8 resulted in 3-fold decrease of inhibitory potency against trypsin, and similarly to HV-BBI was not active against chymotrypsin.¹⁷²

Interestingly, one of its shortest analogs, i.e. HV-BBI(4-16) (Figure 13C), exhibits one of the highest inhibitory potency toward matriptase-1 ($K_i = 8$ nM). Furthermore, inhibitory potency of that peptide was also examined toward bovine β -trypsin ($K_i = 151$ nM) and human plasmin ($K_i = 120$ nM). Besides, such outstanding properties were observed only for the analogue with amide group at the C-terminus. MD studies revealed amide- π interaction between amide group of Phe13 in truncated peptide and an aromatic ring of Phe97 in MT1, which are not present in bicyclic and acidic analogues.¹⁸⁷

2.3. Matriptase-1 inhibitors

As it was mentioned in Section 1.1.3.1. MT1 is a trypsin-like serine protease, which means it cleaves its substrates after the basic amino acids: Arg and Lys. Consequently, diverse MT1 inhibitors block also other proteases exhibiting similar substrate specificity, such as clotting enzymes (thrombin and fXa), the fibrinolytic proteases urokinase-type plasminogen activator (uPA), plasmin and trypsin.¹⁸⁹ First small molecule inhibitors of MT1 were described in 2001 by Enyedy *et al.*¹⁹⁰ These benzamidine analogues were also examined toward thrombin and one of them displayed strong inhibitory potency against MT1 ($K_i = 208$ nM) and weak activity against thrombin ($K_i = 2670$ nM). Structure of this compound is shown in Figure 14.

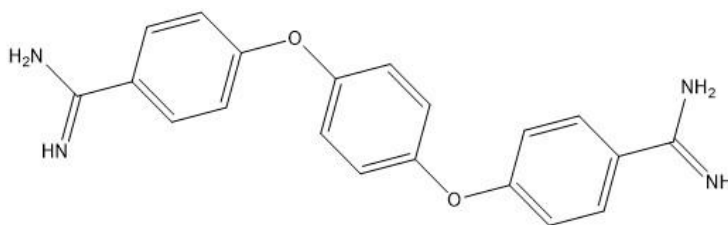


Figure 14. Structure of an analogue of benzamidine, one of the first described potent small-molecule MT1 inhibitor.¹⁹⁰

Various low-molecular-weight MT1 inhibitors, including bis-benzamidines,¹⁹⁰ inhibitors composed of a fixed sulfamoyl benzamidine group linker connected to a second aromatic ring¹⁹¹ or symmetric, achiral bisbenzamidines,¹⁹² symmetrical and nonsymmetrical bis- and trisbenzamidines,¹⁹³ amidinophenylalanines^{194,195,196} have been reported to date. Small molecule compounds, like benzamide derivatives, are most potent and irreversible inhibitors with K_i values at single-digit nanomolar values. However their main disadvantage is lack of or low selectivity toward diverse serine proteases.¹⁹⁷

Boudreault group¹⁹⁸ reported series of potent peptidomimetic inhibitors of MT1 linked with ketobenzothiazole serine trap to form covalent inhibitor. Also Mahoney^{199,200} described novel class of strong covalent small-molecule ketobenzothiazole inhibitors targeting TTSPs, including MT1, however characterized by low specificity.

The design of peptidic inhibitors targeting matriptases is also a focus of research in Prof. Rolka's group. As previously mentioned, SFTI-1 serves as an excellent scaffold for developing inhibitors of various proteases. It has been successfully employed to obtain potent inhibitors of both matriptases.⁹⁶ Two of them are potent ($K_i = 0.433$ μ M and 0.280 μ M) inhibitors of MT2, with 176- and 226-times selectivity over MT1, respectively. Also Fittler *et al.*²⁰¹ reported a series of potent MT1 peptide inhibitors (K_i values even 2.1 nM) using SFTI-1 as a molecular scaffold.

The authors substituted the *N*-terminal Gly with Lys and demonstrated that *C*-terminal amino acids are not required for the efficient interactions between the bicyclic SFTI-1 and MT1. Quimbar *et al.*¹⁷⁹ obtained series of *Momordica cochinchinensis* trypsin inhibitor-II and SFTI-1 analogues. Alanine scanning was performed to assess the importance of individual amino acid residues in SFTI-1. Substitution with Ala at positions Arg2, Thr4, Lys5, Ser6, and Asp 14 resulted in a 7.5-50-fold increase in the K_i value compared with native SFTI-1. Also substitution of Arg2 with Lys resulted in 6-fold increased K_i value compared to the SFTI-1. Li *et al.*²⁰² synthesized a series of analogues, in which amino acid substitutions were introduced based on predicted favorable interactions with the enzyme catalytic site. In the obtained peptides, Arg2 was replaced with citrulline; Lys5 with Arg, hLys or ornithine; Ile10 with Gln; and Phe12 with more bulky Phe analogues. None of the analogues exhibited improved inhibitory activity toward MT1 compared with SFTI-1. Some analogues displayed several-fold increased K_i values and retained similar selectivity toward thrombin. Notably, one analogue displayed remarkable selectivity: replacement of the hydrophobic Ile10 with a more hydrophilic and bulky Gln10 resulted in a $K_i = 2.33 \mu\text{M}$ toward MT1 and over 2500-fold selectivity for MT1 relative to thrombin. It is also worth noting, that SFTI-1 analogues that are potent MT1 inhibitors often exhibit significant inhibitory activity toward MT2, as demonstrated, among others, by Gitlin-Domagalska *et al.*^{96,97} In our recent work²⁰³ we identified a highly selective MT1 inhibitor that does not simultaneously inhibit MT2 and to the best of our knowledge, this is the first report of such a compound. This inhibitor is an analogue of HV-BBI, which is described in detail in a later part of this dissertation (Section 9.2.).

2.4. Furin inhibitors

Various furin inhibitors, including peptidic chloromethyl ketones,²⁰⁴ polyarginines,²⁰⁵ streptamine derivatives²⁰⁶ and peptidomimetics²⁰⁷ have been reported to date. One of the first described furin inhibitors was α_1 -antitrypsin Portland (α_1 -PDX, $K_i = 600 \text{ pM}$) which binds to the active site of the enzyme and inhibits substrate binding in a competitive manner. α_1 -PDX is a variant of the naturally occurring α_1 -antitrypsin serum protease inhibitor which is a member of serpin superfamily. It inhibits both furin and PCSK5 well and it has been shown to prevent the processing of HIV-1 envelope glycoprotein and measles virus fusion glycoprotein *in vitro*.^{208,209,210} In addition to the large molecules, which inhibit furin, there are also smaller compounds. Among them are PDX-derived peptides (known as mini-PDX peptides), which are weaker inhibitors than the full-length protein.²¹¹ Another peptide furin inhibitors are compounds derived from the extended furin cleavage sequence in hemagglutinin (HA) of avian influenza A H5N1, that have been patented in 2009 by Strongin *et al.*²¹² Compounds were designed using as a starting sequence peptide TPQRERRRKRR↓GL but obtained inhibitors were not selective against furin.²¹² Next important group of furin inhibitors are polybasic peptides consisting of numerous arginine

residues (thus known also as polyarginines). An example of such an furin inhibitor is nona-L-Arg (Arg₉) with $K_i = 42$ nM.²⁰⁵

Apart from the peptide inhibitors, peptidomimetics that are potent furin inhibitors were reported.²¹³ Numerous peptides containing multibasic cleavage motif with attached chloromethylketone (CMK) moiety at the C-terminus were described.^{214,204} Unfortunately, beside their relevant inhibitory potency toward furin,²¹⁵ they are not deprived of some certain drawbacks. Their selectivity is poor, they are cytotoxic²¹⁶ and CMK moiety is referred to be unstable in the pH 7.²¹⁷ Taking these all into account they display rather limited therapeutical potential.

In summary, the main limitation of most furin's inhibitors is a simultaneous inhibition of other proteases, including not only members of the PCSK family but also various ubiquitously expressed enzymes across different tissues. Appropriate characterization of their specificity and selectivity is of particular importance, especially in case of PC-selective viruses.^{182,218} Beside the lack of specificity, peptidic furin inhibitors are usually not resistant to proteolysis and are degraded rapidly. Due to proteolysis, opsonization and agglutination, free peptides are not systemically stable without additional modifications.²¹⁹ Construction of poly-D-Arg inhibitors²⁰⁵ seemed to resolve this problem. Due to their high positive charge they were able to permeate cell membrane.²²⁰ Polybasic compounds are well known as one of the groups of cell-penetrating peptides (CPPs). CPPs can be classified, among others, according to their physicochemical properties into cationic, amphipathic, and hydrophobic CPPs. Most cationic CPPs contain more than five basic amino acids in their structures. It has been observed that the internalization capacity of oligoarginines increases with the peptide length, with the optimal range being eight to ten Arg residues.²²¹

Protease inhibitors have already been successfully employed as therapeutic agents against viral diseases. Lopinavir/ Ritonavir (marketed as Kaletra® or Aluvia®) is a potent and selective inhibitor of HIV-1 protease, an essential enzyme required for the maturation of infectious viral particles.^{222,223} Another example is Nirmatrelvir/ Ritonavir (marketed as Paxlovid®), where Nirmatrelvir targets the 3CL-like protease, a key enzyme in viral replication and function, while Ritonavir acts as both a CYP3A inhibitor and a protease inhibitor.²²³

In this context, inhibition of PCSKs has emerged as a promising strategy for the development of novel antiviral therapies. Commercially available furin inhibitors, whose structures are presented in Figure 15, have been investigated for their antiviral potential. The effects of decanoyl-RVKR-chloromethylketone (also known as Dec-RVKR-CMK or furin inhibitor I, Figure 15A) and hexa-D-arginine (D6R or furin inhibitor II, Figure 15B) were studied against the hepatitis B e antigen (HBeAg), a key factor in the progression of chronic hepatitis B virus (HBV) infection. Both inhibitors significantly reduced the extracellular levels of HBeAg and increased intracellular levels of its precursor in HepG2.2.15 cells. However, Dec-RVKR-CMK was found to enhance HBV replication and induce severe cytotoxic effects, whereas D6R did not promote

viral replication and caused only mild cellular damage. Both inhibitors induced cellular damage, but through different mechanisms. Despite its lower toxicity, D6R was less effective than Dec-RVKR-CMK, likely due to limited membrane permeability.²²⁴

Additional studies have explored the antiviral activity of Dec-RVKR-CMK against ZIKV and JEV. Analysis of viral protein expression revealed a significant increase in the prM/E ratio in Vero cells treated with Dec-RVKR-CMK, indicating that the inhibitor effectively blocked prM cleavage and thereby arrested subsequent viral infection.¹³⁶

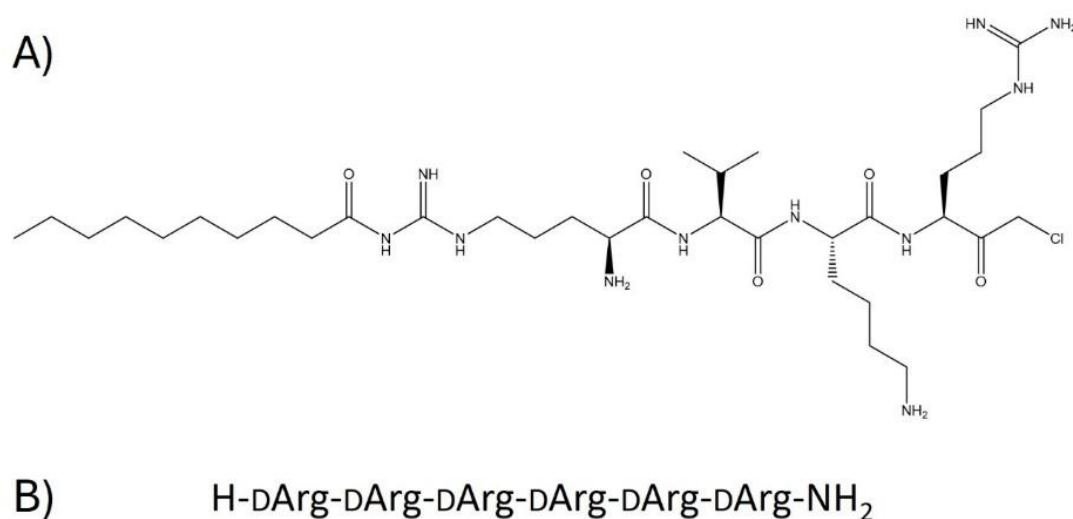


Figure 15. Structures of commercial available furin inhibitors: decanoyl-RVKR-chloromethylketone (Dec-RVKR-CMK, furin inhibitor I; A) and hexa-D-arginine (D6R, furin inhibitor II; B).

3. Molecular imaging - peptide-based molecular probes

As it was mentioned in previous sections, overexpression of MT1 and furin is related, among the others, with various malignancies. This is why visualization of enzyme-expressing cells is an attractive and significant scientific goal. The tools that makes that possible are probes consisting of targeting ligand (for example peptides, antibodies, aptamers) and imaging reagent (for example radioisotopes, fluorophores, photoacoustic imaging reagent). The most commonly used targeting ligands are antibodies. However, due to their huge molecular weight (~ 150 kDa), low specificity and high cost and potential to induce immune responses, antibodies often require alternative molecules for certain applications. The great substitute for antibodies are peptides.²²⁵ Two main

types of the peptide-based molecular probes (PBMPs) may be distinguished: affinity and activity probes.

3.1. Activity-based probes

The peptide-based activity probe consists of three essential parts: a reactive group, a peptidic recognition sequence and a detectable reporter group.²²⁶ In contrast to the above-mentioned affinity probe, it binds covalently with its molecular target (in particular with the protease), either reversibly or irreversibly.²²⁷ Comprehensive summary of serine proteases activity-based probes gives recent review article by Skorenski *et al.*²²⁸ Such mode of interaction is possible due to the presence of the electrophilic warhead located usually at the C-terminal end of peptidic recognition sequence.²²⁹ The activity probes are required to possess two key features: high affinity and specificity. If both conditions are fulfilled, utilization of such agents may guarantee: long duration of labelling resulting in less frequent dosing, successful competition with endogenous ligands, as well as offer a possibility of identification of biological target applying well known laboratory techniques, such as SDS-PAGE electrophoresis and Western Blot. It is worth underlining, however, that the presence of highly reactive warhead is likely to cause a non-specific binding with other enzymes, proteins or nucleic acids. Such covalent modifiers may also react with circulating nucleophiles (e.g. glutathione), and their off-target activity may result in organ toxicity. Moreover, an inappropriate scope and duration of inhibition of some relevant biological targets may also pose a serious threat. Only three fluorescent activity based probes of MT1 and MT2 (Figure 16) have been reported so far.^{230,231,232} In case of MT1, this short list may be extended by addition of two biotinylated probes: tetrapeptide Arg-Gln-Arg-Arg with chloromethyl moiety at C-terminus utilized for detection of active enzyme on cells in culture²³³ and short peptides bearing benzamidine group and C-terminal phosphonate warheads.²³⁴

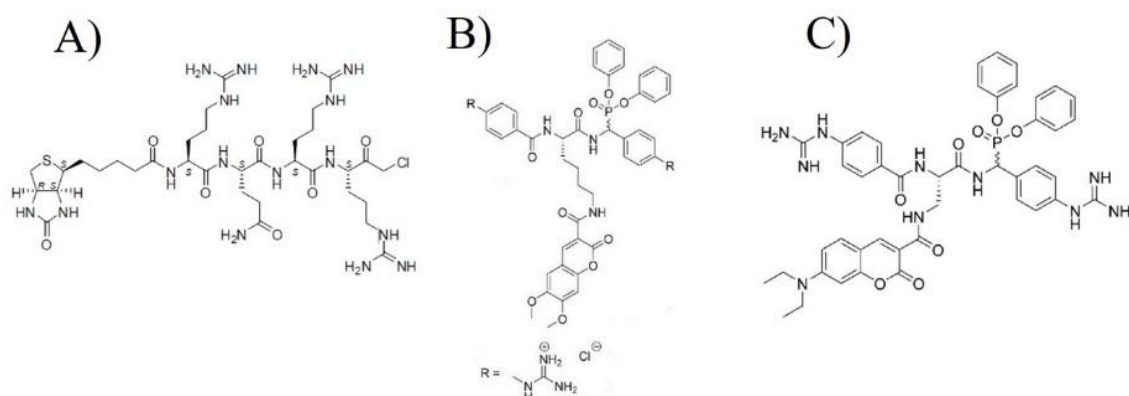


Figure 16. Structures of activity based probes of MT1 and MT2. A) biotin-RQRR-CMK;²³⁰ Activity based probes with phosphono bisbenzguanidines groups: B)²³¹, C)²³²

3.2. Affinity based probes

The affinity probe is composed of a peptide sequence responsible for selective binding to a certain biological target and a detectable fluorophore dye, that allows rapid and reliable imaging. Over the last few years, various applications of such probes have been reported.²³⁵ They were tested in various samples, such as: cell lysates, intact cells and even whole organisms. Regulatory peptides have gained a special interest in cancer imaging. They interact non-covalently and specifically with receptors overexpressed on tumor cells, such as: somatostatin receptors, the transmembrane human growth factor receptor c-Met, cholecystokinin-2 (CCK2)/gastrin receptor, gastrin-releasing peptide receptor (GRPR), epidermal growth factor receptor (EGFR) or integrins ($\alpha_v\beta_3$).²³⁵

Peptide-based affinity probe consists of recognition part (for example peptidic inhibitor), linker and fluorescent tag. General scheme of this probe is shown in Figure 17.

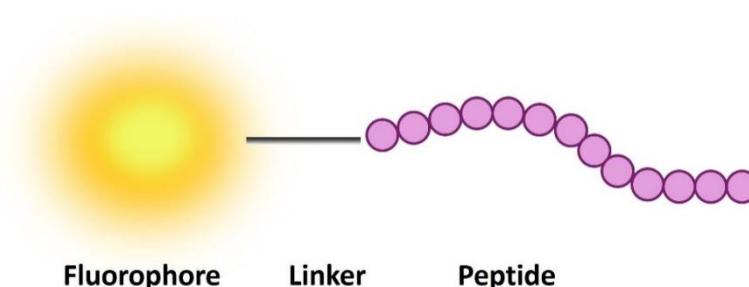


Figure 17. General scheme of peptide-based affinity probe.

Scientific goals of the study

MT1 and MT2 are closely related membrane-anchored serine proteases with distinct physiological and pathological functions. MT1 is broadly expressed in epithelial tissues, where its activity is tightly regulated by autoactivation and the endogenous inhibitors HAI-1 and HAI-2 to maintain epithelial homeostasis. Dysregulation of this balance results in excessive MT1 activity and has been implicated in the progression of multiple epithelial cancers. Elevated MT1 expression correlates with advanced tumor grade, disease stage, and poor prognosis, establishing MT1 as an attractive target for anticancer therapy. In contrast, MT2 is expressed predominantly in hepatocytes and is associated with iron homeostasis. Notably, MT2 expression shows an inverse correlation with cancer development and is considered a favorable prognostic marker, underscoring the need for selective targeting of MT1 without affecting MT2.

Furin is a ubiquitously expressed subtilisin-like serine protease belonging to the proprotein convertase family and is localized mainly to the trans-Golgi network and the cell surface. It is essential for the activation of numerous physiological substrates, including growth factors, hormones, and neuropeptides. Beyond its normal biological roles, furin contributes to cancer progression and facilitates bacterial and viral infections through the proteolytic activation of toxins and viral glycoproteins. Owing to its involvement in tumor growth and pathogen infectivity, furin represents an important molecular target for the development of anticancer and antiviral therapies.

The aim of this work was to design and obtain highly potent, selective, and non-covalent inhibitors of MT1 and furin that remain stable in proteolytic and reducing environments. Emphasis was placed on achieving selective inhibition of the cancer-associated protease MT1 while sparing MT2. Equally important task was to overcome the limitations of existing furin inhibitors, including poor selectivity and high cytotoxicity. The identified compounds are intended to serve both as tools for studying protease function (activity based probes) and as promising lead structures for the development of novel anticancer and antiviral agents.

Developing new MT1 inhibitors is particularly challenging, because of the high structural similarity between MT1 and MT2, which is reflected also in the limited number of publications reporting such compounds. Besides effectively reducing MT1 proteolytic activity, an ideal inhibitor should also exhibit high specificity (i.e. ability to discriminate between structurally related enzymes within the same family) and selectivity (the ability to distinguish between unrelated protease families). These properties minimize the risk of undesirable and potentially harmful off-target effects. This issue is especially pronounced for covalent peptide inhibitors containing highly reactive electrophilic warheads. The combination of high reactivity, inhibitory potency and, sometimes, irreversible mode of binding, may pose a serious threat for living

organism, caused by a non-specific interactions with various enzymes, proteins or even nucleic acids.

Although potent furin inhibitors have been reported, many are limited by critical shortcomings, such as poor selectivity or significant cytotoxicity. These limitations significantly hinder their therapeutic applicability. Given the crucial role of furin in cancer progression and pathogen infectivity, there is a strong demand for new compounds with improved selectivity and safety profiles. Such compounds could enable effective and broadly applicable strategies for anticancer and antiviral therapy.

The structural scaffold for the new MT1 inhibitors was derived from a truncated analogue of HV-BBI (Figure 18A), which exhibits exceptionally strong inhibitory activity against MT1 ($K_i = 8$ nM).¹⁸⁷ The furin inhibitors developed in this work were designed based on the inhibitor reported by Fittler *et. al.*¹⁸², whose structure is shown in Figure 18B.

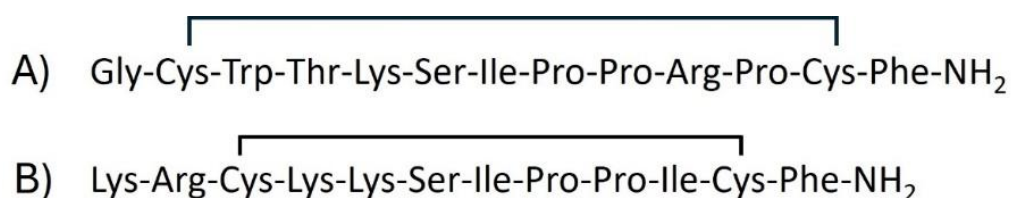


Figure 18. Starting structures used for the development of new MT1 (A) and furin (B) inhibitors.

All peptides synthesized for the purpose of this dissertation, together with their confirmed monoisotopic masses and reaction yields are presented in Table 2.

Table 2. Primary structures, calculated and founded masses, and final yields of obtained peptides.

N°	Sequence	Calculated [M+H] ⁺	Found [M+H] ⁺	Final yield [%]
MT1 inhibitors				
1	Cf-Lys-Arg-Cys(&)-Thr-Arg-NMeSer-Ile-Pro-Pro-Arg-Cys(&)-His-Pro-Asp	2037.906	2037.591	11.3
2	Cf-Lys-Arg-Cys(&)-Thr-Arg-Ser-Ile-Pro-Pro-Arg-Cys(&)-His-Pro-Asp	2021.874	2023.741	28.1
3	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1488.724	1488.789	32.4
4	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-DPhe-NH ₂	1488.724	1488.686	26.9
5	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Dpa-NH ₂	1564.755	1564.713	11.2
6	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Bpa-NH ₂	1592.750	1592.477	23.8
7	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe(4-tBu)-NH ₂	1544.786	1544.710	17.9
8	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe(4-F)-NH ₂	1506.714	1506.719	21.1
9	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe(4-Ph)-NH ₂	1564.755	1564.441	21.0
10	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NPhe-NH ₂	1488.724	1488.501	17.2
11	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe(4-NO ₂)-NH ₂	1533.709	1533.700	26.8
12	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Gly-NH ₂	1398.677	1398.778	23.5
13	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Trp-NH ₂	1527.734	1527.429	24.9
14	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Lys-NH ₂	1469.750	1470.131	23.0
15	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NMePhe-NH ₂	1502.739	1502.478	10.2
16	Phe-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1635.792	1635.480	28.6
17	Ala-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1502.739	1504.363	15.4
18	Gly-Cys(&)-Ala-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1373.681	1373.657	16.8
19	Gly-Cys(&)-Trp-Ala-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1458.713	1458.699	33.1
20	Gly-Cys(&)-Trp-Thr-Ala-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1431.666	1431.539	41.1
21	Gly-Cys(&)-Trp-Thr-Lys-Ala-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1472.729	1472.901	33.4
22	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ala-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1446.677	1447.640	27.0
23	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Ala-Pro-Cys(&)-Phe-NH ₂	1403.660	1403.816	34.7
24	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Ala-Cys(&)-Phe-NH ₂	1462.708	1462.564	48.4
25	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Ala-NH ₂	1412.692	1412.835	25.1
26	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NH ₂	1341.655	1341.588	22.3
27	Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1431.702	1431.643	43.4
28	Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NH ₂	1284.634	1284.575	42.9
29	Gly-Cys(&)-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1302.644	1302.626	28.1
30	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Cys(&)-Phe-NH ₂	1391.671	1391.643	32.5
31	Gly-Cys(&)-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Cys(&)-Phe-NH ₂	1205.592	1205.575	40.0

32	Gly-Cys(&)-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NH ₂	1155.576	1155.695	29.8
33	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Cys(&)-NH ₂	1244.602	1244.567	38.2
34	Gly-Cys(&)-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Cys(&)-NH ₂	1058.523	1058.532	24.7
35	Gly-Aha(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Pra(&)-Phe-NH ₂	1505.812	1505.824	18.0
36	Cf-Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂	1846.771	1848.880	24.3
MT2 inhibitor				
37	& ¹ Gly-Arg-Cys(& ²)-Thr-Arg-Ser-Ile-Pro-Pro-Ile-Cys(& ²)-Phe-Pro-Lys(Cf)& ¹	1912.851	1913.200	8.9
Furin inhibitors				
38	& ¹ Arg-Arg-Cys(& ²)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Cys(& ²)-Phe& ¹	1427.776	1427.766	24.0
39	Lys-Arg-Arg-Cys(&)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Cys(&)-Phe-NH ₂	1572.897	1572.898	32.3
40	& ¹ Lys-Arg-Arg-Cys(& ²)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Cys(& ²)-Phe& ¹	1555.871	1556.119	25.2
41	& ¹ Lys-Arg-Arg-Cys(& ²)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Cys(& ²)-Lys(Cf)& ¹	1894.945	1895.916	10.2
42	Arg-Arg-Arg-Cys(&)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Cys(&)-Phe-NH ₂	1600.904	1602.062	46.0
43	Cf-Arg-Arg-Arg-Cys(&)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Cys(&)-Phe-NH ₂	1958.951	1961.446	29.5
44	Arg-Arg-Arg-Aha(&)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Pra(&)-Phe-NH ₂	1617.992	1617.992	13.5
45	Arg-Arg-Arg-Sec(&)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Sec(&)-Phe-NH ₂	1696.793	1696.738	16.1
46	& ¹ Arg-Arg-Arg-Cys(& ²)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Cys(& ²)-Phe& ¹	1583.877	1584.096	29.3
47	& ¹ Arg-Arg-Arg-Cys(& ²)-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Cys(& ²)-Phe(4-NH-Cf)& ¹	1956.936	1958.923	9.4
48	Arg-Arg-Arg-Ala-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Ala-Phe-NH ₂	1538.975	1538.900	22.0
49	&Arg-Arg-Arg-Ala-Lys-Lys-Ser-Ile-Pro-Pro-Ile-Ala-Phe&	1521.949	1522.497	19.0

Cyclization is showed as “&” symbol according to Spengler *et. al.* nomenclature.²³⁶ Cf means 5(6)-carboxyfluorescein.

Isolated yield is a final yield of obtained peptides after purification.

In the subsequent sections of my dissertation, when discussing or referring to specific peptides, the numbering assigned to the inhibitors in Table 2 will be used.

Experimental section

4. Materials used in the synthesis and purification of inhibitors

4.1. Reagents and solvents

- *N,N*-Dimethylformamide (DMF), hexane, acetic acid (AcOH), diethyl ether, ascorbic acid were purchased from POCH, Poland.
- 1-methyl-2-pyrrolidone (NMP) was purchased from Scharlau, Spain.
- Methylene chloride (DCM), isopropanol (IsOH), methanol (MeOH) were purchased from STANLAB, Poland. DCM was dried over anhydrous K_2CO_3 .
- Acetonitrile (ACN) and MeOH for HPLC were purchased from VWR.
- *N,N,N',N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) were purchased from BioSynth, Slovakia.
- *N,N'*-Diisopropylcarbodiimide (DIC) was purchased from Iris Biotech, Germany.
- *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) were purchased from GLS, China.
- *N*-methylmorpholine (NMM), *N,N*-diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), trifluoroethanol (TFE), benzylamine, 2,2'-dithiobis(5-nitropyridine) (DTNP), dithiothreitol (DTT), triethylamine (TEA), 1,8-Diazabicyclo(5.4.0)undec-7-en (DBU), *o*-nitrobenzenesulfonyl chloride (*o*-NBS-Cl), 2-mercaptoethanol were purchased from Sigma-Aldrich, Germany.
- 5(6)-carboxyfluorescein (Cf) and dimethyl sulfate were purchased from ThermoScientific, USA.
- Bromoacetic acid and 4-dimethylaminopyridine (DMAP) were purchased from Acros Organics, USA.
- Triisopropylsilane (TIPS) was purchased from AmBeed, USA
- $CuSO_4 \cdot 5H_2O$ was purchased from Chempur, Poland.
- Thioanisole was purchased from Fluka, Germany.
- Bovine β -trypsin which was used for test described in Section 6.7.2.1.2. was purchased from Genaxxon bioscience, Germany.

4.2. Amino acids and derivatives

All amino acid derivatives that were used in the syntheses are presented in the Table 3.

Table 3. Amino acid derivatives which were used for syntheses.

Abbreviation	Amino acid	Protected derivative, which was used for the synthesis	Supplier
Aha	L-Azidohomoalanine	Fmoc-L-Aha	AmBeed, USA
Ala	L-Alanine	Fmoc-Ala	GLS, China
Arg	L-Arginine	Fmoc-Arg(Pbf)	Angene, UK
Asp	L-Aspartic acid	Fmoc-Asp(OtBu)	GLS, China
Bpa	Benzoyl-L-phenylalanine	Fmoc-Bpa	AmBeed, USA
Cys	L-Cysteine	Fmoc-Cys(Trt)	GLS, China
Dpa	3,3-diphenyl-L-alanine	Fmoc-Dpa	AmBeed, USA
D-Phe	D-Phenylalanine	Fmoc-D-Phe	GLS, China
Gly	Glycine	Fmoc-Gly	GLS, China
His	L-Histidine	Fmoc-His(Trt)	GLS, China
Ile	L-Isoleucine	Fmoc-Ile	GLS, China
Lys	L-Lysine	Fmoc-Lys(Boc)	GLS, China
Phe	L-Phenylalanine	Fmoc-Phe	GLS, China
Phe(4-F)	4-fluoro-L-phenylalanine	Fmoc-Phe(4-F)	AmBeed, USA
Phe(4-NH ₂)	4-amino-L-phenylalanine	Fmoc-Phe(4-NH ₂)	AmBeed, USA
Phe(4-NO ₂)	4-nitro-L-phenylalanine	Fmoc-Phe(4-NO ₂)	GLS, China
Phe(4-Ph)	4-phenyl-L-phenylalanine	Fmoc-Phe(4-Ph)	Fluorochem, UK
PheTrt	4-tert-butyl-L-phenylalanine	Fmoc-Phe(4-tBu)	Fluorochem, UK
Pra	L-Propargylglycine	Fmoc-Pra	AmBeed, USA
Pro	L-Proline	Fmoc-Pro	GLS, China
Sec	L-Selenocysteine	Fmoc-Sec(Mob)	Angene, UK
Ser	L-Serine	Fmoc-Ser(tBu)	Iris Biotech, Germany
Thr	L-Threonine	Fmoc-Thr(tBu)	GLS, China
Trp	L-Tryptophan	Fmoc-Trp(Boc)	GLS, China

4.3. Resins

Bicyclic peptides were synthesized on 2-chlorotrityl chloride resin (loading of Cl atoms: 1.46 mmol/g, GL Biochem Shanghai). Peptides with amide group at the C-termini were synthesized on Rink amide methylbenzhydrylamine resin (Fmoc-MBHA, GL Biochem, Shanghai; loading: 0.646 mmol/g).

5. Analytical methods

The progress of cyclization and purity of obtained compounds were examined by RP-HPLC. Almost all analyses were performed using Shimadzu Prominence-I LC-2050C 3D equipped with a Kinetex XB-C18 (150 × 4.6 mm, 5 μm) column or Dr. Maisch ReproSil-Pur Basic-C18 (150 × 4.6 mm, 5 μm) column and UV-Vis detector (detection at 214 nm, 30°C and/or 50°C for selected furin inhibitors). Attachment of the 5(6)-carboxyfluorescein (Cf) to the Fmoc-Lys (described in detail in Section 6.9.2.1.) was monitored using Varian with ProStar System equipped with Kinetex XB-C18 (150 × 4.6 mm, 5 μm) column, detection at 226 nm. For both HPLC sets flow rate was 1 ml/min, linear gradient 10-90% phase B for 20 or 40 minutes. Eluent A was 0.1% TFA in H₂O and eluent B was 0.1% TFA in 80% ACN. Crude peptides and other compounds were dissolved in H₂O/ACN, filtrated through 0.45 μm regenerated cellulose filters, prior to injection. Purified peptides were dissolved in H₂O and subjected for analyses.

The mass spectrometry (MS) analyses were carried out using MALDI MS (Autoflex maX MALDI-TOF spectrometer, Bruker Daltonics, Germany) using 2,5-dihydroxybenzoic acid matrix (DHB) for all compounds except peptide **11**, which was analyzed using HCT Ultra spectrometer (Bruker Daltonics, Germany).

During optimization of the *N*-methylation method, one-dimensional ¹H and ¹³C NMR spectra were recorded (Bruker Avance III 500 MHz under the frequencies of 500 MHz (¹H) and 126 MHz (¹³C), respectively, in CD₃OD at room temperature). The spectra were processed and analyzed by dr Mladena Glavas from Department of Organic Chemistry and Biochemistry, Ruder Boskovic Institute using MestReNova version 0.2-5475, Mestrelab Research S,L; 2009 software.

MS and NMR analyses were carried out by staff members of Physical-Chemical Measurements Section of Faculty of Chemistry University of Gdańsk.

6. Methodology of peptide synthesis

All peptides were synthesized according to the solid phase peptide synthesis (SPPS) protocol using Fmoc chemistry. Peptides were synthesized manually and using automatic (Prelude Peptide Synthesizer, Gyros Protein Technology Inc., USA) or semi-automatic synthesizers (Biotage

Initiator+SP Wave synthesizer, Biotage, Sweden). In case of peptides with backbone cyclization, the first amino acid was attached to the resin, according to the procedure described in Section 6.1. Peptides chains were elongated in consecutive cycles of deprotection (the removal of Fmoc) and coupling (elongation of peptide4 chain).²³⁷

Before synthesis resin was swelled in *N,N*-dimethylformamide (DMF). For deprotection 20% solution of piperidine in DMF (v/v) was used. For coupling two types of coupling reagents were used: *N,N,N',N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) or *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt), which were dissolved in DMF with base (*N,N*-diisopropylethylamine, DIPEA or *N*-methylmorpholine, NMM) and Fmoc-AA in molar ratio:

- HBTU/ HOBt/ base /Fmoc-AA, molar ratio 1:1:2:1
- HATU/ HOAt/ base/ Fmoc-AA, molar ratio 1:1:2:1

Every amino acid derivative was coupled twice, first with 3 equivalent in relation to the resin loading and the second time with 1.5 equivalent. After deprotection or coupling cycle, resin was washed and the progress of these steps was monitored by the Kaiser and/or chloranil tests, described in detail in Section 6.5.

Depending on the synthesis method (manual or automatic), different reaction times, coupling reagents, bases and solvents for washing the resin were used, as described in the following chapters.

6.1. Attachment of the C-terminal Fmoc-amino acid derivative to the 2-chlorotirityl resin

The first Fmoc-protected amino acid (2 mmol) was attached to the resin (1 g) in the presence of an equimolar amount of DIPEA in anhydrous dichloromethane (DCM) for 2 h and 45 min. After this time MeOH was added to cap any remaining reactive chloride groups and mixed for 15 min. Then, the solvent was removed and the resin was washed on the Shott funnel with methanol (MeOH) and DCM.⁹⁶

The resin was dried on the air (overnight) and then the resin loading was determined. About 10 mg of Fmoc-amino acid resin was suspended in 1.3 ml of 30% piperidine in DMF for 30 min. After this time, absolute ethyl alcohol was added to obtain 50 ml mixture. The absorbance of dibenzofulvene-piperidine adduct was measured at 300 nm (4-5 measurements) and the resin loading was calculated with the following equation:

$$Loading = \frac{A \times V}{\epsilon \times m \times l} \left[\frac{mmol}{g} \right]$$

A – absorbance at 300 nm wavelength (average of at least 4 measurements)

V – volume of the prepared solution [cm³]

ε – extinction coefficient at 300 nm (6402 mM⁻¹ × cm⁻¹)

m – exact weight of the resin [g]

l – the path length of the sample (width of the cuvette) [cm]

Amino acid derivatives which were attached to the resin were Fmoc-Cys(Trt), Fmoc-Asp(OtBu) and Fmoc-Phe. Example calculation of the resin loading is shown below (for the resin with Fmoc-Phe attached):

$$A_1 = 0.880$$

$$A_2 = 0.885$$

$$A_3 = 0.884$$

$$A_4 = 0.882$$

$$A_{\text{average}} = 0.883$$

$$Loading = \frac{0.883 \times 50}{6402 \times 0.01005 \times 1} = \mathbf{0.686 \frac{mmol}{g}}$$

$$m = 10.05 \text{ mg} = 0.01005 \text{ g}$$

6.2. Solid phase peptide synthesis – manual synthesis

All manual syntheses were performed in polypropylene syringes, equipped with a filter, in the ultrasonic bath (Polsonic Sonic 2 with ultrasonic power (peak/period) of 2×100W and ultrasonic frequency of 40 kHz), according to procedure described by Wołczański *et al.*²³⁸ The deprotection was carried out for 3 min and repeated for 5 min. The coupling reaction was carried out for 15 min and it was repeated twice. The coupling mixtures were:

- HBTU/ HOBt/ DIPEA /Fmoc-AA, molar ratio 1:1:2:1, for the first coupling;
- HATU/ HOAt/ DIPEA /Fmoc-AA, molar ratio 1:1:2:1, for the second coupling.

After each coupling and deprotection the resin was washed with DMF (3 × 1 min), isopropanol (IsOH, 3 × 1 min) and DCM (3 × 1 min).

6.3. Solid phase peptide synthesis using automatic synthesizer

All syntheses performed using automatic synthesizer were carried out using Prelude Peptide Synthesizer (Gyros Protein Technology Inc., USA). The deprotection was carried out for 4 minutes and it was repeated twice. The coupling reaction was carried out for 1 h and it was repeated twice. The coupling mixture was:

- HBTU/ HOBt/ NMM /Fmoc-AA, molar ratio 1:1:2:1

After each coupling and deprotection the resin was washed with DMF (5×1 min).

6.4. Solid phase peptide synthesis using semi-automatic synthesizer

All syntheses performed using semi-automatic synthesizer were carried out using microwaves synthesizer (Biotage Initiator+SP Wave synthesizer, Biotage, Sweden) in dedicated polypropylene, open reactor vessel, with polytetrafluoroethylene (PTFE) frit (Biotage, Sweden) at 40°C and with rotational frequency 900 RPM. The deprotection was carried out for 4 min and repeated for 8 min (40°C, 900 RPM).

The coupling mixtures were:

- HBTU/ HOBt/ DIPEA /Fmoc-AA, molar ratio 1:1:2:1, for the first coupling;
- HATU/ HOAt/ DIPEA /Fmoc-AA, molar ratio 1:1:2:1, for the second coupling.

After each coupling and deprotection the resin was washed with DMF (4×1 min).

6.5. Monitoring of peptide coupling and deprotection reactions

The coupling and deprotection steps were monitored using two colorimetric tests: the Kaiser test and the chloranil test. Both methods detect free amino groups.

Kaiser test, also known as the ninhydrin test, relies on the reaction of ninhydrin with free primary amines. For this purpose three solutions were prepared: ninhydrin (5% in ethanol), phenol (80% in ethanol), KCN in H₂O diluted with pyridine (2 ml 0.001 M KCN solution diluted to 100 ml with pyridine). A small amount of washed peptidyl resin (several resin beads) was placed in a glass tube, followed by the addition of 20 µl of each solution. The tube was then heated at 100°C for 5 minutes. The resin beads turned dark blue in the presence of primary amines, confirming deprotection. A slight blue coloration of the beads after coupling indicated the need for a second coupling step. The Kaiser test does not produce the characteristic dark blue color with residues such as Ser, Asp, Asn and Pro.²³⁹

Chloranil test relies on the reaction of amino groups with 2,3,5,6-tetrachloro-1,4-benzoquinone (chloranil) in the presence of acetaldehyde or acetone. Several resin beads were placed in a glass tube, followed by the addition of 50 μ l of a saturated solution of chloranil in toluene and 200 μ l of acetaldehyde (for the detection of primary amino groups) or 200 μ l of acetone (for the detection of secondary amino groups). The presence of free amino groups resulted in the formation of green color (primary amino groups) or an orange color (secondary amino groups).^{240,241}

6.6. Cleavage of peptide from solid support

After completed synthesis, peptides were cleaved from the resin. Monocyclic peptides were cleaved with simultaneous removal of protecting groups from side chains of amino acids using the mixture of TFA/ phenol/ H₂O/ triisopropylsilane (TIPS) (88:5:2:5, v/v/v/v) and 3 h agitation. Then they were precipitated in ice-cold diethyl ether. The precipitate was centrifuged, dissolved in water and lyophilized. Peptides with backbone cyclization were cleaved from the resin by 2 h agitation with a mixture of acetic acid/2,2,2-trifluoroethanol/DCM (2:2:6, v/v/v). This cocktail enables preservation of side-chain protecting groups. After that time, resin was removed during filtration. The solution was vacuum evaporated with addition of hexane to remove AcOH. Obtained oily product was suspended in water and lyophilized.^{96,242}

6.7. Synthesis of cyclic peptides

6.7.1. Formation of disulfide bridge

After cleaving from the resin, unprotected, linear peptides were subjected to disulfide bonds formation in solution under highly diluted conditions to prevent formation of dimers and promote intramolecular cyclization. Crude peptides, were dissolved in MeOH and AcOH (0.1 mmol of peptide was dissolved in 1 l of MeOH and 30 ml of AcOH). Mixture was stirred and 0.1 M iodine in MeOH was added dropwise until a light yellow color emerged. After completing the oxidation, ion exchange resin Dowex (Type 1 $\times$ 4, Mesh size: 50-100, counter ion COO⁻) was added to remove excess of the iodine. After the yellow color disappears resin was removed by filtration, while the solution was concentrated by vacuum distillation. Cyclic peptides were then dissolved in water and lyophilized.^{97,243}

The oxidation efficiency was monitored by HPLC and MS analyses. Monoisotopic masses of reduced peptides are approximately 2 Da higher compared to the oxidized compounds. Example of reduced and oxidized peptide HPLC chromatograms and MS spectra are shown on Figure 19.

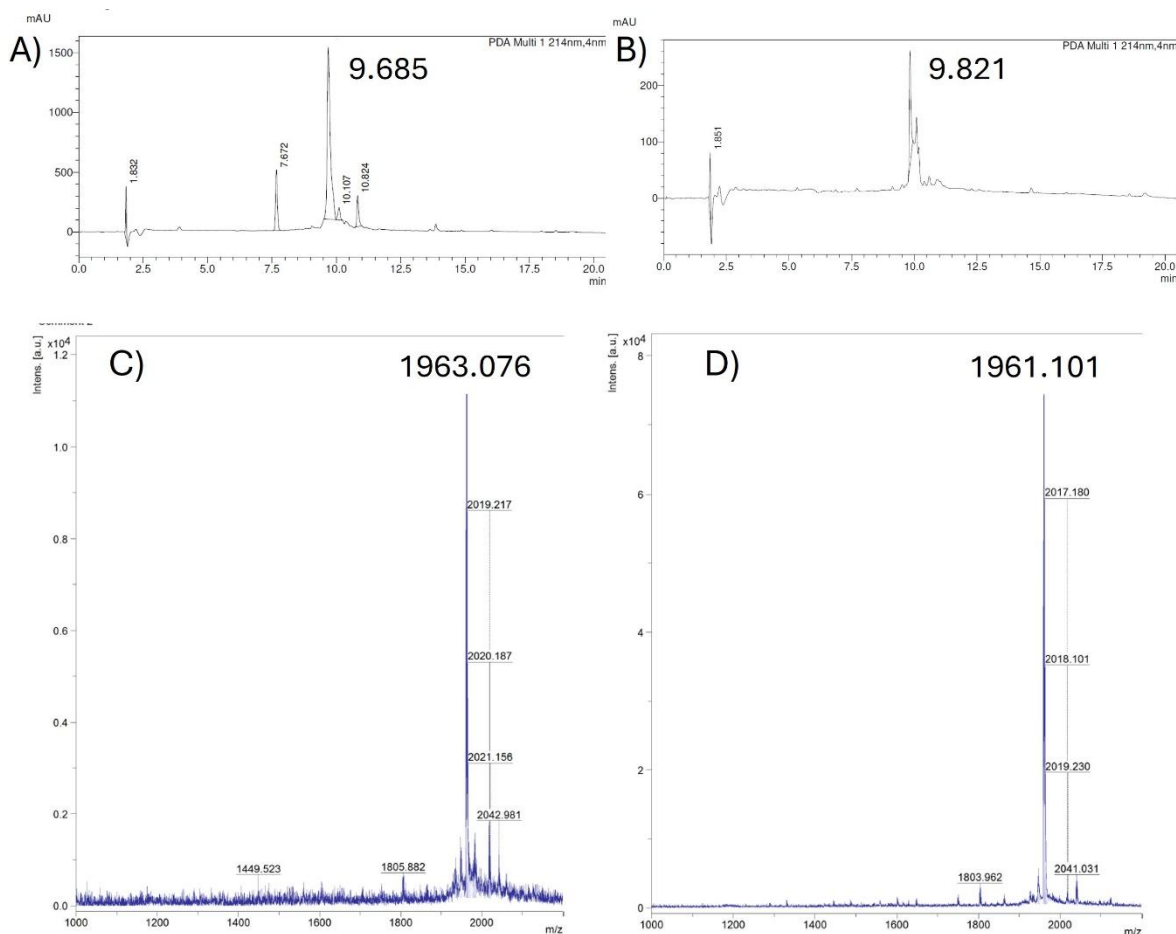


Figure 19. HPLC chromatograms of reduced (A) and oxidized (B) peptide 43. MS analysis of reduced (C) and oxidized (D) peptide 43.

$M_{\text{reduced}} = 1959.960$; Calculated $[M+H]^+ = 1960.967$; Found $[M+H]^+ = 1963.076$.

$M_{\text{oxidized}} = 1957.944$; calculated $[M+H]^+ = 1958.951$; Found $[M+H]^+ = 1961.101$.

Shimadzu Prominence-I HPLC equipped with a Kinetex column; 10-90% B in 20 min.

6.7.2. Formation of the triazole bridge applying “click” chemistry

Both peptides with the triazole bridge (**35** and **44**) were synthesized according to standard procedure described in Section 6. Instead of Cys residues, azidohomoalanine (Aha, positioned closer to the *N*-terminus) and proparglycine (Pra, positioned closer to the *C*-terminus) were introduced. This modification enabled to formation of a triazole bridge, following the procedure described below. The same protocol was applied to peptides **35** and **44**, however detailed amounts of reagents, chromatograms and MS analyses refer to peptide **44**. Analyses of peptide **35** are shown in Figure 24.

5mg (0.003 mmol) of pure, unprotected linear peptide was dissolved in 3 ml of mixture of H₂O/ *tert*-butanol (2:1, v:v). Then 10.8 mg of CuSO₄ · 5H₂O (14 eq., 0.099 mmol) and 7.1 mg of ascorbic acid (13 eq., 0.092 mmol) were dissolved in 0.9 ml H₂O. These reagents were mixed together to reduced Cu²⁺ to Cu⁺, while the ascorbic acid (Figure 20A, retention time (*t*_R) = 2.064 min) was reduced to dehydroascorbic acid (Figure 20B, *t*_R = 5.209 min).

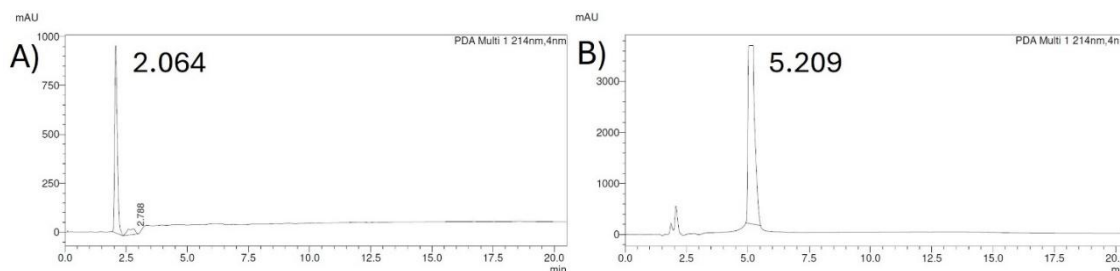


Figure 20. HPLC chromatograms of ascorbic acid (A) and dehydroascorbic acid (B). Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 20 min.

The mixture was added to the dissolved peptide. Reaction was carried out 24 h and then solvents were removed under reduced pressure. Crude product was dissolved in water and lyophilized.²⁴⁴ HPLC chromatograms of pure linear and cyclic forms of peptide **44** are shown in Figure 21. Their retention times are almost the same. For linear peptide it is *t*_R = 9.024 min (Figure 21A) and for cyclic peptide it is *t*_R = 9.098 min (Figure 21B). Such a small difference in retention time may result from analytical variability rather than from the analysis of different compounds. Therefore, additional confirmation was required, and an HPLC analysis of the mixture of linear and cyclic peptides was performed (Figure 21C) in longer gradient method (10-90% B in 40 min). Obtained chromatogram indicated two peaks which confirmed presence of two different compounds.

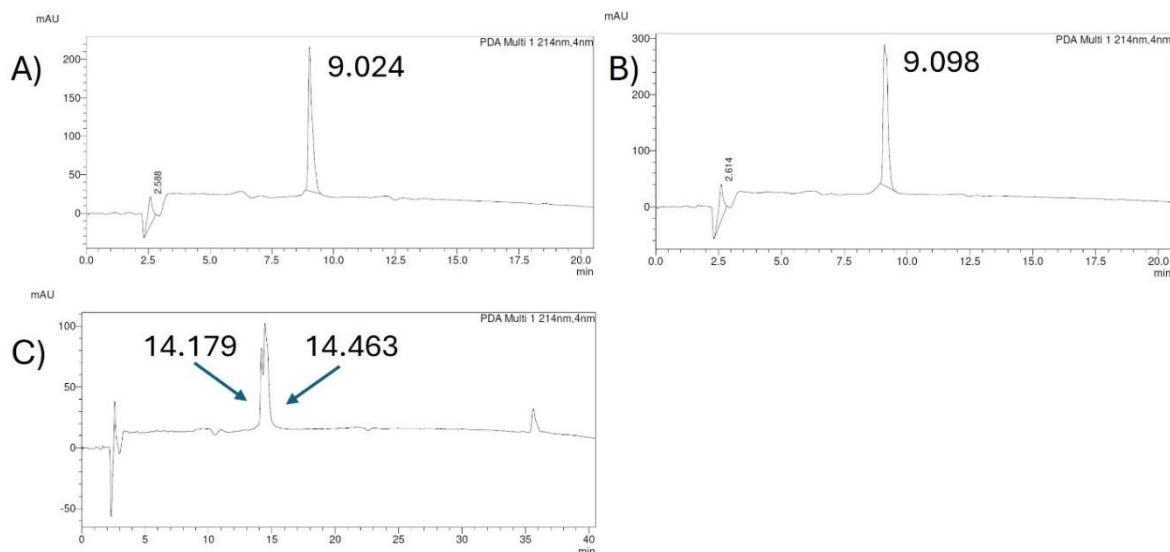


Figure 21. HPLC chromatograms of: linear (A), cyclic (B) peptide 44 and their co-injection (C). Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; Chromatograms A and B were recorded in method 10-90% B in 20 min, chromatogram C was recorded in method 10-90% B in 40 min.

The reaction was monitored by HPLC (Figure 22). After 15 min from start of the reaction a significant signal from dehydroascorbic acid appeared (Figure 22A, $t_R = 5.224$ min). However, after 1 h the signal decreased noticeably (Figure 22B, $t_R = 5.288$ min) and a peak from peptide increased (Figure 22B, $t_R = 8.935$ min). Chromatogram of the mixture after 24 h (Figure 22C) did not differ from that after 1 h. As it was mentioned before, retention times of linear and cyclic peptides are almost the same. Furthermore, monoisotopic masses of these compounds are the same (Figure 23). Therefore reaction was carried out 24 h to ensure that the triazole bridge was formed with satisfying efficiency.

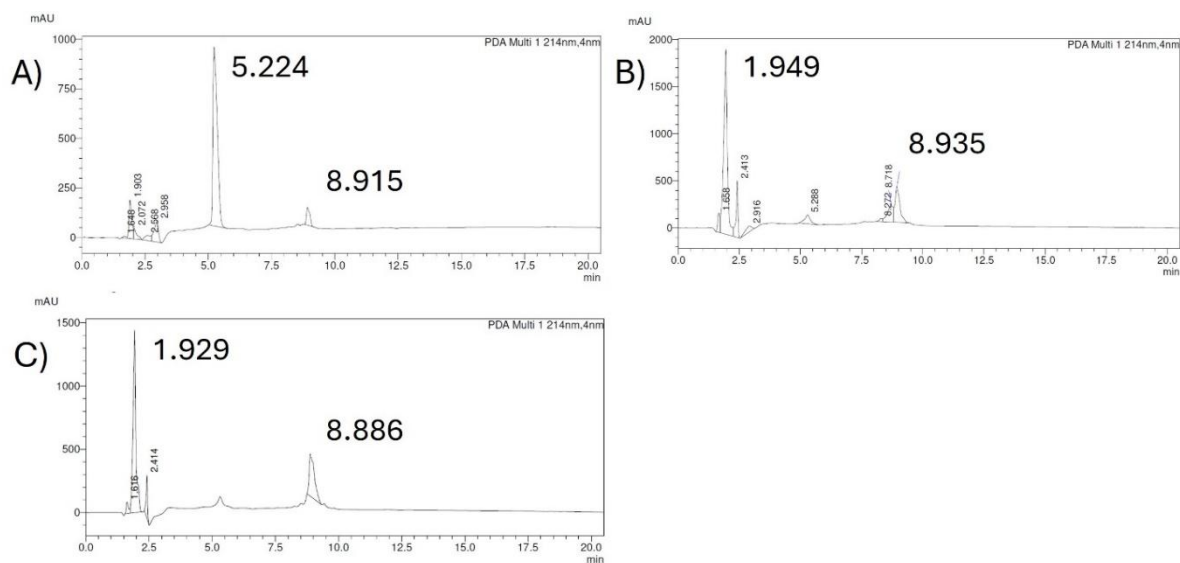


Figure 22. HPLC chromatograms of reaction mixture during synthesis of peptide 44 after: 15 min (A); 1 h (B); 24 h (C) of reaction.

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 20 min.

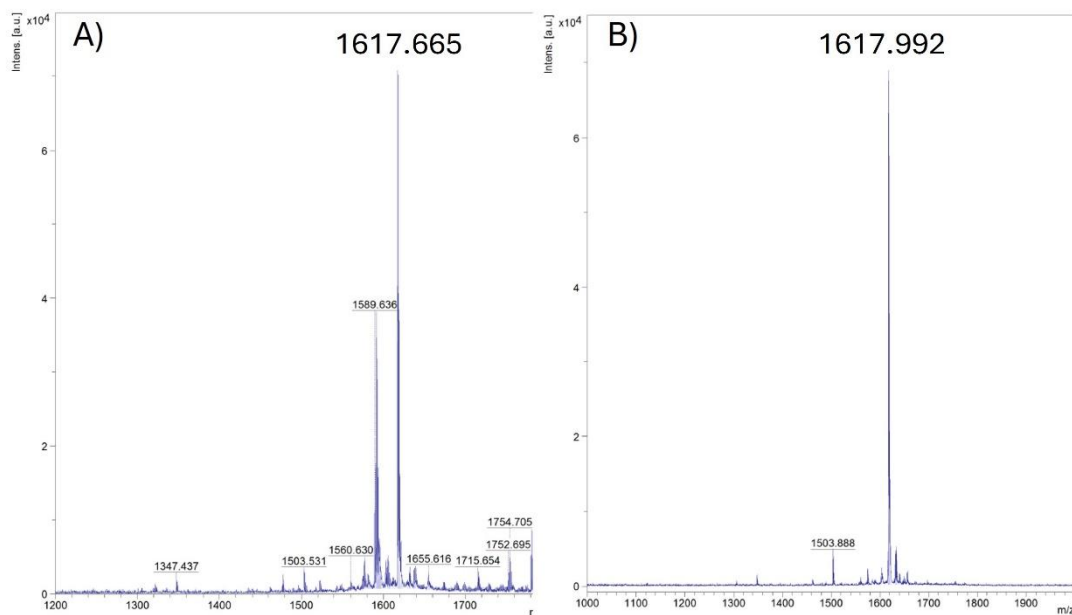


Figure 23. MS analyses of linear (A) and cyclic (B) peptide 44.

For both peptides: $M = 1616.985$; Calculated $[M+H]^+ = 1617.992$.

Found $[M+H]^+ = 1617.665$ (linear peptide); Found $[M+H]^+ = 1617.992$ (cyclic peptide).

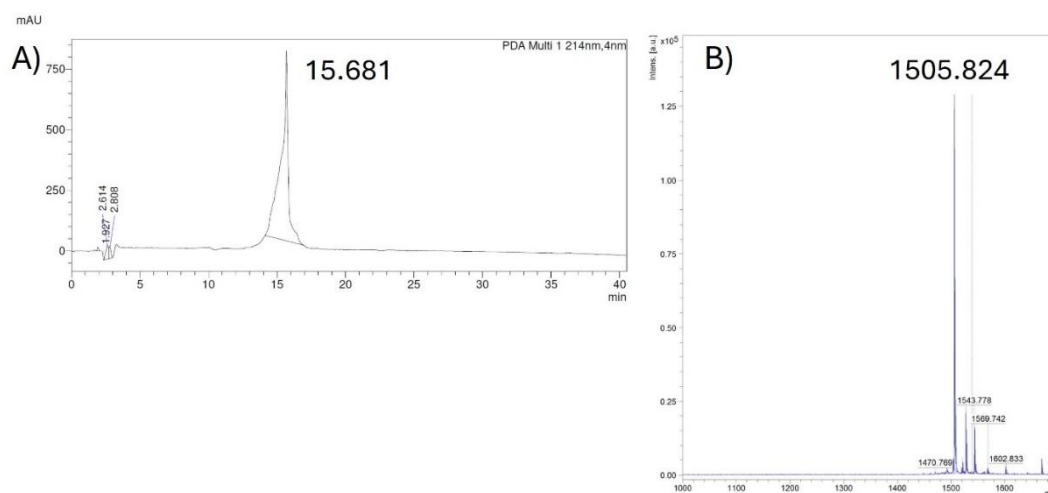


Figure 24. HPLC chromatogram (A) and MS analysis (B) of peptide 35.

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 20 min.

M = 1504.805; Calculated $[M+H]^+ = 1505.812$; Found $[M+H]^+ = 1505.824$.

6.7.2.1. Confirmation of the presence of triazole bridge

As it was mentioned in Section 6.7.2. peptides with triazole bridge exhibit the same monoisotopic mass as their linear analogues (with azidohomoalanine and propargylglycine in the structure). Furthermore, their retention times are so similar that distinguishing between their chromatographic signals is not feasible. Consequently, it is not possible to reliably assess the success of cyclization using either MS or HPLC. Verification whether the applied cyclization conditions resulted in effective triazole bond formation required additional testing. Two methods were employed for this purpose: test with DTT²⁴⁵ and trypsin digestion.¹⁸⁵ Since peptide **44** was synthesized before peptide **35**, the above-mentioned tests were performed on peptide **44**.

6.7.2.1.1. Test with DTT

In the linear peptide, residual azide groups are expected to be reduced to amine groups upon reaction with a reducing agent such as dithiothreitol (DTT). This transformation can be detected using HPLC and MS analyses, as it results in changes in both monoisotopic mass and retention time. In contrast, DTT should have no effect on the cyclic peptide, where the azide group is involved in triazole bond formation. For this purpose, I applied the method described by Zhang *et al.*²⁴⁶ who reduced poly(ethylene glycol) azide with DTT.

First, 2.2 mg (0.00136 mmol) of cyclic peptide, 0.25 mg (0.00163 mmol) of DTT and 0.38 μ l (0.00272 mmol) of TEA were dissolved in 500 μ l H₂O, then 5 μ l of 10% solution of NaOH was added to obtain pH 9-10. The mixture was shaken for 3 hours. Linear peptide with DTT and TEA were dissolved in analogical manner and molar ratio and with addition of NaOH. In both cases HPLC and MS analyses were performed.

The HPLC analyses of mixture of cyclic peptide with DTT showed no changes, regardless of the reaction time. (Figure 25A). Also MS analysis (Figure 25C) confirmed only the mass of peptide **44** ($M = 1616.985$), and the mass corresponding to the peptide with azide group reduced to an amine ($M = 1590.995$), was not found.

In case of the reaction of the linear peptide with DTT, there is a noticeable new signal with retention time slightly lower than corresponding to the linear compound ($t_R = 13.756$ min). It appeared at the beginning of the reaction (Figure 25B, $t_R = 13.376$ min) and increased as the reaction progressed. MS analysis of this specific signal (Figure 25D) confirmed that it corresponds to the peptide in which the azide group has been reduced to an amine.

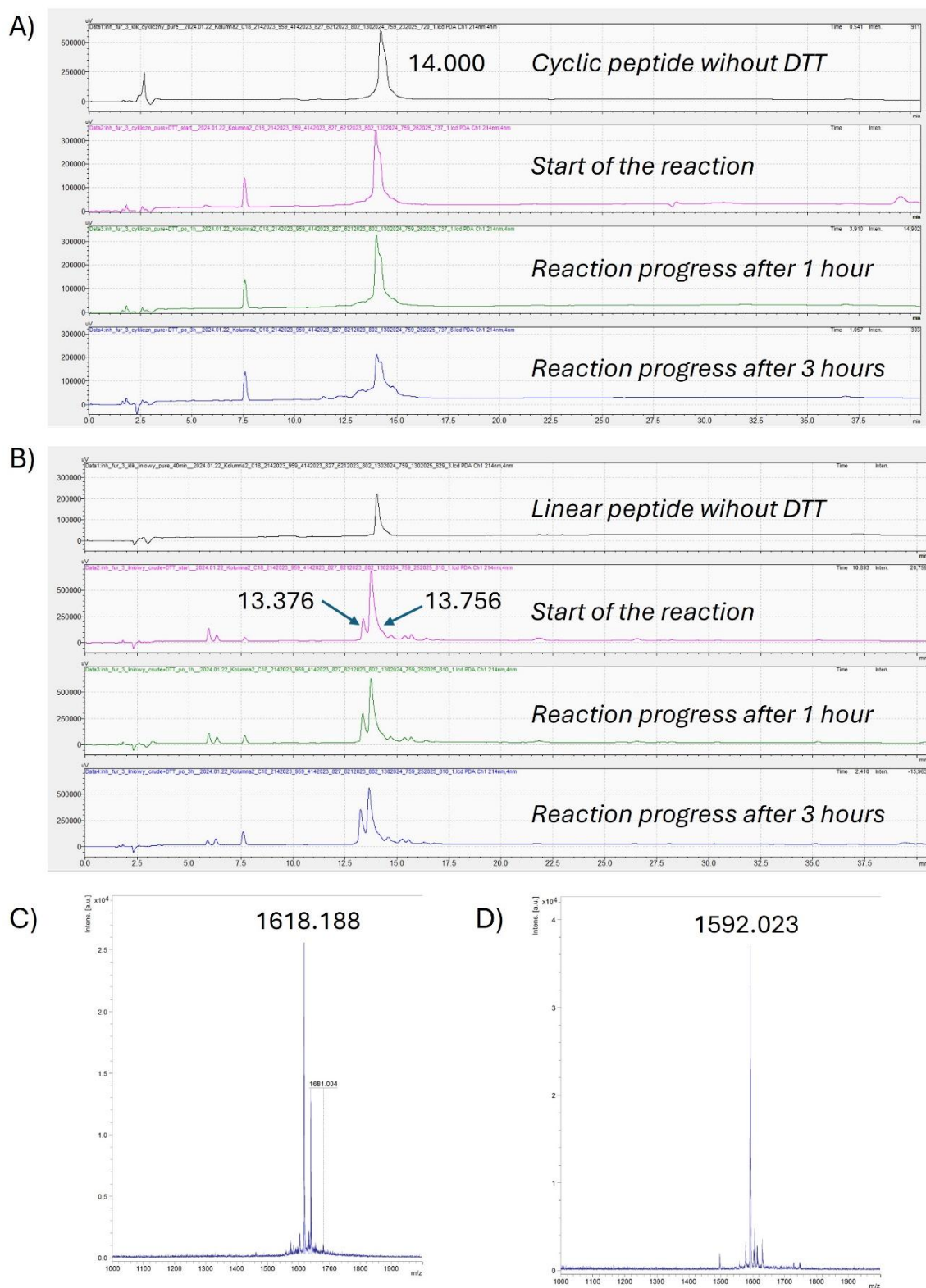


Figure 25. Monitoring progress of reaction of cyclic peptide (A) and linear peptide (B) using HPLC. MS analyses of cyclic (C) and linear (D) peptide with DTT.

Peptide **44**: M = 1616.985; Calculated $[M+H]^+$ = 1617.992; Found $[M+H]^+$ = 1618.188.

Linear analogue of peptide **44** with reduced azide group to amine group: M = 1590.995; Calculated $[M+H]^+$ = 1592.002; Found $[M+H]^+$ = 1592.023.

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 40 min.

6.7.2.1.2. Incubation with trypsin

An additional method used to confirm successful formation of triazole bridge was incubation of peptide **44** with trypsin. The formation of the bridge would be confirmed by the detection, in MS analyses, of a monoisotopic mass corresponding to a peptide with hydrolyzed peptide bond or bonds, but with a noticeable mass of a linear peptide connected by a triazole bridge. In contrast, if the “click” reaction had failed and the peptide remained linear without the triazole linkage, we expected to observe only short peptide fragments in the MS spectra. For this purpose, 0.6 mg of peptide **44** (0.000371 mmol) was dissolved in 937 μ l of 50 mM Tris-Cl buffer (pH 8.3) containing 20 mM CaCl_2 and 0.005% Triton X-100. The 63 μ l of solution of bovine β -trypsin in the buffer (3.0×10^{-3} M) was added. Samples were taken and the reaction was quenched using AcOH at following time points: the start of the reaction and after 15, 30, 60, 120 and 180 min. Then HPLC and MS analyses were performed.¹⁸⁵

MS analysis of peptide **44** dissolved in buffer, without trypsin, is shown in Figure 26A. After the addition of the enzyme significant decomposition of the peptide was noticeable and just at the beginning of the reaction monoisotopic mass from hydrolyzed peptide was observed (Figure 26B). That immediate proteolysis is also confirmed by HPLC analysis (Figure 26C). MS analysis indicated on the adduct with K^+ (calculated $[\text{M}+\text{K}]^+ = 1205.655$; found $[\text{M}+\text{K}]^+ = 1205.675$) corresponding to the peptide without three Arg residues and with hydrolyzed peptide bond between amino acids in binding loop (probably between Lys and Ser residues). Structure of peptide **44** and observed scissile sites are marked schematically in Figure 26D.

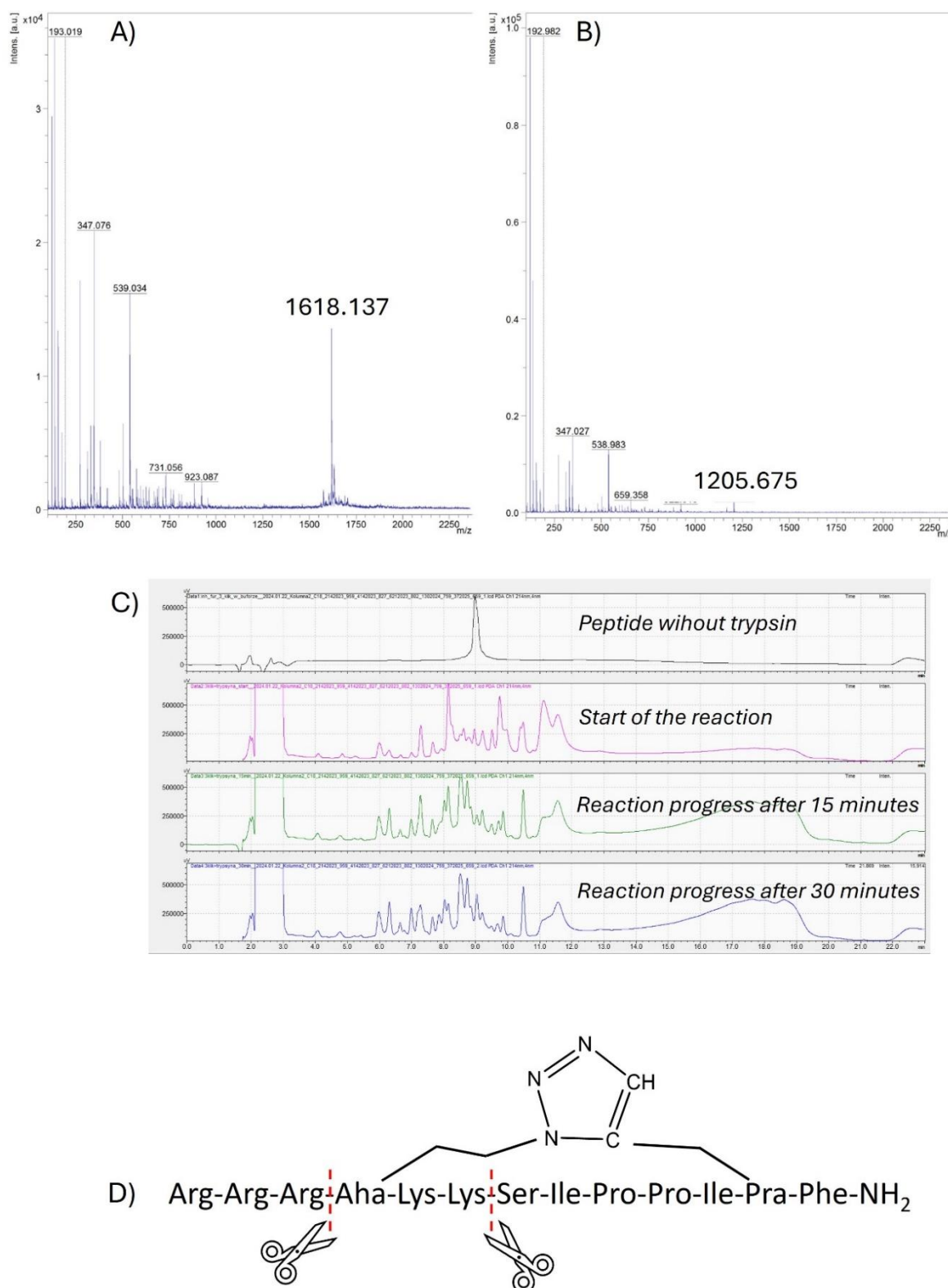


Figure 26. MS analyses of peptide 44 dissolved in buffer (A) and after addition of trypsin (B). Progress of hydrolysis showed using HPLC (C). Structure of inhibitor 44 with schematically marked observed scissile sites by red crossed lines and scissors icons (D).

Peptide **44**: M = 1616.985; Calculated $[M+H]^+$ = 1617.992; Found $[M+H]^+$ = 1618.137.

Hydrolyzed peptide **44** (without three Arg residues and with hydrolyzed peptide bond inside binding loop): M = 1166.692; Calculated $[M+K]^+$ = 1205.655; Found $[M+H]^+$ = 1205.675.

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 20 min.

6.7.3. Formation of the diselenide bridge

Among the synthesized peptides, diselenide bridge was present only in peptide **45**. This particular peptide was synthesized by dr hab. Dawid Dębowski, prof. UG from Department of Molecular Biochemistry, Faculty of Chemistry, University of Gdańsk. However, this peptide was purified by me using semipreparative HPLC and included in my research topic.

Peptide was synthesized according to the methodology described in Section 6.3, however the cleavage from the resin was performed differently. Selenocysteine (Sec) derivatives side chain was protected using *p*-methoxybenzyl (Mob) groups. The peptide was cleaved from the resin with simultaneous removal of protecting groups and formation of diselenide bridge. For this purpose mixture of TFA/ TIPS/ thioanisole/ water (90:7.5:2.5:5, v/v/v/v) with 1.3 eq. 2,2'-dithiobis(5-nitropyridine) (DTNP) was used.²⁴⁷ The peptidyl resin was agitated with this mixture for 3 hours and filtrated on Schott funnel. The peptide was precipitated with ice cold diethyl ether and centrifuged. Then the peptide was dissolved in water and lyophilized. HPLC chromatograms of crude and purified peptide **45** and MS analysis are shown in Figure 27.

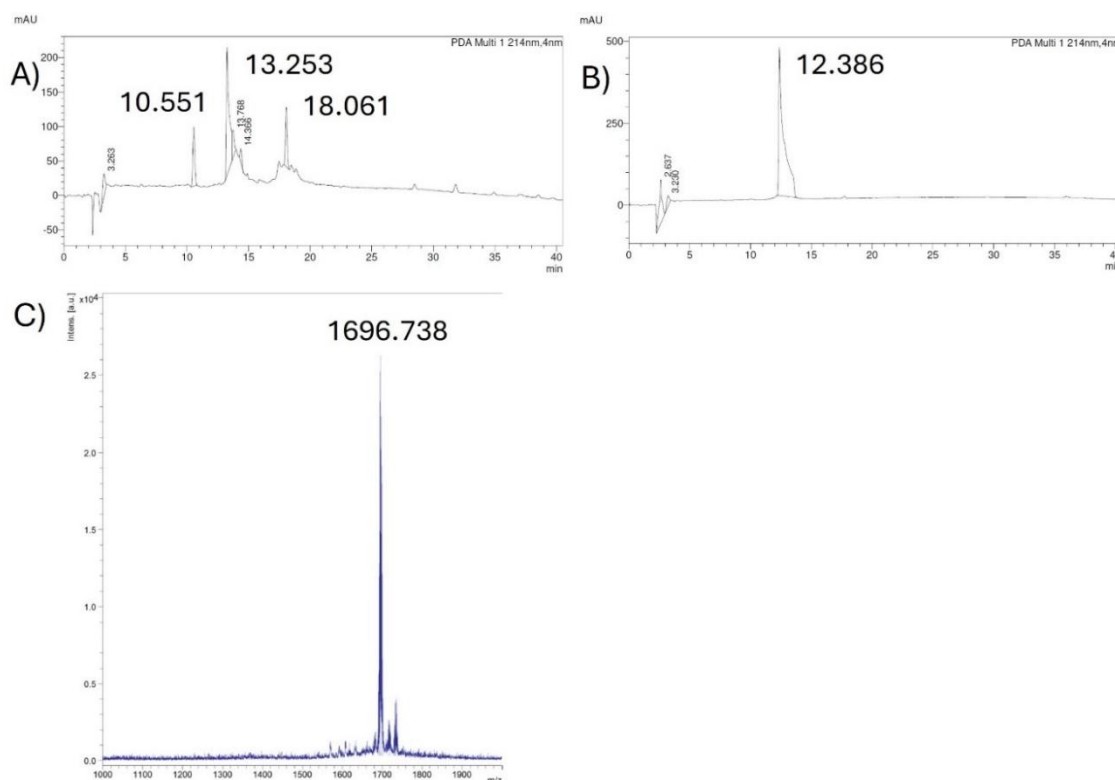


Figure 27. HPLC chromatograms of crude (A) and pure (B) peptide 45. MS analysis of pure peptide 45 (C).

M = 1695.786; Calculated $[M+H]^+$ = 1696.793; Found $[M+H]^+$ = 1696.738.

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 40 min.

6.7.4. Backbone cyclization

Peptides with cyclic backbone were first cleaved from the resin preserving side-chain protecting groups, according to procedure described in Section 6.6. Peptides were cyclized in a presence of PyBOP and DIPEA in molar ratio: peptide/ benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)/ DIPEA (1:1.2:2.4).^{97,248}

PyBOP was dissolved in DMF (65 ml of solvent per 100 mg of protected peptide). Then DIPEA was added and mixture was cooled in ice-bath to 0°C. Protected peptide, with free *N*- and *C*-termini, was dissolved in DMF (100 mg of protected peptide in 6 ml DMF). Afterwards, the dissolved peptide was dropped into stirring solution of PyBOP and DIPEA in DMF. The mixture was stirred for 48 h in room temperature. After backbone cyclization, the DMF was vacuum evaporated and protecting groups were removed using mixture of TFA/ H₂O/ phenol/ TIPS (88:5:5:2, v/v/v/v) as described in Section 6.6.

Result of backbone cyclization was monitored by HPLC and MS analyses. An example of HPLC chromatogram and MS spectra of peptide with cyclic backbone is shown in Figure 28.

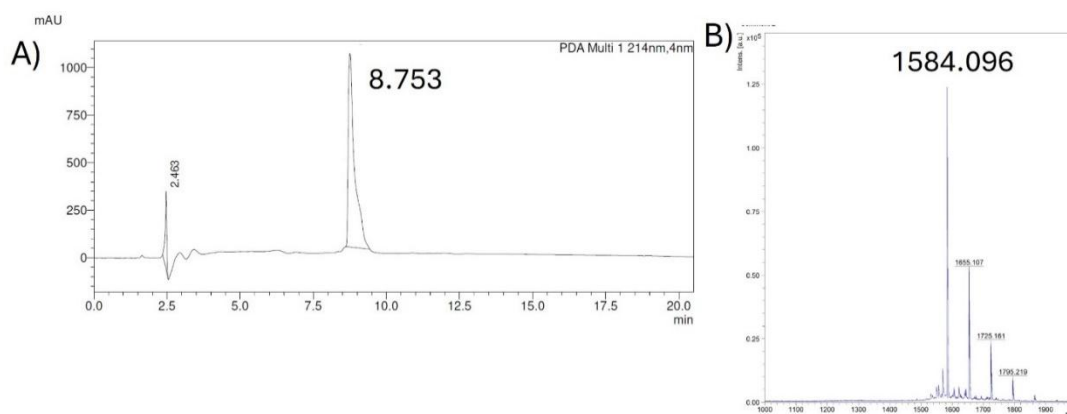


Figure 28. HPLC chromatogram (A) and MS analysis (B) of peptide 46.

M = 1582.870; calculated $[M+H]^+ = 1583.877$; Found $[M+H]^+ = 1584.096$.

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 20 min.

6.8. Synthesis of peptomer

The only peptomer synthesized during my research is peptide **10**. *N*-benzylglycine (NPhe) monomer was introduced (as the *C*-terminal residue) in two-steps approach. First, 89.76 mg of bromoacetic acid (5 eq., 0.646 mmol) and 121.5 μ l of *N,N'*-diisopropylcarbodiimide (DIC) (6 eq., 0.775 mmol) were dissolved in 4 ml of DMF and preactivated for 10 minutes. Then the

mixture was added to the 0.2 g of RINK amide MBHA resin (loading: 0.646 mmol/g) with free α -amino groups and shaken for 30 min. The procedure was repeated twice and then the resin was washed three times with DMF. Successful coupling was confirmed by chloranil and Kaiser tests. In the second step 282.0 μ l of benzylamine (20 eq., 2.584 mmol) was diluted in 4 ml DMF and agitated with resin for 24 h. In this step nucleophilic substitution of bromine with primary amine occurred. Then the resin was washed with DMF (3×1 min), IsOH (3×1 min) and DCM (3×1 min) and completeness of this step was confirmed by chloranil and Kaiser tests. Following amino acid (i.e. Cys) was coupled applying the standard protocol described in Section 6.2. HPLC chromatogram and MS analysis of peptide **10** are shown in Figure 29.

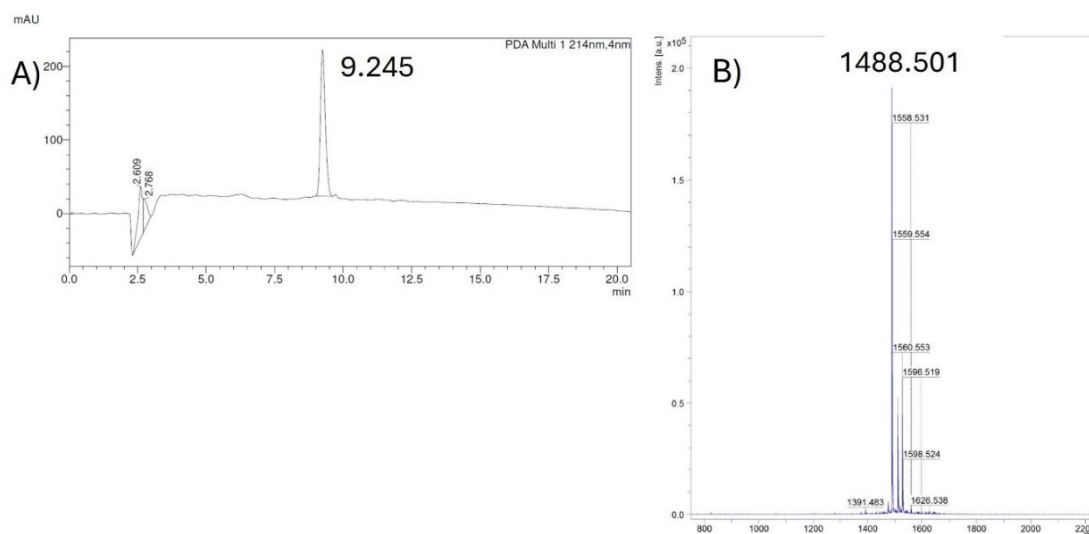


Figure 29. HPLC chromatogram (A) and MS analysis (B) of peptide 10.

M = 1487.717; Calculated $[M+H]^+ = 1488.724$; Found $[M+H]^+ = 1488.501$.

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 20 min.

6.9. Labeling peptides – introduction of fluorescent tag

One of the most popular and broad-based fluorescent tag is 5(6)-carboxyfluorescein (Cf) (Figure 30), which is commonly used for molecular imaging. It has some disadvantages, such as susceptibility to photobleaching and range of emission (400-600 nm), which may interfere with tissue and components of the body (hemoglobin, deoxyhaemoglobin and water) autofluorescence.^{229,249} However, despite its limitations, Cf is inexpensive and usually effortless to introduce in peptides so its drawbacks are of minor meaning for initial *in vitro* research.

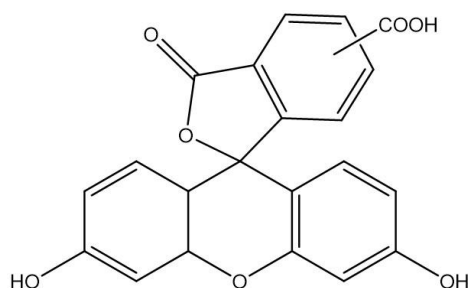


Figure 30. Structure of 5(6)-carboxyfluorescein.

6.9.1. Labeling of monocyclic peptides – introduction of fluorescent tag in the *N*-termini

In monocyclic peptides (**1**, **2**, **36**, **43**) Cf was attached on resin to their *N*-termini as last step before cleavage from the solid support. After Fmoc-removal from the *N*-terminal residue and washing of the peptidyl-resin with DMF, Cf was introduced. For this purpose 73.4 mg (0.195 mmol) of Cf (3 equivalents in relation to the resin loading) with 26.3 mg (0.195 mmol) of HOBt and 30.6 μ l (0.195 mmol) of DIC were dissolved in 3 ml DMF and added to the 0.1 g of RINK amide MBHA resin (loading: 0.646 mmol/g) resin. The reaction was carried out for 24 h in the dark and then the resin was washed with 20% solution of piperidine in DMF (2×30 minutes) to remove ester-bound carboxyfluorescein adducts, which is a side product of the reaction.²⁵⁰ The visual confirmation of this step was the change of resin color from yellow to red. After, resin was washed with DMF (3×1 min), IsOH (3×1 min) and DCM (3×1 min). Peptidyl-resin was dried on air and labeled peptide was cleaved from the solid support according to procedure described in Section 6.6. Afterwards peptide was cyclized according to the procedure described in Section 6.7.1.

In HPLC analyses there are two peaks with similar retention times (but giving a single peak in MS spectrum) which is effect of attaching mixture of 5-carboxyfluorescein and 6-carboxyfluorescein and thus obtaining two isomers of peptide. As an example of such phenomenon, analysis of peptide **36** is shown below (Figure 31).

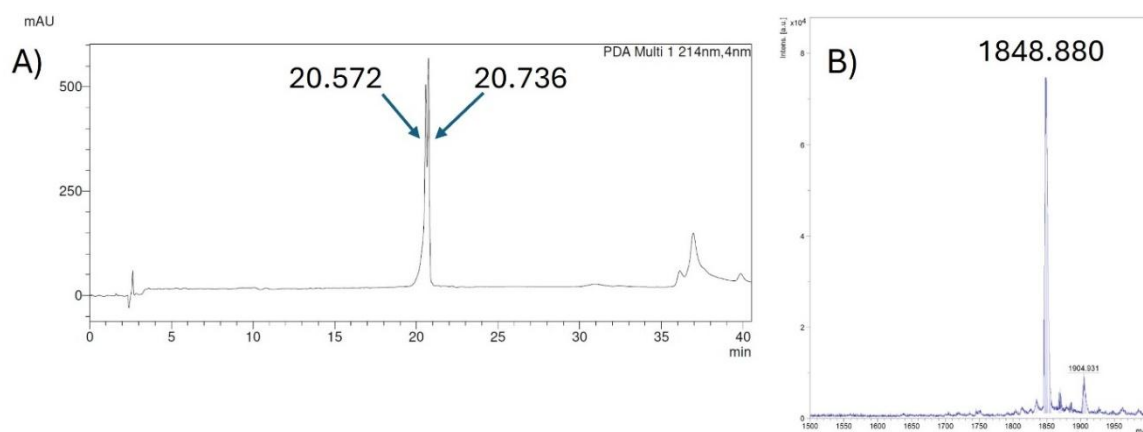


Figure 31. HPLC chromatogram (A) and MS analysis of peptide 36 (B).

M = 1845.764; Calculated $[M+H]^+$ = 1846.771; Found $[M+H]^+$ = 1848.880.

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 40 min.

6.9.2. Labeling of bicyclic peptides – introduction of fluorescent tag in the peptide side chain

To obtain labeled bicyclic peptides (**37**, **41**, **47**), it was necessary to synthesize amino acids derivatives containing Cf attached to the side chains, due to the lack of free N-terminal amino group. Thus, Cf was introduced onto ϵ -amino group of Fmoc-Lys and amino group attached to aromatic ring of Fmoc-Phe(4-NH₂).

6.9.2.1. Attachment of 5(6)-carboxyfluorescein to Fmoc-Lys

To obtain Fmoc-Lys(Cf) I decided to use the derivative that is routinely used in our laboratories for standard syntheses of peptides containing Lys in their structure, namely Fmoc-Lys(Boc). First step was removal of Boc group. For this purpose 300 mg (0.640 mmol) Fmoc-Lys(Boc) was dissolved in 7 ml DCM and diluted with 10 ml DCM on ice bath. Then 2.5 ml TFA was carefully added and the mixture was stirred for 2 h. After that time DCM and TFA was vacuum evaporated. The oily Lys derivative was washed with MeOH and hexane to remove traces of TFA. Solvents were vacuum evaporated. The resulting product was suspended in water and lyophilized. The successful removal of Boc and obtaining of Fmoc-Lys was confirmed by HPLC and MS analyses (Figure 32) 214 mg (0.580 mmol) of Fmoc-Lys was produced (yield of reaction = 90%).

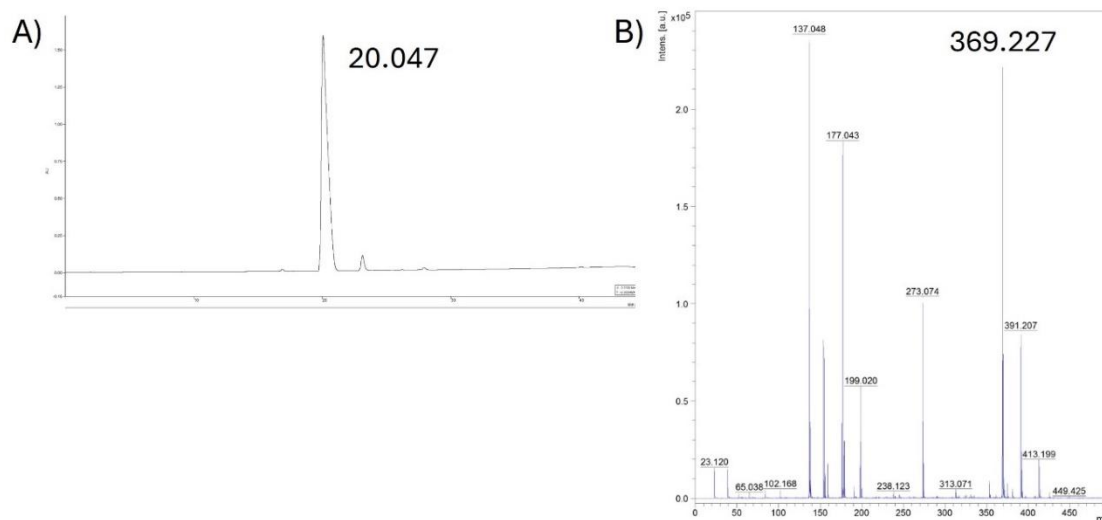


Figure 32. HPLC chromatogram (A) and MS analysis of Fmoc-Lys-OH (B).

M = 368.170; Calculated $[M+H]^+ = 369.177$; Found $[M+H]^+ = 369.227$.

Varian HPLC equipped with a Kinetex column; 10-90% B in 40 min.

The next step was introduction of Cf. For this purpose 102 mg of Cf (0.271 mmol), 36.9 mg of HOAt (0.271 mmol) and 45.5 μ l of DIC (0.271 mmol), in molar ratio 1:1:1, were dissolved in 15 ml DMF and preactivated in 45°C for 15 minutes. 99.8 mg of (0.271 mmol) Fmoc-Lys-OH was dissolved in 8 ml DMF with 47.2 μ l (0.271 mmol) of DIPEA in molar ratio 1:1 and the mixture was added dropwise to the previously activated mixture of Cf, HOAt and DIC. The reaction was carried out 24 h. After that time 45 ml H₂O was added resulting in precipitation of the crude product that was centrifuged. Then, sediment was repeatedly washed with water in order to remove remains of unattached Cf, suspended in water and lyophilized.²⁵¹ HPLC analysis (Figure 33) indicates on minor presence of Cf in the mixture ($t_R = 18.242$ min) and significant superiority of Fmoc-Lys(Cf) in product ($t_R = 29.182$ min). 116 mg (0.160 mmol) of crude Fmoc-Lys(Cf) was produced (yield of reaction = 59%), that was later used in peptide synthesis (peptides 37, 41).

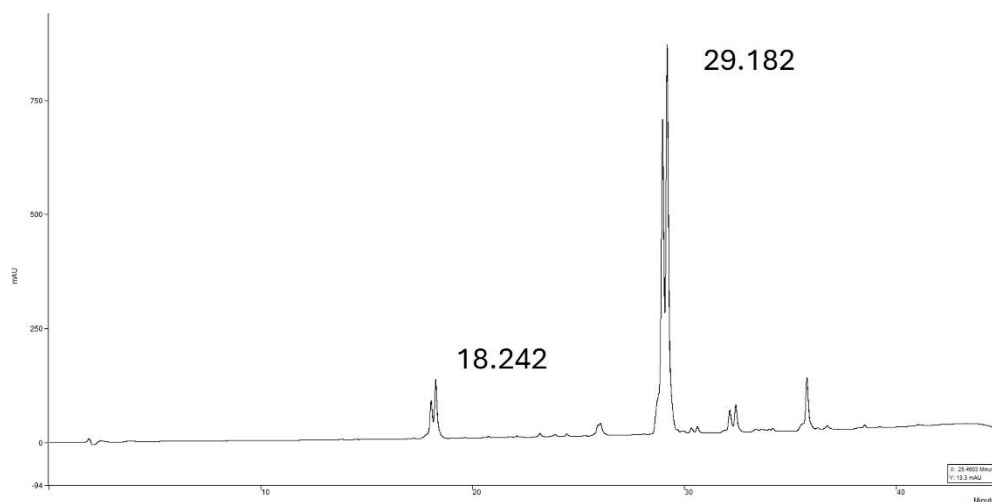


Figure 33. HPLC analysis of Fmoc-Lys(Cf).

$t_R = 18.242$ min (Cf), $t_R = 29.182$ (Fmoc-Lys(Cf)).

Varian HPLC equipped with a Kinetex column; 10-90% B in 40 min.

In case of peptide **41**, after completing the peptide chain elongation, 10 mg of peptidyl resin was used for peptide cleavage. MS analysis (Figure 34) revealed monoisotopic mass of linear peptide **41** and verified the successful synthesis of Fmoc-Lys(Cf).

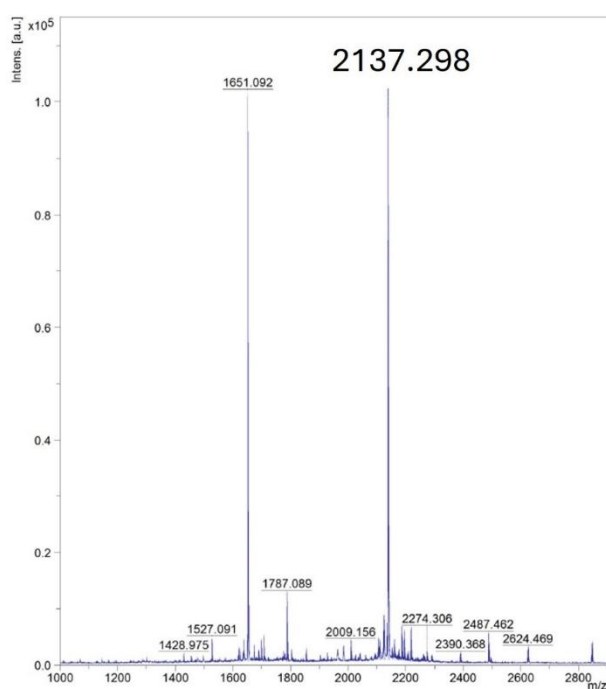


Figure 34. MS analysis of linear peptide 41.

$M = 2136.033$; Calculated $[M+H]^+ = 2137.040$; Found $[M+H]^+ = 2137.298$

6.9.2.2. Attachment of 5(6)-carboxyfluorescein to Fmoc-Phe(4-NH₂)

Peptide **45** was labeled by introduction of Cf on the Phe(NH₂) side chain. Methodology of attachment Cf to the amine group of Fmoc-Phe(4-NH₂) was analogous to procedure described in Section 6.9.2.1 – using equimolar mixture of all reagents (Cf, HOAt, DIC, Fmoc-Phe(4-NH₂), DIPEA). First, 1 mmol of Cf (376.3 mg), HOAt (136.1) and DIC (157 μ l) were preactivated in 15 ml DMF in 45°C for 15 min. 402.5 mg of Phe derivative and 174.1 μ l of DIPEA were dissolved in 15 ml DMF and added to the preactivated mixture. The reaction was monitored using HPLC. After 24 h 70 ml of water was added to the mixture to precipitate the product. After centrifugation, the solid was washed several times with water, suspended in water and lyophilized.²⁵¹ HPLC analysis of sediment is shown in Figure 35A. MS analysis (Figure 35B) of the signal $t_R = 17.917$ min confirmed obtaining of the desired product Fmoc-Phe(4-NH-Cf). In this procedure 541 mg (0.711 mmol) of the crude product (yield of reaction = 71 %) was obtained. Crude product was used for synthesis.

In case of peptide **45**, after completing the peptide chain elongation, 10 mg of peptidyl resin was used for peptide cleavage. MS analysis (Figure 35C) revealed monoisotopic mass of linear peptide **45** and verified the successful synthesis of Fmoc-Phe(4-NH-Cf).

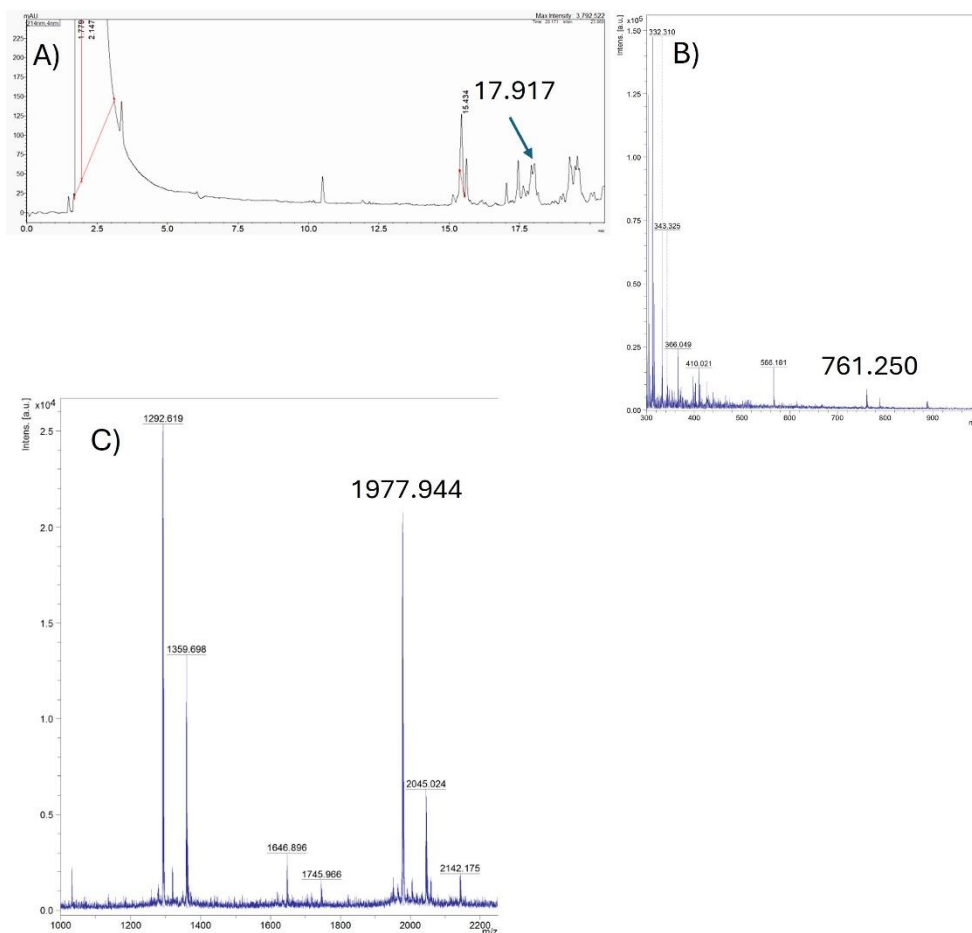


Figure 35. HPLC analysis of Fmoc-Phe(4-NH-Cf) (A) and MS analysis of peak with $t_R = 17.917$ min (B). MS analysis of linear peptide 45 (C).

Shimadzu Prominence-I HPLC equipped with a Kinetex column; 10-90% B in 20 min. Fmoc-Phe(4-NH₂): M = 760.206; Calculated $[M+H]^+ = 761.213$; Found $[M+H]^+ = 761.250$. Linear peptide **45**: M = 1975.955; Calculated $[M+H]^+ = 1976.962$; Found $[M+H]^+ = 1977.944$.

6.10. N-methylation of peptides

Peptides (**1**, **15**) were N-methylated on solid support according to procedure described in my recent publication²⁵² which is optimized, shortened method described previously by Naoum *et al.*²⁵³ General procedure was schematically shown in Figure 36. Optimization of the method is described in Section 12.

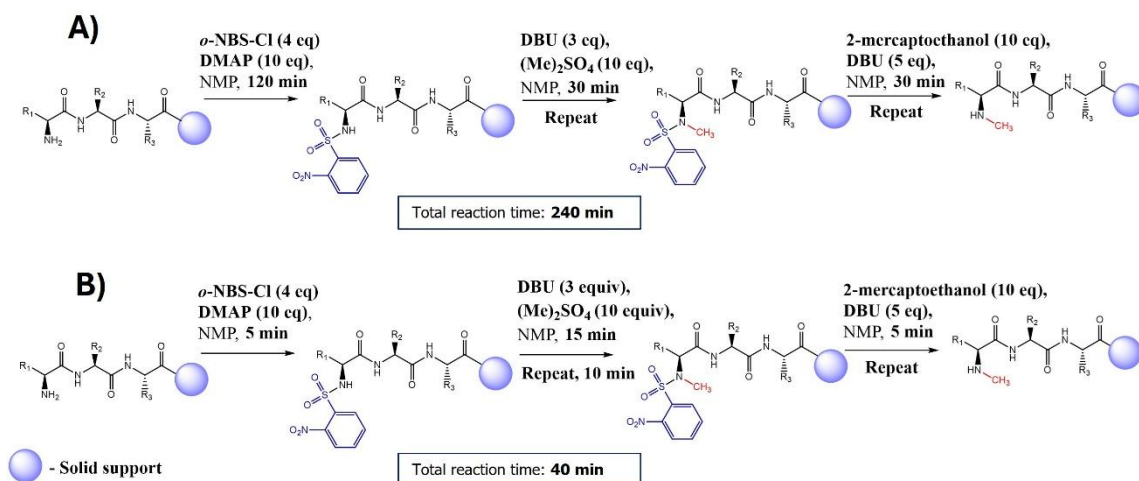


Figure 36. General *N*-methylation procedure described by Naoum et al. (A)²⁵³ and method optimized by our group(B).²⁵²

After coupling of the Fmoc-protected amino acid derivative that is supposed to be *N*-methylated, Fmoc was removed using 20% solution of piperidine in DMF. Then the *o*-NBS protecting group was introduced on α -amine group. For this purpose 28.8 mg of *o*-nitrobenzenesulfonyl chloride (*o*-NBS-Cl, 10 eq., 0.130 mmol) and 39.7 mg of 4-dimethylaminopyridine (DMAP, 4 eq., 0.325 mmol) were dissolved in 2.5 ml of NMP and preactivated for 1 minute. Then the mixture was added to the 50 mg of RINK amide MBHA resin (loading: 0.646 mmol/g) and agitated for 5 minutes. After that time resin was washed with NMP (5×1 min).

Second step was methylation. In order to deprotonate the amine group, 14.6 μ l of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 3 eq., 0.098 mmol) was diluted in 1.5 ml of NMP, added to the resin and agitated for 3 minutes. Next, 30.7 μ l of dimethyl sulfate (10 eq., 0.325 mmol) was diluted in 1.5 ml NMP and added to the cocktail of resin and DBU solution. Whole mixture was stirred with resin for 15 minutes and after that time washed with NMP (3×1 min). The procedure was repeated but the time of reaction was 10 minutes. At the end resin was washed with NMP (5×1 min).

Final step was *o*-NBS removal. A solution of 22.8 μ l of 2-mercaptoethanol (10 eq., 0.325 mmol) and 24.3 μ l of DBU (5 eq., 0.163 mmol) was preactivated for 1 minute, added to the resin and agitated for 5 minutes. Then the resin was washed with NMP (3×1 min), the procedure was repeated and the resin was finally washed with NMP (5×1 min).

After introducing methyl group consecutive coupling of following amino acid residue was performed according to procedure described in Section 6.2.

6.11. Purification of peptides

Crude peptides were purified by RP-HPLC on PLC 2050 Gilson HPLC (Gilson Glider Prep. Software, Gilson, France) equipped with ReproSil-XR 300 C18 column (20 × 250 mm, 10 µm, 300 Å). The solvent system was 0.1% TFA in H₂O (phase A) and 0.1% TFA in 80% ACN (phase B). Depending on purified peptide, diverse linear gradients were applied (flow rate 15 ml/min, detection at 226 nm).

7. Enzymatic studies

Enzymatic studies were conducted with assistance with dr Agata Gitlin-Domagalska from Department of Molecular Biochemistry, Faculty of Chemistry, University of Gdańsk.

7.1. Materials and methods used in enzymology studies

7.1.1. Enzymes

- Recombinant human matriptase-1/ST14 catalytic domain was purchased from R&D Systems, USA.
- Recombinant human matriptase-2 was purchased from Enzo Life Sciences, USA.
- Recombinant human furin was kindly gifted by Dr Anna Kwiatkowska from the group of Prof. Robert Day from Université de Sherbrooke or it was purchased from R&D Systems, USA.

7.1.2. Substrates

Fluorogenic substrates with C-terminal 7-amino-4-methylcoumarin (AMC) group for MT1 and MT2 was Boc-Gln-Ala-Arg-AMC (Biosynth, Switzerland or Peptide Institute. Inc., Japan) and for furin was Pyr-Arg-Thr-Arg-AMC (PeptaNova, Germany).

7.1.3. Others

- 96-well black plates were purchased from BRAND, Germany.
- Trizma® hydrochloride (Tris-Cl), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Aldrich, Germany.

- Triton X-100 was purchased from Thermo Scientific, USA.
- NaCl was purchased from STANLAB, Poland.
- CaCl₂ was purchased from EUROCHEM BGD, Poland.

Inhibitory activity of all synthesized peptides was examined *in vitro* against human recombinant enzymes, depending on the inhibitor these were: MT1, MT2 or furin. All measurements were carried out using Fluostar Omega microplate reader (BMG Labtech, Germany) and 96-well black plates.

7.1.4. Procedure for measuring the inhibitory activity of MT1 and MT2 inhibitors

The assay buffer was prepared: 50 mM Tris-Cl; pH 8.0 supplemented with 150 mM NaCl and 0.1% BSA. Peptides were dissolved in water to obtain concentration 1.54 mM and diluted 100-times. Then samples were diluted with buffer without BSA to obtain following concentrations in well: 642 nM, 321 nM, 160 nM. Enzymes (MT1 and MT2) were diluted with buffer without BSA to obtain final concentration 0.25 nM. The stock solution of fluorogenic substrate was prepared in DMSO and then diluted with buffer without BSA to final concentration in well 10 µM. During the assay the final concentration of DMSO never exceeded 2%. In every well 120 µl of assay buffer was preheated at 37°C. Then 50 µl inhibitor, 20 µl of substrate (Boc-Gln-Ala-Arg-AMC) and 10 µl of enzyme (MT1 or MT2) were added. Following release of fluorescent product, AMC, was monitored for at least 30 minutes at 37°C.

7.1.5. Procedure for measuring the inhibitory activity of furin inhibitors

The assay buffer was 25 mM TRIS with 1 mM CaCl₂ and 1.8 mg/ml BSA pH 9.0,. Peptides were dissolved in water to obtain concentration 1.54 mM and diluted 100-times. Then samples were diluted with buffer without BSA to obtain following concentrations in well: 2.4 nM, 24 nM, 24 nM or 0.077 nM, 0.77 nM, 7.7 nM. Furin was diluted with buffer without BSA to obtain final concentration 1.1 nM. The stock solution of fluorogenic substrate was prepared in DMSO and then diluted with buffer without BSA to final concentration in well 20 µM. In every well 120 µl of assay buffer was preheated at 37°C. Then 50 µl inhibitor, 20 µl of substrate (Pyr-Arg-Thr-Arg-AMC) and 10 µl of enzyme were added. Following release of fluorescent product, AMC, was monitored for at least 30 minutes at 37°C.

7.1.6. Procedure of a IC₅₀ determination

The IC₅₀ (half-maximal inhibitory concentration) values were calculated from plots of enzyme activity (% of control sample) *versus* the inhibitor's concentration using a four-parameter fit model (GraFit 5.0.12 Erithacus Software Ltd.). The IC₅₀ values were determined from triplicated measurements with at least seven different concentrations of inhibitor.

7.1.7. Procedure of K_m determination

The Michaelis-Menten constant (K_m) was determined by incubating the enzyme with its substrate in concentrations in range 0.1-10 K_m , basing on the K_m reported in literature.²⁵⁴ Substrate concentration *versus* initial reaction velocity were analyzed by the Michaelis-Menten equation and plotted using GraFit 5.0.12 Erithacus Software Ltd. Double reciprocal. Lineweaver-Burk plot was used to determine the K_m values.

7.1.8. Procedure of inhibition constant (K_i) determination

The K_i values were calculated using the Cheng-Prusoff equation, assuming the competitive mode of inhibition, according to the following equation:

$$IC_{50} = K_i \left(1 + \frac{[S]}{K_m} \right)$$

IC₅₀ – half-maximal inhibitory concentration
K_i – inhibition constant
[S] – substrate concentration
K_m – Michaelis-Menten constant

8. Study of inhibitors' proteolytic stability

Selected peptides were evaluated for their stability in human serum (purchased from Sigma-Aldrich, USA). The experiments were carried out according to procedures described by Gitlin-Domagalska *et al.*⁹⁷

A stock solution of the peptide was prepared by dissolving the inhibitor in water to achieve a final concentration of 1.6 mM. This solution was then diluted 10-fold with serum pre-warmed to 37°C. The resulting mixture was incubated at 37°C, and samples were collected at 0, 1, 2, 3 and 24 h. At each time point, 150 µl of mixture was taken and mixed with 150 µl ACN to quench hydrolysis and precipitate serum proteins. Then sample was centrifuged for 10 min at 13 400 RPM. The supernatant was then placed in ice bath for 1 h to promote further protein precipitation, followed by a second centrifugation step (10 min at 13 400 RPM). The prepared sample was analyzed using HPLC and MS to monitor peptide degradation.

Each peptide was evaluated twice under the same conditions. The progress of hydrolysis was determined as the percentage of the area under the peak using RP-HPLC (Shimadzu Prominence-I LC-2050C 3D equipped with a Kinetex XB-C18 (150 × 4.6 mm, 5 µm); detection at 30°C and 214 nm).

Results and discussion

9. Design and enzymatic evaluation of MT1 inhibitors

List of all obtained peptides is included in Section “Scientific goals of the study” in Table 2. Mentioned table comprises two groups of MT1 inhibitors: analogues of SFTI-1 (compounds **1** and **2**) and analogues of HV-BBI (compounds **3-36**).

9.1. Analogues of SFTI-1

The first two peptides I designed based on the scaffold of a potent MT1 inhibitor ($K_i = 4.3$ nM)⁹⁷. This peptide, named here as inhibitor A, was designed in our group and its structure is shown in Figure 37A. This monocyclic peptide exhibited nearly 74-fold selectivity toward MT1, compared to MT2. Its first analog, inhibitor **1**, contains an attached Cf at *N*-terminus and it has *N*-methylated Ser residue (Figure 37B), while inhibitor **2** is Cf- labeled parent compound (Figure 37C). The methyl group in inhibitor **1** was introduced to enhance proteolytic stability and to prevent cleavage of the scissile peptide bond between Arg and Ser.

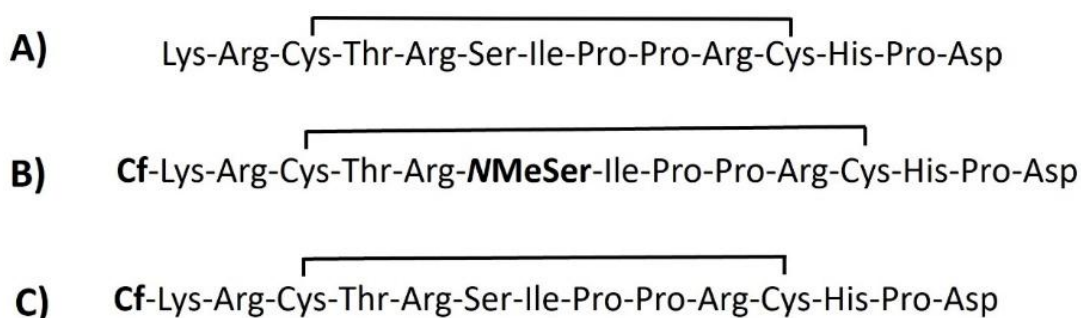


Figure 37. Structures of MT1 inhibitor⁹⁷ (A) and its analogues: inhibitors **1 (B) and **2** (C).** Introduced modifications are marked by bold.

Preliminary inhibition assays of these peptides were conducted by the research groups of Prof. Pierre-Luc Boudreault and Prof. Richard Leduc from Department of Pharmacology-Physiology, Faculty of Medicine and Health Sciences, Université de Sherbrooke in Canada. Both groups

provided exceptional and well-documented experience in search for MT1 and MT2 inhibitors^{71,198,255,256} and their further applications in biological context.^{64,257} The inhibitors were tested against MT1 and MT2. Recombinant enzymes were incubated with the compounds at two concentrations (0.1 and 10 μ M), and their activity was monitored using fluorogenic substrate containing AMC group at the C-terminus (Boc-Gln-Ala-Arg-AMC). The results (Figure 38) confirmed our findings that the inhibitor A exhibits selectivity toward MT1 over MT2. The activity measurements obtained at 0.1 μ M indicated a decrease in MT1 enzymatic activity for the tested inhibitors (respectively **1** and **2**). Inhibitor A almost completely inhibited MT1 at 10 μ M, whereas MT2 activity was reduced by approximately 50% at the same concentration. Inhibitor **1** displayed a similar inhibitory activity toward MT1 at 10 μ M as inhibitor A, however, it also inhibited MT2. The enzymatic evaluation of inhibitor **2** at 0.1 μ M demonstrated that it was inactive toward both enzymes. However, at the higher concentration, inhibitor **2** inhibited MT1 almost as effectively as the other inhibitors, while showing no inhibitory effect on MT2.

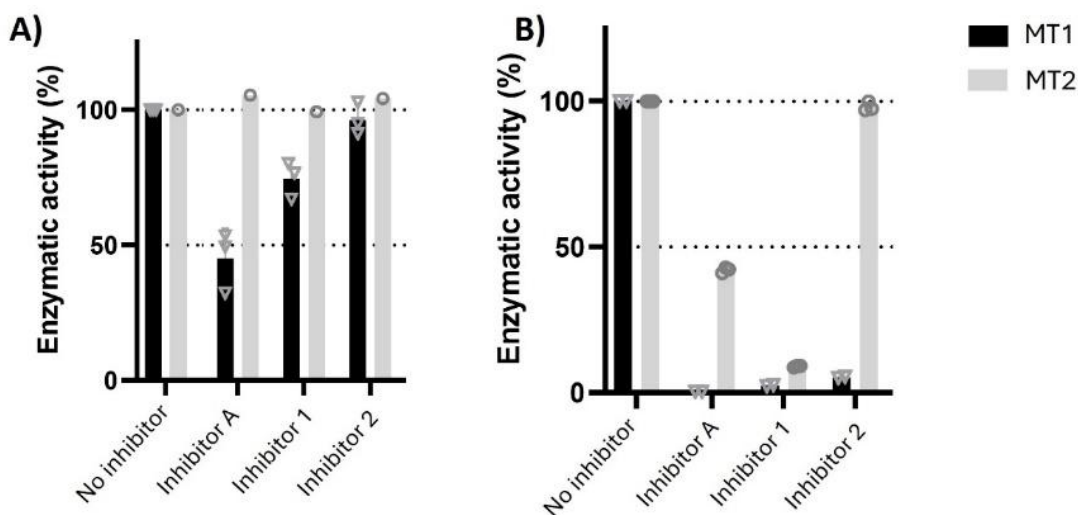


Figure 38. Activity of inhibitors 1 and 2 at 0.1 μ M (A) and 10 μ M (B) against MT1 and MT2.

9.2. Analogues of HV-BBI

As mentioned in Section 2.2.1.2., the gradual shortening of the frog-skin derived HV-BBI primary structure resulted in highly potent and selective MT1 inhibitor.¹⁸⁷ In the referenced study, an interesting difference in inhibitory potency toward MT1 was observed among analogues differing at the C-terminus. Only the peptide containing an amide moiety (Figure 39A) exhibited strong inhibitory activity, whereas its analogues with a carboxylic group (Figure 39B) and with backbone cyclization (Figure 39C) were considerably less active. In addition, our recently

published research²⁰³ revealed that this inhibitor demonstrates unprecedented selectivity toward MT1 over the closely related MT2. The nanomolar inhibitory constant (K_i) is 929-fold lower for MT1 than for MT2. Such significant selectivity, combined with high inhibitory potency, has not been reported for any MT1 inhibitor. Inhibition constants were also determined for the other two analogues. All analogues exhibited moderate activity toward MT2. However, only the analogue with an amide group revealed such an exceptional selectivity. Peptides with carboxylic group and with backbone cyclization showed 96-fold and 65-fold preferences for MT1 over MT2, respectively. The K_i values of the three HV-BBI analogues are presented in Figure 39 alongside the peptide structures.²⁵⁸

		K_i [nM]	
		MT1	MT2
A)	Gly-Cys-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys-Phe-NH ₂	8.5 ± 1.1	7900 ± 900
B)	Gly-Cys-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys-Phe-OH	470 ± 50	45000 ± 9000
C)	Gly-Cys-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys-Phe	530 ± 70	34000 ± 7000

Figure 39. Structures and K_i values against MT1 and MT2 of truncated analogues of HV-BBI differing in their C-termini: amide group (A), carboxyl group (B) and backbone cyclisation (C).

The Canadian group carried out molecular dynamics (MD) studies of complexes of above-mentioned peptides with MT1.²⁵⁸ Analyses of molecular interactions revealed that, in the peptide containing a carboxylic group, a salt bridge forms between the terminal residues Gly1 and Phe13. This interaction contributes to a strong and stable hydrogen bonding network, effectively locking the peptide in a constrained conformation. Consequently, the orientation of Arg10 is significantly altered compared to the peptide with an amide moiety, which hinders optimal interactions with the MT1 surface. This conformational rigidity also affects the position of Ser6 and Pro9, which are shifted relative to the same residues in the more flexible analogue containing an amide group. The interaction network of these two peptides in complex with MT1 are shown in Figure 40. Overall, these conformational changes reduce interactions with regions crucial for catalytic activity, and therefore, decrease the binding affinity to the enzyme.²⁵⁸ The obtained results are in

good agreement with enzymatic studies explaining the importance of *C*-terminal moiety in interaction of inhibitor with MT1.

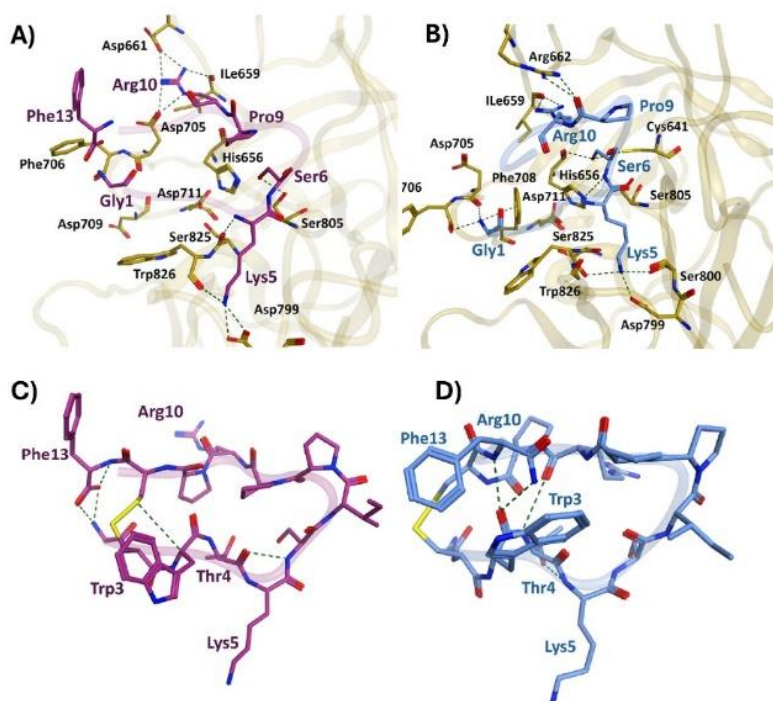


Figure 40. Visualization of enzyme-peptide interaction maps between the analogue with a carboxylic group (A) and the analogue with an amide group at the *C*-terminus (B) in complex with the MT1 active site. Intramolecular hydrogen-bond networks are shown for the analogue with a carboxylic group (C) and the analogue with an amide group (D).²⁵⁸

MT1 is represented as a beige ribbon with key residues displayed in stick format. The inhibitors are shown as colored sticks: the analogue with a carboxylic group in blue and the analogue with an amide group in magenta.

All three peptides were also tested against other serine proteases, including representatives of TTSPs (TMPRSS2, hepsin) and coagulation proteases (thrombin, factor Xa). The inhibitors were examined by a Canadian research group at two concentrations: 10 μ M and 100 μ M, and their activity was monitored using fluorogenic substrate Boc-QAR-AMC (Figure 41). At 10 μ M, none of the inhibitors exhibited activity toward any protease except MT1 (Figure 41A). At 100 μ M, the peptide with an amide group at the *C*-terminus slightly inhibited hepsin activity, and all three compounds showed low activity toward TMPRSS2. No activity against thrombin or factor Xa was observed for any of the peptides (Figure 41B). These results confirm the exceptional selectivity of the peptide with an amide group, as well as its strong potency at low concentration (10 μ M). Moreover, it does not inhibit coagulation proteases, which could otherwise pose a significant obstacle to potential therapeutic application.²⁵⁸

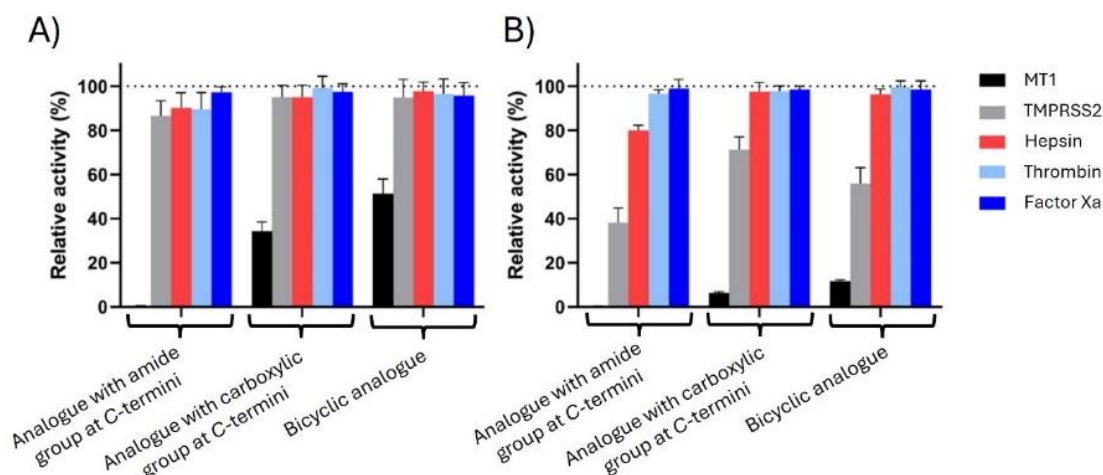


Figure 41. Inhibitory activity of HV-BBI analogues against selected proteases. Enzymatic studies performed at 10 µM (A) and 100 nM (B) concentrations of peptide.²⁵⁸

Due to its high inhibitory potency and unusual selectivity, the compound with C-terminal amide group was selected for further investigation. The peptide was re-synthesized (named in further parts of this thesis as inhibitor **3**) and its structure served as the scaffold for subsequent modifications. These peptides became subject of my studies and obtained results are discussed here as part of my dissertation. Modifications introduced into the structure of HV-BBI are summarized in Figure 42 and described in details in the following Sections.

MD studies carried out by Canadian group revealed conformational changes in peptide **3** when complexed with MT1 (PDB ID: 3P8F) and MT2 (Homology model). These results led to design of analogues with a Phe13 substitution, as described in Section 9.2.1. However, these findings did not confirm Phe13 as a major determinant of inhibitory activity of peptide **3**, prompting alanine scanning (Section 9.2.2.) to identify which amino acids play a pivotal role in MT1 binding. The conclusions drawn from alanine scanning contributed to the design of peptides with reduced molecular weight (Section 9.2.3.). Furthermore, an analogue in which the disulfide bridge was replaced with its mimetic, a triazole bridge, was synthesized (Section 9.2.4.).

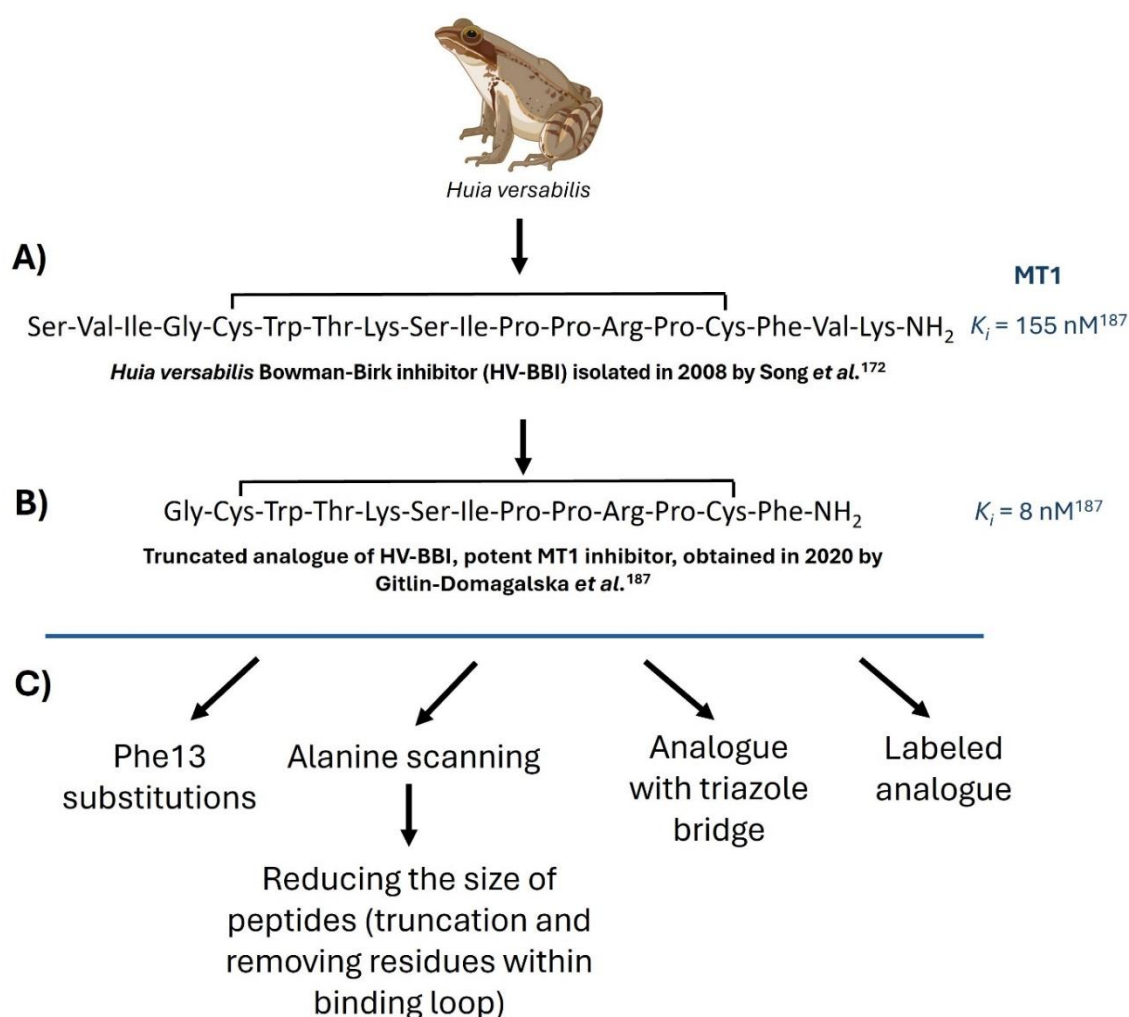


Figure 42. Structures of the *Huia versabilis* Bowman-Birk inhibitor (HV-BBI) and its analogues and schematic representation of the development of novel MT1 inhibitors based on HV-BBI. The modifications that are subject of my work are shown below the blue line.

- A) Trypsin inhibitor isolated in 2008 by Song *et al.*¹⁷² from Chinese bamboo odorous frog *Huia versabilis*;
- B) Truncated analogue of HV-BBI obtained by Gitlin-Domagalska *et al.*¹⁸⁷
- C) Modifications of peptide **3** that are part of my dissertation.

9.2.1. Replacement of Phe13 in peptide 3

As mentioned in the previous section, MD studies (Figure 43), performed by Canadian group of Prof. Pierre-Luc Boudreault and Prof. Richard Leduc, indicated that the Lys5 located in P1 position in inhibitor **3** forms a stable hydrogen bond with Asp799 of MT1 at the bottom of S1 pocket, indicating a favorable interaction at this binding site. This observation is in accordance with results published by Sisay *et al.*⁷⁰, where MT1 preference for Lys in P1 was explained. Moreover, inhibitor's C-terminal Phe13 is located in close distance to Phe706 at the left side of

the MT1 S4 pocket (Figure 43A). This arrangement can promote hydrophobic interactions between both aromatic phenyl rings and contributes to π - π and van der Waals interactions.

In MT2 Asp756 relocates from its initial position, moving away from the P1 Lys5 (Figure 43B). This structural shift results in the loss of the interaction between Lys5 of the inhibitor and Asp756 within the S1 enzyme's pocket, which in turn may explain inefficient and weak inhibition. Importantly, Sisay *et al.*⁷⁰ explain MT2's preference for Arg at this position, which arises from the presence of Ala757 instead of Ser at the bottom of the S1 pocket and enables the formation of a salt bridge between the guanidinium group of Arg and the carboxyl group of Asp. This mechanism is described in detail in Section 1.1.3.3. The hydrophobic interaction between Phe residues in inhibitor and MT1, mentioned above, is not observable in MT2, due to the presence of hydrophilic Asp663 in the analogous position (Phe706 in MT1).

It is worth noting that this study indicates dynamic behavior of C-terminal region in inhibitor 3, which may undergo conformational changes to optimize interactions with the enzyme. Additionally, the orientation of inhibitor's Arg10 exhibits comparable changes in both enzyme complexes. Notably, the presence of the C-terminal amide group affects the overall conformation of the inhibitor 3, subsequently influencing interactions with neighboring residues.

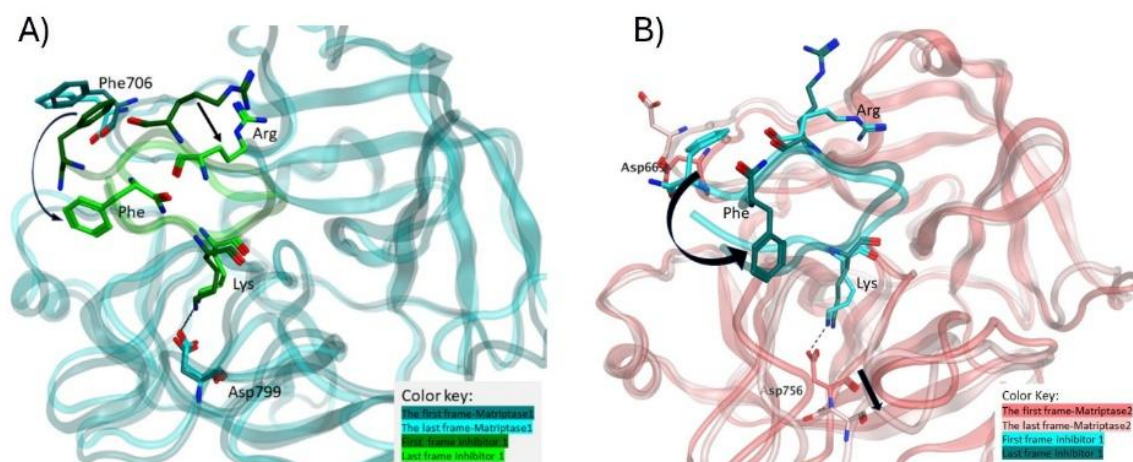


Figure 43. Visualization of conformational changes in inhibitor 3 in complex with MT1 (PDB ID: 3P8F) (A) and MT2 (Homology model) (B).

GROMACS: MD simulation; 500ns. MOE: Analysis and Visualization. The method was validated using SFTI-1 – MT1 complex (PDB:3P8F). "The first frame" and "The second frame" refer to the initial and last molecular dynamics poses of the inhibitors in the active sites of the MT1 and MT2 receptors.

The provided results, particularly these showing differences in the molecular environment around inhibitor's Phe13 in MT1 compared to MT2, as well as the dynamic behavior of the C-

terminal tails, prompted the design of series of inhibitor **3** analogues. They included peptides in which Phe13 was replaced with more hydrophobic amino acids (including various Phe derivatives), as well as hydrophilic amino acids as control. According to the objectives, the introduction of a more hydrophobic component than Phe would provide stronger hydrophobic interactions and contribute to improved binding to the enzyme. In this position various natural and unnatural amino acids have been introduced (Figure 44) and sequences of obtained inhibitors are presented in Table 4. First analogue, inhibitor **4**, has D-Phe instead of Phe13. Some of the designed compounds have amino acids with bulky side chains with large hydrophobic surface area instead of Phe13 (i.e. Dpa, Bpa, Phe(4-tBu), Phe(4-Ph); compounds **5-7** and **9**, respectively), which may facilitate stronger hydrophobic interactions with MT1 than Phe. Interestingly, according to MD studies, Phe13 can move away from MT1 Phe706 and bent inward, toward enzyme's active site. This situation potentially alters its interaction with surrounding residues. Consequently, Phe13 was replaced with Gly, which lacks any side chain (peptide **12**), another hydrophobic proteinogenic amino acid Trp (peptide **13**), and a basic, more polar residue, Lys (peptide **14**). Purpose of introducing NMePhe (peptide **15**) and NPhe (peptide **10**) was to increase the tendency of *cis/trans* isomerization, which could influence the direction and mobility of C-terminal residue of inhibitor **3**. Moreover, there are two analogues with small substituent groups in the *para* position on the phenyl group of Phe: Phe(4-F) (peptide **8**) and Phe(4-NO₂) (peptide **11**). In peptide **16** Phe replaced Gly1, to check if additional Phe may increase inhibitory strength.

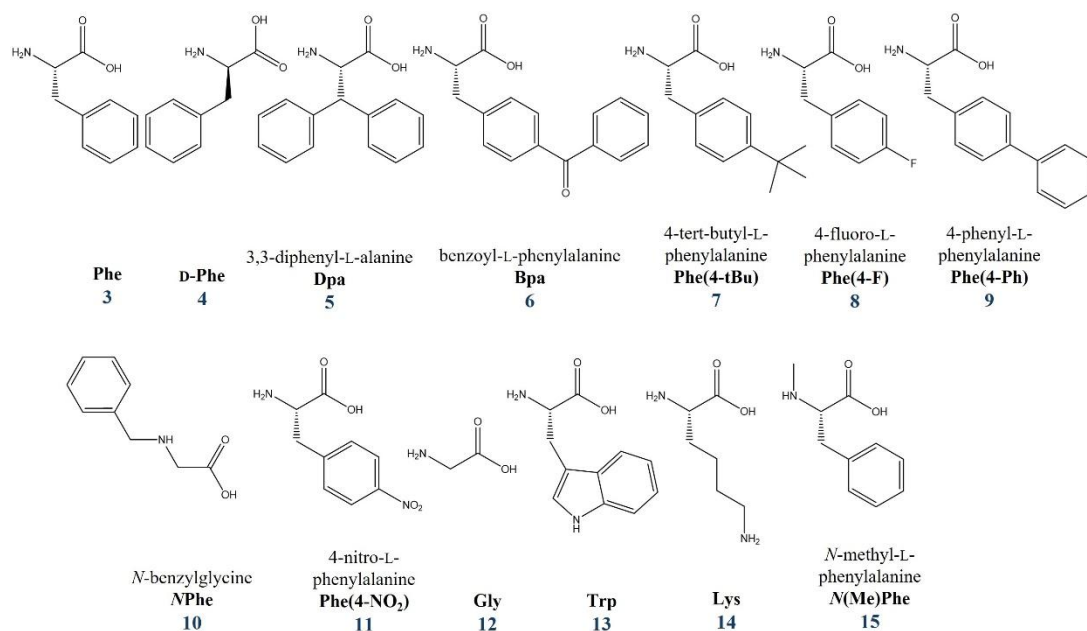


Figure 44. Amino acids introduced instead of Phe13 in inhibitor **3.**
Numbers corresponding to the following inhibitors are marked in blue.

Table 4. Sequences of peptide 3 analogues with substituted Phe13. The introduced modifications indicated in bold.

N°	Sequence
3	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
4	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- D Phe-NH ₂
5	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Dpa -NH ₂
6	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Bpa -NH ₂
7	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Phe(4-tBu) -NH ₂
8	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Phe(4-F) -NH ₂
9	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Phe(4-Ph) -NH ₂
10	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- N Phe-NH ₂
11	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Phe(4-NO₂) -NH ₂
12	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Gly -NH ₂
13	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Trp -NH ₂
14	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Lys -NH ₂
15	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- NMePhe -NH ₂
16	Phe -Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂

Peptides **4-16** were evaluated against MT1 and MT2 at three concentrations (160, 321, 642 nM) (Figure 45). None of the analogues exhibited higher activity toward MT1 than inhibitor **3** (Figure 45A). Most inhibitors demonstrated activity comparable to the starting compound, regardless of the substituted amino acid, including analogues with aromatic and hydrophobic Phe(4-F) (peptide **8**), hydrophobic and bulky Phe(4-Ph) (peptide **9**), or even hydrophilic and positively charged Lys (peptide **14**). A moderate loss of activity was observed in peptides containing hydrophobic and bulky Dpa (peptide **5**), NMePhe (peptide **15**), and the D-enantiomer D-Phe (peptide **4**). The obtained results, unlike suggested by MD studies, indicate that C-terminal Phe13 residue is not a critical determinant of inhibitor **3**'s affinity and selectivity toward MT1.

Remarkably, none of the inhibitor **3** analogues affected MT2 activity (Figure 45B), and all the peptides failed to exhibit inhibitory activity at the tested concentrations.

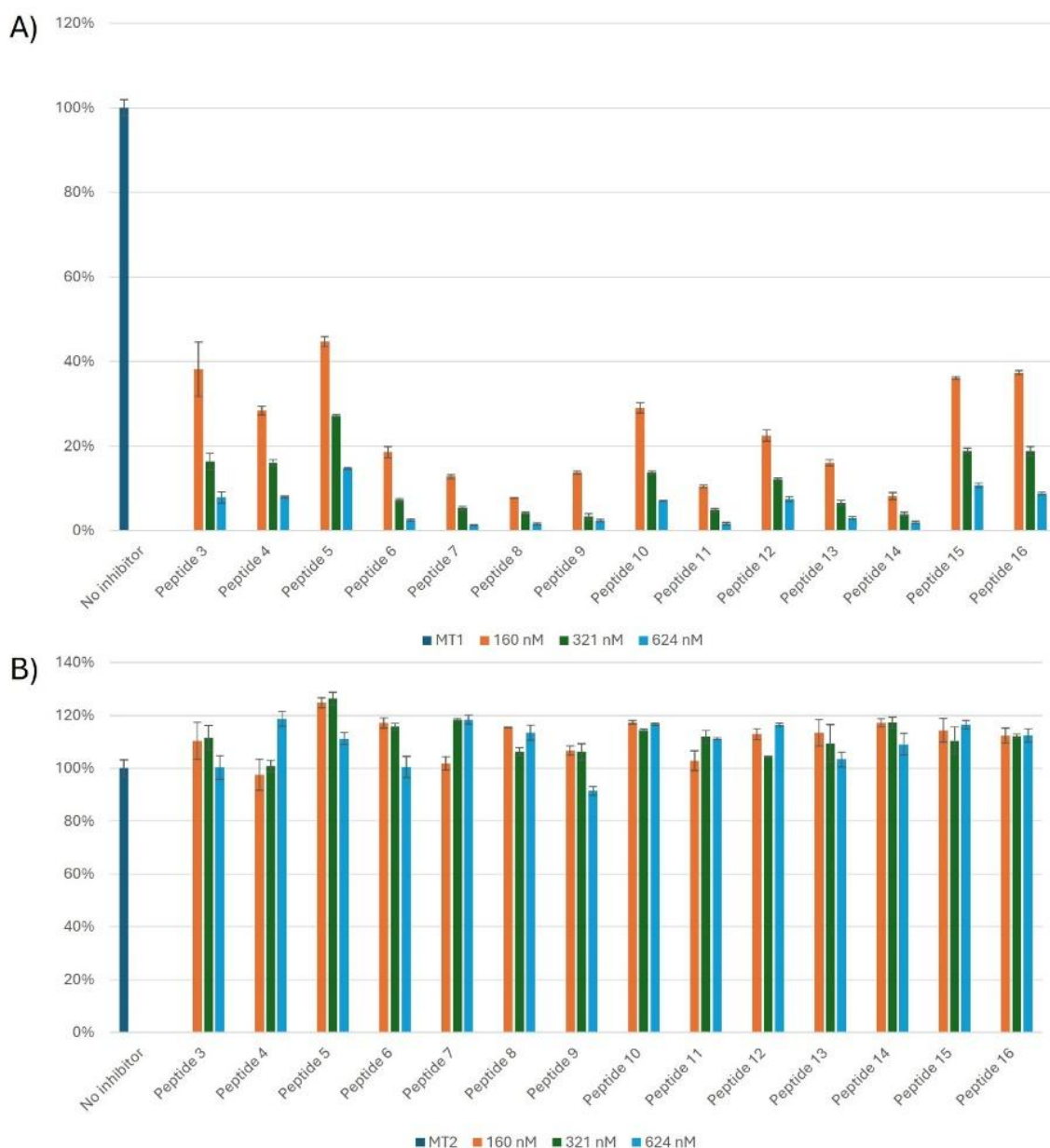


Figure 45. Results of enzymatic evaluation of inhibitor 3 and its analogues with substituted Phe13 residue toward MT1 (A) and MT2 (B).

Inhibitors were tested at three concentrations: 160 nM (orange), 321 nM (green) and 624 nM (blue).

9.2.2. Alanine Scanning in peptide 3

Substitution of Phe13 in inhibitor 3 with various amino acids did not validate the hypothesis that this residue is a key determinant of the inhibitor's unique properties. Consequently, an alanine scanning approach was undertaken to systematically assess the contribution of individual residues to its activity and selectivity. Consecutive amino acids were replaced by Ala. Exceptions were

Cys2 and Cys12 residues, which are forming disulfide bridge crucial for inhibitory activity. Moreover, Pro residues (Pro8 and Pro9) were preserved, as they are located in highly conservative motif Thr-Lys-Ser-Ile-Pro-Pro found in all Bowman-Birk inhibitors (at inhibitor positions P2 to P4').²⁵⁹ These Pro residues induce turn, which is critical for canonical conformation of the binding loop and inhibitory activity.²⁶⁰

Table 5. Sequences of peptide 3 analogues obtained through alanine scanning. The introduced Ala residue is indicated in bold.

N°	Sequence
3	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
17	Ala -Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
18	Gly-Cys(&)- Ala -Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
19	Gly-Cys(&)-Trp- Ala -Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
20	Gly-Cys(&)-Trp-Thr- Ala -Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
21	Gly-Cys(&)-Trp-Thr-Lys- Ala -Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
22	Gly-Cys(&)-Trp-Thr-Lys-Ser- Ala -Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
23	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro- Ala -Pro-Cys(&)-Phe-NH ₂
24	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg- Ala -Cys(&)-Phe-NH ₂
25	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)- Ala -NH ₂

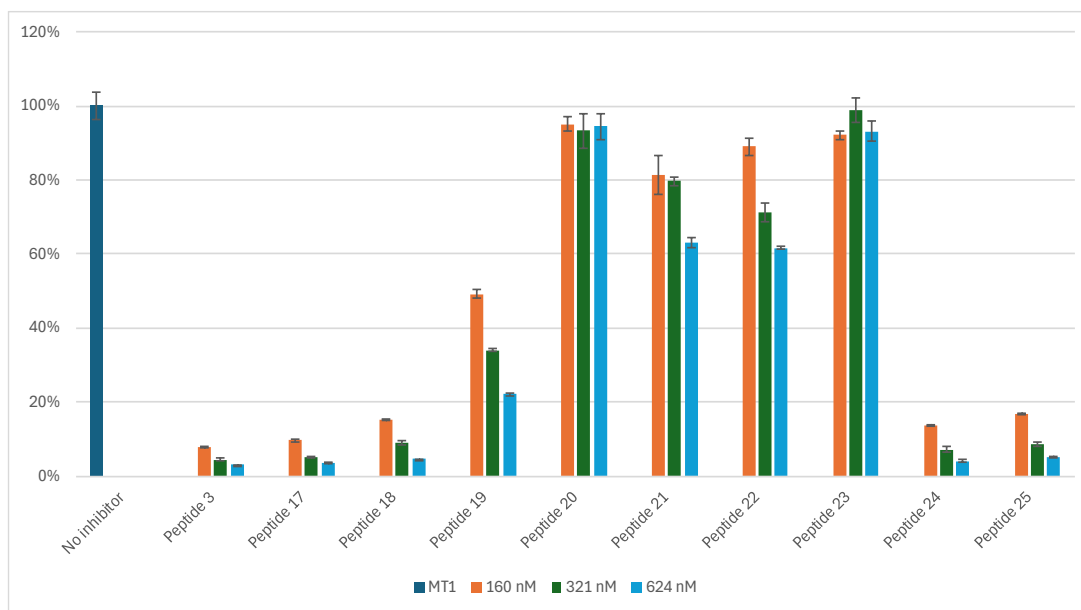


Figure 46. Results of enzymatic evaluation of inhibitor 3 and its analogues obtained through alanine scanning.

Inhibitors were tested at three concentrations: 160 nM (orange), 321 nM (green) and 624 nM (blue).

Analogues **17-25** were named accordingly, and their sequences are presented in Table 5. All peptides were tested against MT1 at three concentrations (160, 321, and 624 nM) (Figure 46). The results confirmed the crucial role of the P1 Lys5 residue (which largely determines inhibitor's specificity) in MT1 recognition, as replacement of this amino acid in peptide **20** with Ala resulted in a dramatic reduction in activity toward MT1. Moreover, substitution of other residues located in the binding loop with Ala (Ser6 in peptide **21**, Ile7 in peptide **22**, Arg10 in peptide **23**) i.e. at positions P1', P2', P5' had a drastic and decisive impact on enzymatic activity- these analogues exhibited no inhibitory effect. In contrast, replacing Thr4 (peptide **19**) with Ala had only a moderate effect. Furthermore, substitutions near the *N*-terminus (peptides **17** and **18**) and *C*-terminus (peptides **24** and **25**) had progressively less impact on inhibitory potency, and these analogues displayed activity comparable to that of the starting compound inhibitor **3**.

As expected, removal of residues located in the highly conserved binding loop dramatically decreased inhibitory potency, whereas substitutions near *N*- and *C*-termini resulted in activity similar to that revealed by peptide **3**. These findings suggest that terminal residues may be modified to improve the inhibitor's pharmacokinetic properties or even removed to reduce molecular weight of the peptides.

9.2.3. Reduction and optimization of the molecular size of inhibitor **3**

It is well described that short-chain peptides are efficiently absorbed from the human gastrointestinal tract, whereas longer and more complex peptides are prone to hydrolysis by enterocyte peptidases.²⁶¹ A commonly used parameter for evaluating transcellular transport is the apparent permeability coefficient (P_{app}).²⁵⁶ P_{app} value decreases with growing peptide chain length and simultaneously molecular weight, while bioactive peptides with lower molecular weight exhibit higher intestinal permeability. Experiments using Caco-2 cell monolayers demonstrated that compounds with masses <500 Da had a bioavailability of approximately 16%, compounds with masses in the range of 500 - 1000 Da showed approximately 9%, and larger peptides (1300 - 1600 Da) permeated only at approximately 10%. Smaller peptides were transported *via* peptide transporter 1 (PepT1), whereas larger peptides relied on the paracellular route, which is less efficient than transcellular transport.²⁶¹ Moreover, longer peptides are more susceptible to hydrolysis due to the presence of proteases,²⁶¹ and truncation of sequences is a well-known strategy to overcome this limitation. One example, already mentioned in this dissertation, is the truncation of HV-BBI performed by Gitlin-Domagalska *et al.*¹⁸⁷ which yielded an analogue degraded by 50% in human serum after 24 h of incubation, whereas the original HV-BBI peptide was completely absent after the same period.

Considering the potential therapeutic application of the obtained MT1 inhibitors, it is essential that these compounds demonstrate stability in a proteolytic environment and good bioavailability. Results from alanine scanning (Section 9.2.2.) indicated residues that are not crucial for inhibitory activity and interaction with MT1, thus opening the possibility to design peptides with reduced molecular weight. First, Gly1 and Phe13 were deleted (Section 9.2.3.1.), followed by the removal of Trp3 and Pro11 (Section 9.2.3.2.).

9.2.3.1. Truncation of peptide **3**

Inhibitor **3** was initially truncated at both, the *N*- and *C*-termini. Analogues lacking Phe13 (peptide **26**), Gly1 (peptide **27**) and both of these residues (peptide **28**) were synthesized. Their sequences are presented in Table 6.

Table 6. Sequences of truncated peptide **3 analogues.**

N°	Sequence
3	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
26	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NH ₂
27	Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
28	Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NH ₂

Inhibitors were tested against MT1 at three concentrations (160, 321, 624 nM) (Figure 47). Enzymatic evaluation revealed that the inhibitor lacking Phe13 (peptide **26**) exhibited activity comparable to that of the starting compound **3**. The activity of the other two analogues (peptides **27** and **28**) was slightly lower than that of inhibitor **3**. Therefore, peptide **26** was selected for further modifications, aimed at additional downsizing, as described in Section 9.2.3.2.

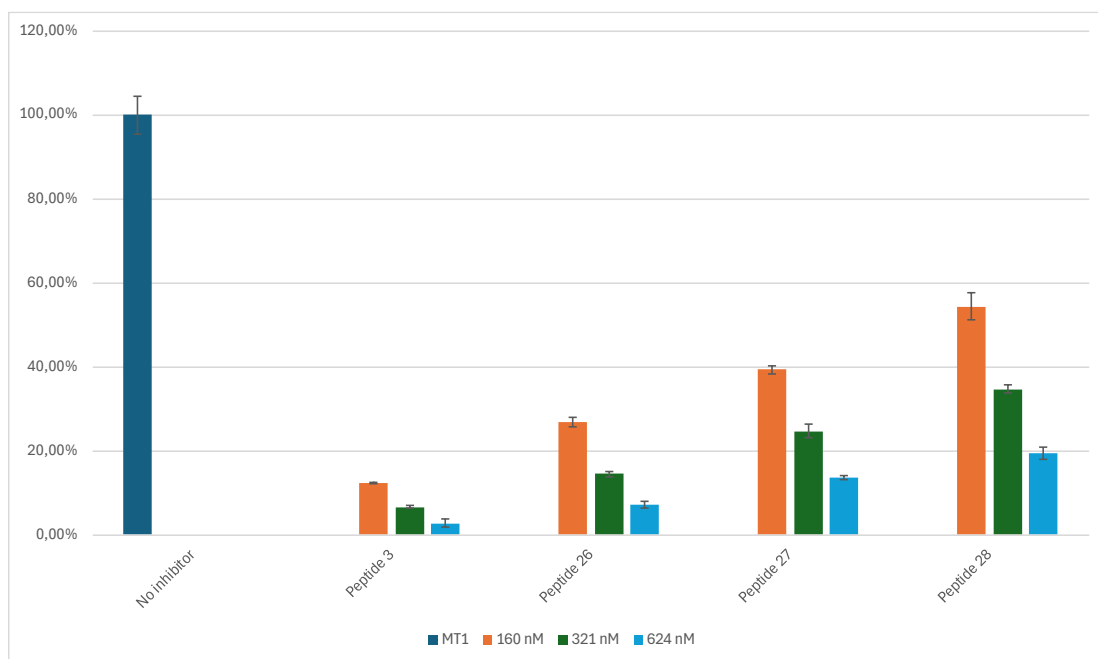


Figure 47. Results of enzymatic evaluation of peptide 3 and its truncated analogues 26-28. Inhibitors were tested at three concentrations: 160 nM (orange), 321 nM (green) and 624 nM (blue).

9.2.3.2. Deletion of Trp and Pro residues in peptide 3

Alanine scanning results revealed that replacing selected amino acids within the binding loop of inhibitor **3** does not necessarily exert a dramatic impact on its inhibitory potency. Substitution of Trp3 and Pro11 resulted in inhibitory activity moderately similar to that of the starting compound. Therefore, a series of truncated analogues was synthesized. Three of them were based on inhibitor **3**, i.e. peptide **29** with removed Trp3, peptide **30** deprived Pro11, and peptide **31** lacking both residues. Corresponding analogues of truncated peptide **26** were designed and synthesized, i.e. peptides lacking Trp3 (peptide **32**), Pro11 (peptide **33**), or both residues (peptide **34**). The sequences of these truncated inhibitors are presented in Table 7.

Table 7. Sequences of peptide 3 and its analogues with deleted residues located inside the binding loop.

N°	Sequence
3	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
29	Gly-Cys(&)-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-Phe-NH ₂
30	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Cys(&)-Phe-NH ₂
31	Gly-Cys(&)-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Cys(&)-Phe-NH ₂
26	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NH ₂
32	Gly-Cys(&)-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Pro-Cys(&)-NH ₂
33	Gly-Cys(&)-Trp-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Cys(&)-NH ₂
34	Gly-Cys(&)-Thr-Lys-Ser-Ile-Pro-Pro-Arg-Cys(&)-NH ₂

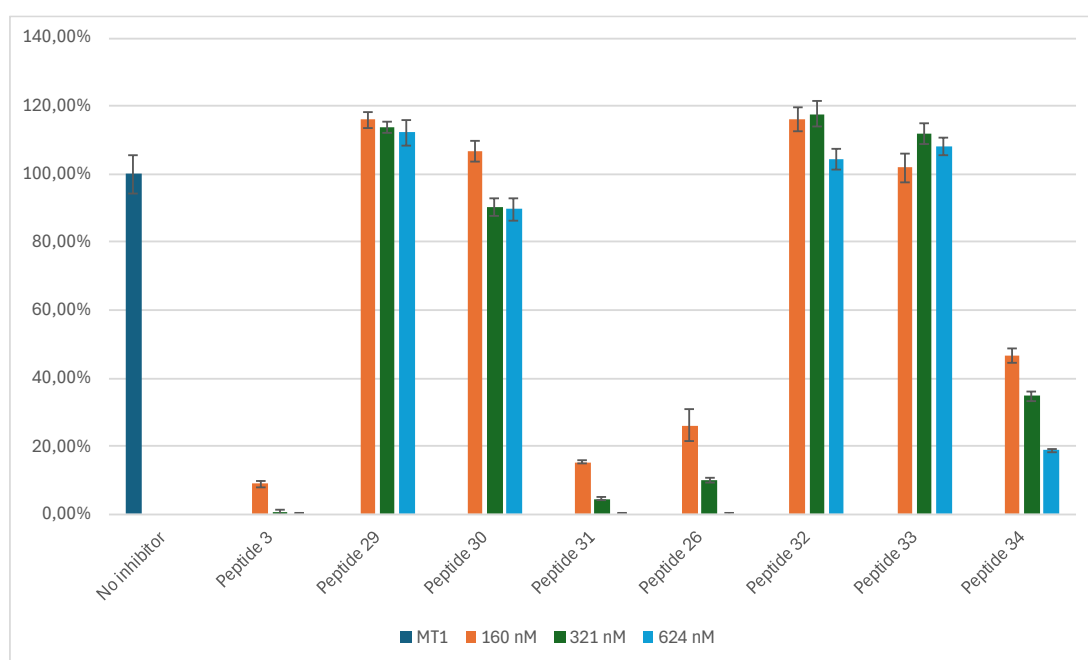


Figure 48. Results of enzymatic evaluation of peptide 3 and its analogues (26 and 29-34) with deleted residues located inside the binding loop.

Inhibitors were tested at three concentrations: 160 nM (orange), 321 nM (green) and 624 nM (blue).

Enzymatic evaluation was performed against MT1 at three concentrations (160, 321, 624 nM) (Figure 48). For reference, peptides **3** and **26** were tested. The results indicated that removal of individual amino acids (either Trp or Pro) caused a dramatic reduction in inhibitory potency toward MT1, both in analogues of inhibitor **3** (i.e. peptides **29** and **30**) and in analogues of inhibitor **26** (i.e. peptides **32** and **33**). Interestingly, only peptide **31**, lacking both Pro and Trp, exhibited activity comparable to that of the starting compound. An analogous elimination in the truncated peptide **26**, yielding peptide **34**, did not produce a compound with satisfactory inhibitory

potency, however it was more active than analogues with single deletions. These findings indicate that the simultaneous removal of both Trp3 and Pro11 from inhibitor **3** yields a more favorable outcome than eliminating mentioned residues individually. This suggests, that combined modifications within the binding loop can have a synergistic effect on inhibitory potency and may promote a more favorable conformational adjustment. Comparable results were obtained also for truncated peptide **26**, where simultaneous removal of both amino acids yielded better outcome than the deletion of single amino acids.

9.2.4. Analogue of peptide **3** with triazole bridge

Given the potential therapeutic application of MT1 inhibitors, in addition to stability in a proteolytic environment, the inhibitor should also exhibit stability under reducing conditions. As mentioned in Section 2.2.1.2., the presence of a disulfide bridge is required to maintain the proper conformation of the inhibitory binding loop. Any disruption in that spatial structure could result in loss of bioactivity. However, the disulfide bridge is prone to rapid cleavage in reducing environment, driven either by various molecules containing free thiol groups or by enzymes. Plasma proteins with free thiol groups, such as albumin, may also contribute to disulfide bond reduction.²⁶² A similar effect can be exerted by reduced glutathione, the most abundant non-protein thiol source in mammals, with a concentration in blood plasma of approximately 10 μM .²⁶³ To overcome the limitations of disulfide bonds in reductive environment I decided to introduce a triazole bridge as a more resilient alternative, maintaining the inhibitory loop conformation.

The triazole bridge was synthesized using click chemistry, as described in detail in Section 6.6.2. A schematic representation of this reaction is shown in Figure 49, where the structure of triazole bridge is presented.

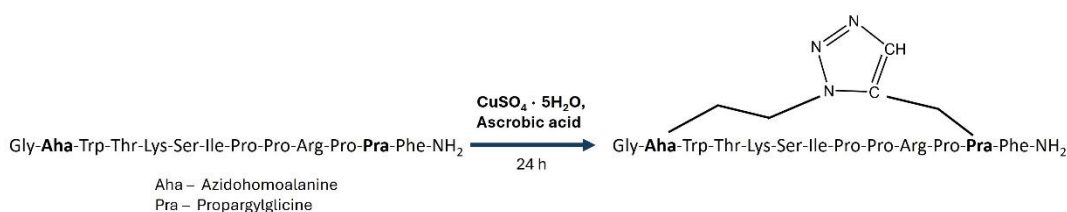


Figure 49. Scheme of synthesis of peptide 35.
 Aha - azidohomoalanine; Pra - propargylglycine

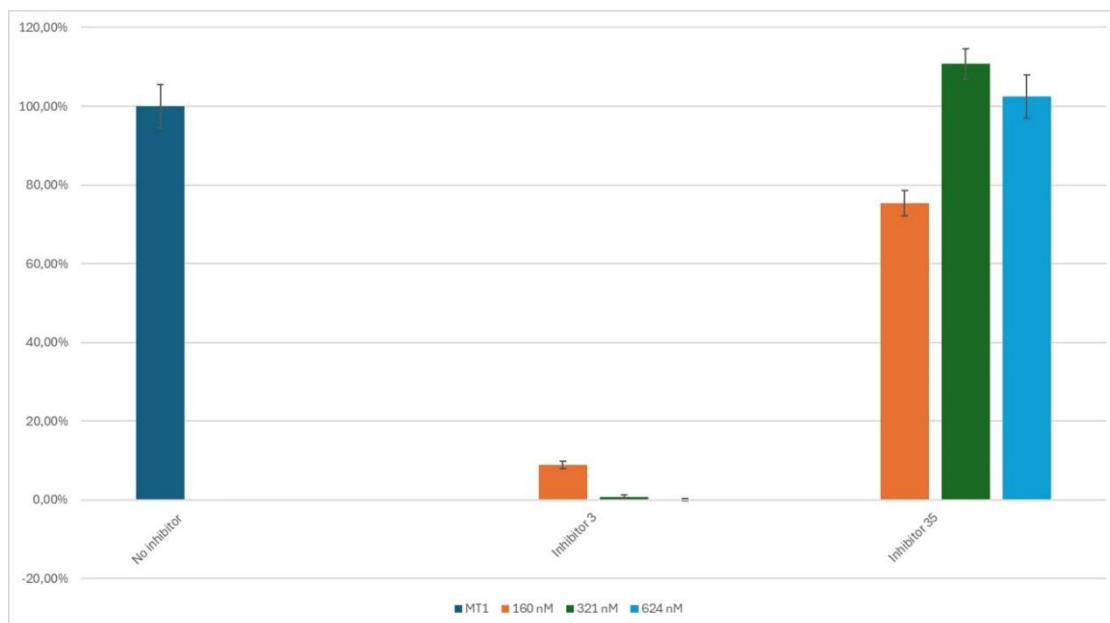


Figure 50. Results of enzymatic evaluation of inhibitor 3 and its analogue, peptide 35, with triazole bridge.

Inhibitors were tested at three concentrations: 160 nM (orange), 321 nM (green) and 624 nM (blue).

The obtained analogue (peptide **35**) was tested against MT1 at three concentrations (160, 321, 624 nM) (Figure 50). Unfortunately, compared to inhibitor **3**, replacement of the disulfide bridge had a negative impact on inhibitory activity, and peptide **35** did not reveal even similar activity to the parent compound.

Nevertheless, peptides **3** and **35** are currently tested for its potential anticancer properties toward MCF7 cell line, by dr inż. Natalia Maciejewska from Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University Technology. MCF7 cell line is well described human breast cancer cell line, in which both MT1 and MT2 are strongly overexpressed, as it was described in Sections 1.1.3.1. and 1.1.3.2.⁹⁰ This cell line was derived from a pleural effusion of a 69-year old Caucasian metastatic adenocarcinoma in 1970. It has estrogen, progesterone and glucocorticoid receptors and it is valuable in *in vitro* breast studies due to its ability to processing of estrogen in the form of estradiol, *via* estrogen receptors in the cytoplasm, which is a feature of mammary epithelium.²⁶⁴

The labeled analogue of inhibitor **3** (peptide **36**) will soon be provided to dr Natalia Maciejewska to evaluate its potential for imaging cancer cells.

9.2.5. Study of MT1 inhibitors' stability in serum

The following peptides were tested: **3**, **31**, **34**, **35**. The stability of inhibitor **3** had already been evaluated and described in 2020 by Gitlin-Domagalska *et al.*¹⁸⁷ However, due to its role as the lead compound for designing analogues in my dissertation, I also examined inhibitor **3** with its analogues under the same conditions. Peptides **31** and **34** were selected because they were shortened analogues and exhibited similar inhibitory activity (peptide **31**) or slightly lower (peptide **34**) to that of the parent compound. Whereas peptide **35** was chosen to assess whether the presence of a triazole bridge would affect proteolytic stability. The results of the proteolysis of the tested analogues are shown in the Table 8 and are graphically presented in Figure 51.

Table 8. Progress of proteolysis expressed as the percentage of the area under the peak corresponding to selected inhibitors.

Time of incubation [h]	Percentage of the area under the peak corresponding to the MT1 inhibitor			
	Peptide 3	Peptide 31	Peptide 34	Peptide 35
0	100	100	100	100
1	88.7 ± 3.7	96.0 ± 5.7	79.2 ± 0.1	93.5 ± 9.3
2	71.3 ± 6.9	78.4 ± 5.1	69.4 ± 1.6	65.7 ± 4.8
3	57.7 ± 0.3	70.9 ± 9.3	61.3 ± 2.3	32.2 ± 7.0
24	3.1 ± 0.6	21.6 ± 5.5	0	0

The hydrolysis results are the average of two replicates.

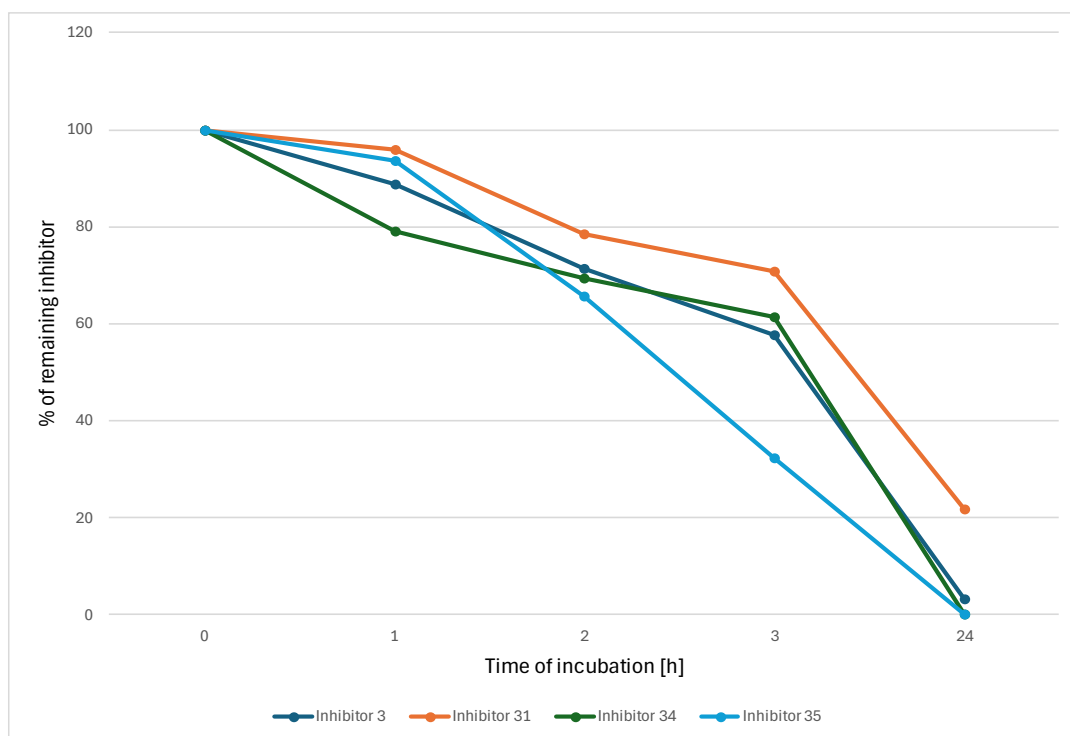


Figure 51. Stability of MT1 inhibitors (3, 31, 34, and 35) in human serum.

Results obtained after incubation inhibitor **3** with serum revealed that nearly half of the initial peptide amount was degraded after 3 h, and its presence after 24 h was negligible. An example of the hydrolysis progression for inhibitor **3** is shown in Figure 52. MS analyses performed at the beginning of the reaction and after 24 h are presented in Figure 53. The only identifiable hydrolysis product detected after 24 h corresponded to the peptide lacking the C-terminal Phe residue.

The stability assessment repeated in my dissertation did not confirm findings described in the aforementioned publication¹⁸⁷, where it was reported that after 3 h of incubation 23% of the initial amount of inhibitor **3** was hydrolyzed, and approximately 50% remained in serum after 24 h. This discrepancy may be attributed to serum source.

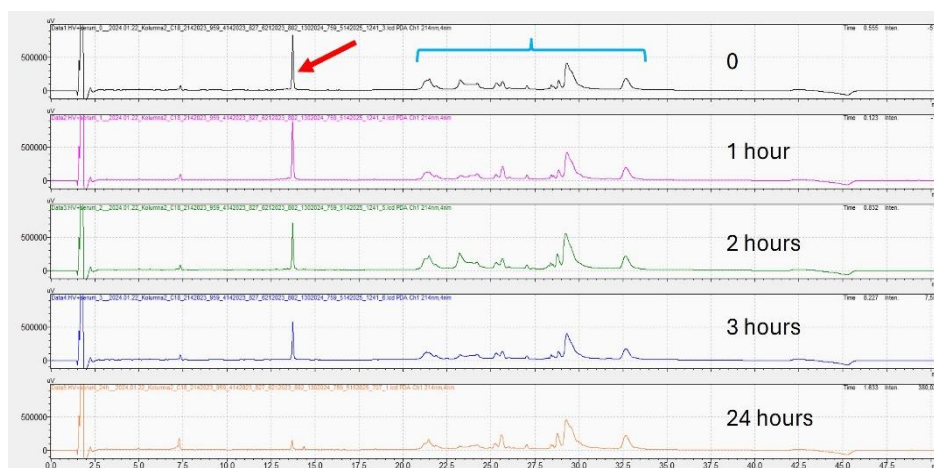


Figure 52. The progress of inhibitor 3 hydrolysis in serum.

Red arrow indicates inhibitor **3**, blue bracket indicates peaks from serum.

Shimadzu Prominence-I HPLC equipped with a Kinetex column; 10-90% B in 40 min.

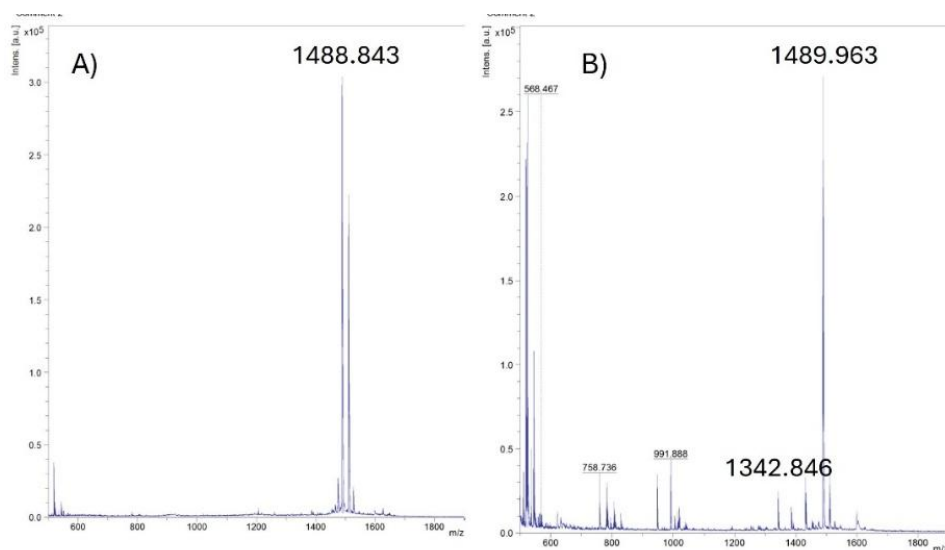


Figure 53. MS analyses of peptide 3 in serum at the initiation of the reaction (A) and after 24 h (B).

Peptide **3**: M = 1487.717; Calculated $[M+H]^+$ = 1488.933; Found $[M+H]^+$ = 1488.843.

Peptide **3** lacking Phe residue at C-terminus: M = 1341.632; Calculated $[M+H]^+$ = 1342.639; Found $[M+H]^+$ = 1342.846.

Peptide **31** (the analogue lacking Trp3 and Pro11) was the most stable among the tested peptides. After 3 h, approximately 71% of the peptide remained in serum, and after 24 h almost 21% of the initial peptide concentration was still detectable. Among the studies, this result was the most favorable. The other peptides were almost completely degraded after this incubation period. The MS analysis performed at the beginning of the reaction is shown in Figure 54A. The only identifiable hydrolysis product was a peptide lacking the *N*-terminal Gly residue, which was detected after 1 h (Figure 54B).

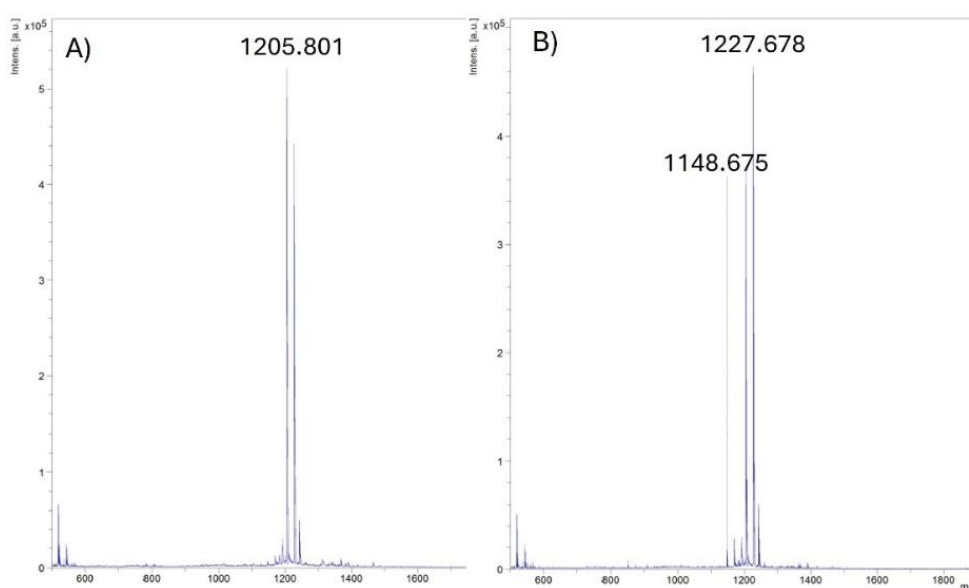


Figure 54. MS analyses of peptide 31 in serum at the initiation of the reaction (A) and after 1 h (B).

Peptide **31**: M = 1204.585; Calculated $[M+H]^+$ = 1205.812; Found $[M+H]^+$ = 1205.801; Calculated $[M+Na]^+$ = 1227.574; Found $[M+Na]^+$ = 1227.678

Peptide **31** lacking Gly residue at *N*-terminus: M = 1147.563; Calculated $[M+H]^+$ = 1148.570; Found $[M+H]^+$ = 1148.675.

Peptide **34** (the analogue lacking Trp3, Pro11, and Phe13) exhibited slightly worse stability compared to inhibitor **31**. The MS analysis performed at the initiation of its hydrolysis is shown in Figure 55A. The only identifiable hydrolysis product was a peptide lacking the *N*-terminal Gly residue, detected after 1 h (Figure 55B). After 24 h peptide **34** was completely hydrolyzed.

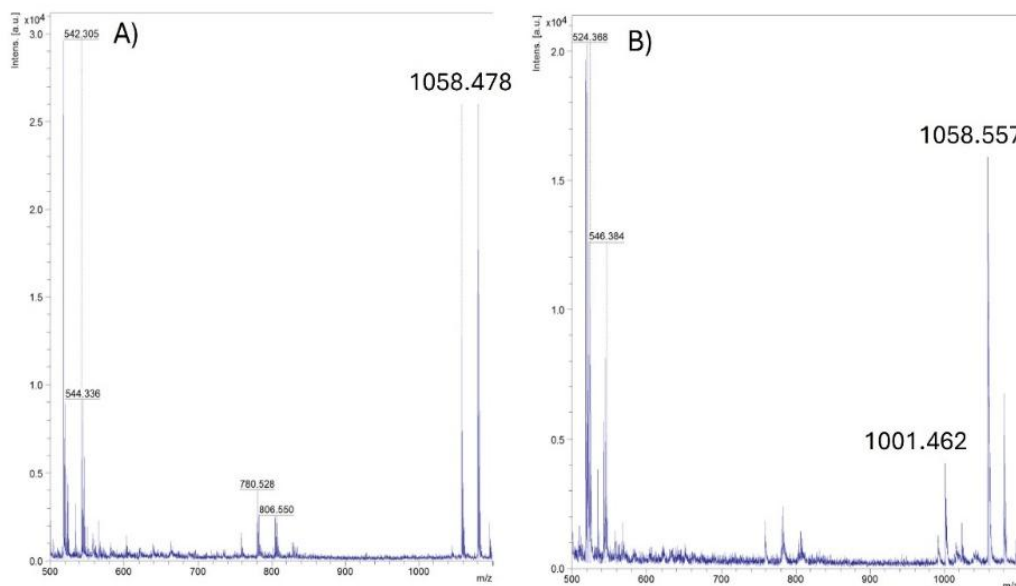


Figure 55. MS analyses of peptide 34 in serum at the initiation of the reaction (A) and after 1 h (B).

Peptide **31**: $M = 1057.516$; Calculated $[M+H]^+ = 1058.523$; Found $[M+H]^+ = 1058.478$;

Peptide **31** lacking Gly residue at *N*-terminus: $M = 1000.495$; Calculated $[M+H]^+ = 1001.502$; Found $[M+H]^+ = 1001.462$.

Interestingly, in contrast to inhibitor **3**, its triazole bridge analogue (inhibitor **35**) underwent hydrolysis within the binding loop. The identifiable hydrolysis products were peptides lacking the Lys5 and Ser6 residues ($M = 1307.689$) and lacking Trp3, Thr4, Lys5 and Ser6 ($M = 1020.562$), both detected after 1 h. Notably, no peptides missing terminal amino acids were observed. However, after 3 h it remained at only 32% and after 24 h it was completely hydrolyzed. MS analyses performed at the start of the hydrolysis reaction and after 1 h are shown in Figure 56.

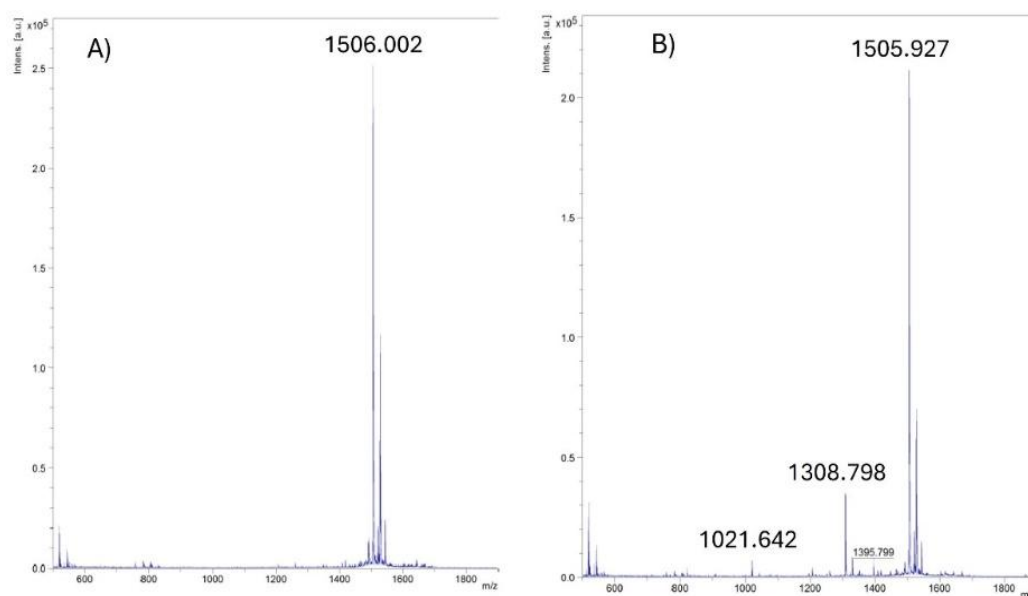


Figure 56. MS analyses of peptide 35 in serum at the initiation of the reaction (A) and after 1 h (B).

Peptide **35**: $M = 1504.805$; Calculated $[M+H]^+ = 1505.812$; Found $[M+H]^+ = 1506.002$;
 Peptide **35** lacking Lys5 and Ser6: $M = 1307.689$; Calculated $[M+H]^+ = 1308.696$; Found $[M+H]^+ = 1308.798$;
 Peptide **35** lacking Trp3, Thr4, Lys5 and Ser6: $M = 1020.562$; Calculated $[M+H]^+ = 1021.562$; Found $[M+H]^+ = 1021.642$;

To sum up, among 33 analogues of inhibitor **3** twelve contained a Phe13 substitution (peptides **4-15**), one had Gly1 replaced by Phe (peptide **16**), nine were obtained for alanine scanning (peptides **17-25**), and nine were designed to reduce the molecular weight (peptides **26-34**). In addition, one analogue had disulfide bridge replaced with a triazole bridge (peptide **35**), and one was labeled with Cf (peptide **36**). Among these derivatives, the shortened analogues (peptides **26** and **31**) exhibited activity comparable to that of inhibitor **3**. Notably, the lower-molecular-weight analogue (**31**) was also the most stable in human serum among all compounds examined.

10. Synthesis of MT2 inhibitor

Inhibitor **37** is the only compound in the series specifically designed to target MT2. This SFTI-1 analogue is labeled derivative of a potent MT2 inhibitor with $K_i = 19$ nM, previously described by Gitlin-Domagalska *et al.* in 2015 (Figure 57A).⁹⁶ Peptide **37** (Figure 57B) features a Lys residue with a coupled Cf in its side chain, replacing the original Asp.



Figure 57. Structures of potent MT2 inhibitor⁹⁶ (A) and its new, labeled analogue, inhibitor 37 (B).

Modified position in inhibitor **37** is marked by bold.

The starting compound and its analogue, similarly to MT1 inhibitors **1** and **2** were evaluated for their inhibitory potency by Canadian group at two concentrations: 0.1 and 10 μM toward MT1 and MT2. The obtained results (Figure 58) indicated that both compounds exhibit comparable inhibitory potency.

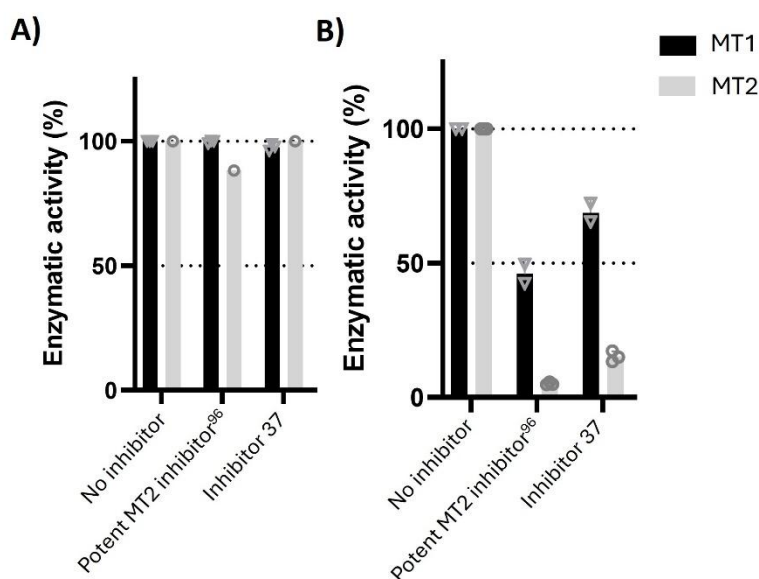


Figure 58. Preliminary inhibition assay of inhibitor 37 at 0.1 μM (A) and 10 μM (B).

The purpose for obtaining this inhibitor was to achieve potential molecular probe for imaging breast cancer cells. As it was mentioned previously, in Section 1.1.3.2., expression of MT2 was found only in a few breast cancer cell lines: MCF7 and ZR-751.⁹⁰ Inhibitor **37** was tested by dr Anna Kloska from Department of Medicinal Biology and Genetics of Faculty of Biology University of Gdańsk on MCF7 cells. Preliminary fluorescence microscopy studies have shown that inhibitor binds to the membranes of MCF7 breast cancer cells (Figure 59). This finding highlights peptides **37** potential as a molecular probe for targeted imaging applications.

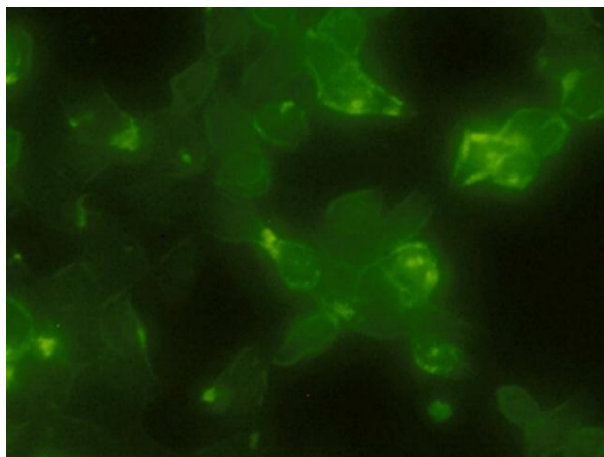


Figure 59. Fluorescence microscopy image showing peptide 37 attachment to the membrane of MT2-rich MCF7 cells.

11. Design and enzymatic evaluation of furin inhibitors

Another enzyme that was subject of my doctoral research was furin. In 2015 Fittler *et al.*¹⁸² published a potent furin inhibitor based on the SFTI-1 structure (Figure 60A). Using the crystal structure of furin (PDB: 1P8J), which reveals a highly negative charge surrounding the active site, combined with molecular dynamics studies, they developed its inhibitor with remarkable potency ($K_i = 0.49$ nM), the structure of which is shown in Figure 60B. The presence of Lys residue in the P1 position enabled electrostatic interactions between its positively charged side chain and Asp258 and Asp306 located in the S1 pocket of furin.¹⁸²

Fittler's results were utilized by our group²⁴⁸ to generate a peptide library using a combinatorial chemistry approach with iterative deconvolution. Our primary goal was to identify preferred amino acids at the P1, P2, P4 and P5 positions (Figure 60C). At the P1 position only two proteinogenic amino acids (Lys and Arg) were introduced, whereas at the other positions, ten different residues were evaluated: Lys, D-Lys, ornithine (Orn), Phe(4-NH₂), 4-guanidinophenylalanine (Gnf), Arg, D-Arg, homoarginine (HArg), 2-amino-3-guanidinopropionic acid (Agb), citrulline (Cit). We screened each sublibrary at a concentration of 2.2 ng/ml for inhibitory activity against human recombinant furin using the fluorogenic substrate Pyr-Arg-Thr-Lys-Arg-AMC (where Pyr denotes pyroglutamic acid).

The first library consisted of 10 sublibraries, each containing a single amino acid in P5 position. After screening we observed no significant preferences. Only Arg exhibited slightly better activity than the other residues and was therefore selected. The next library, with Arg fixed at the P5 position, again comprised 10 sublibraries. In this step Arg and Orn demonstrated the most potent activity toward furin. However, more detailed analysis revealed that Arg at the P4

position provided nearly 2-4 times stronger inhibition of the enzyme, leading to its selection for that position. In the subsequent step, analysis of the P2 position was performed. Screening revealed preferences for Orn and Lys, with a slight advantage for Lys. Due to the comparable properties of both variants, both were selected for further evaluation.

Finally, we screened the P1 position with only two residues examined based on known furin substrate specificity. The most active was inhibitor containing Lys at the P1 position. Subsequently, peptides with Arg fixed at P5 and P4 positions, Lys or Orn at P2 position, and Lys at P1 were tested. It was found that the analogue with Lys at P2 was 2-3 times more active than that with Orn. Consequently, the peptide presented in Figure 60D was selected for further modifications.²⁴⁸

According to the structural data published by Dahms *et al.*²⁶⁵, who indicated that the presence of a basic amino acid residue at the P6 position may enhance inhibitor interaction with furin, I synthesized analogues with Lys or Arg introduced at the *N*-terminus. Moreover, many natural furin substrates and some synthetic inhibitors (such as polyarginines and polylysines described in Section 2.4.) contain basic amino acids at the P5 and P6 positions. Another modification was additional backbone cyclization, aimed at obtaining peptides more resistant to proteolysis.²⁴⁸ The structures of peptides synthesized by me are presented in Figure 60E and labeled analogues obtained for biological studies are shown in Figure 60F.

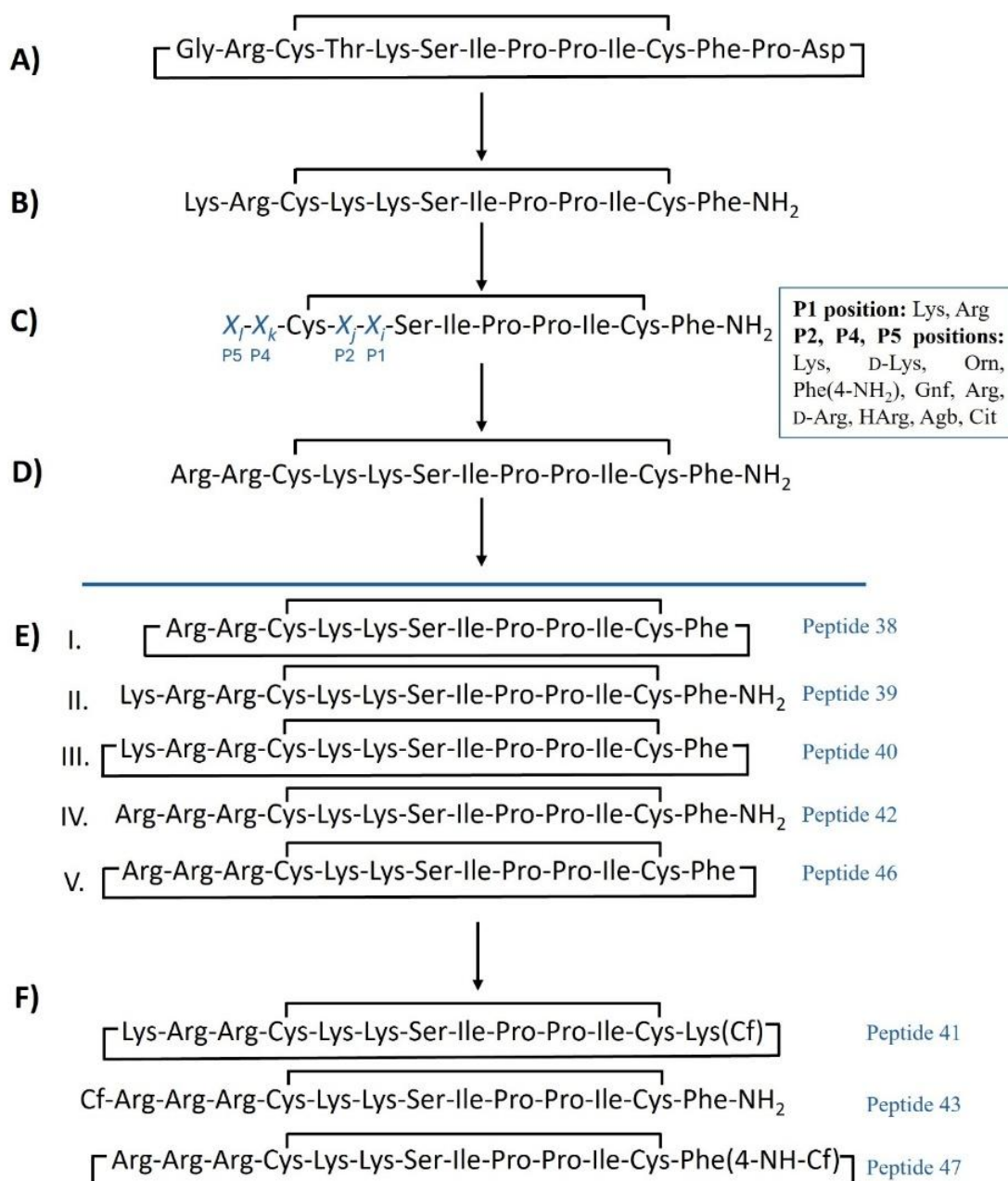


Figure 60. Schematic representation of the development of novel furin inhibitors. Structures of sequentially modified peptides: SFTI-1 (A); furin inhibitor obtained by Fittler et al.¹⁸² (B); general formula of the peptide amide library with presented amino acids which were introduced in positions P1, P2, P4, P5 (C); peptide selected through combinatorial chemistry (D); peptides I-V with an additional basic amino acid at P6 position and/or backbone cyclization (E); labeled analogues for biological studies (F). Inhibitors obtained in this dissertation are shown below the blue line.
 Orn - ornithine; Phe(4-NH₂) - 4-amino-L-phenylalanine; Gnf - 4-guanidinophenylalanine; Arg, D-Arg, HArg – homoarginine; Agb - 2-amino-3-guanidinopropionic acid; Cit - citrulline

I performed the enzymatic assay under the supervision of dr Agata Gitlin-Domagalska. Peptides were screened at three concentrations (2.4, 24.0, and 77.0 nM), and the results of this evaluation are presented in Figure 61. Additionally, the inhibitor described by Fittler *et al.*¹⁸² (inhibitor B) and the inhibitor selected through combinatorial chemistry (inhibitor D) were tested. Inhibitors B and D exhibited similar inhibitory activity toward furin. The weakest inhibitor was peptide **38**, which was the only compound unable to completely inhibit enzyme activity at 77.0 nM. Peptides sharing the same sequence, but differing in the presence of backbone cyclization (**42** and **46**) exhibited similar activity. However, another pair of peptides (**39** and **40**) did not show such comparable inhibitory potency. For the most promising inhibitors (**40**, **42**, **46**) and for the starting compound (inhibitor B) K_i were calculated (Table 9).²⁴⁸

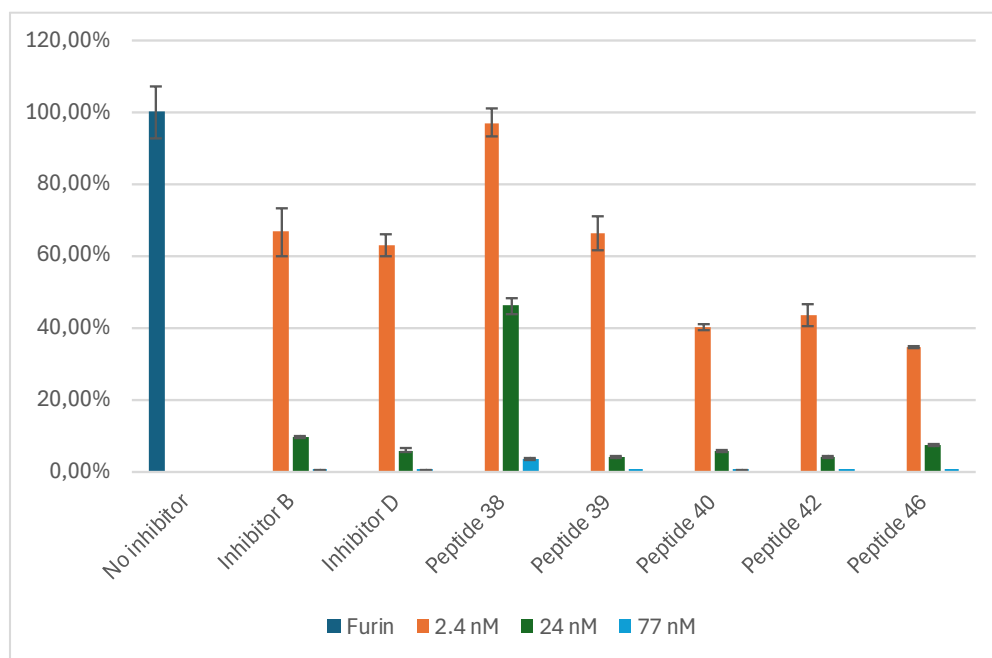


Figure 61. Results of enzymatic evaluation of furin inhibitors.

Inhibitors were tested at three concentrations: 2.4 nM (orange), 24 nM (green) and 77 nM (blue).

Table 9. Inhibitory constants (K_i) calculated for selected furin inhibitors.

Peptide	K_i [nM]
Inhibitor B	0.38 ± 0.02
40	0.21 ± 0.01
42	0.27 ± 0.02
46	0.25 ± 0.01

All tested inhibitors exhibited subnanomolar inhibitory activity. Moreover, inhibitors **40**, **42**, **46** demonstrated improved potency compared with the starting compound (inhibitor B). Their K_i

values were, averaging around 0.2-0.3 nM. The most potent inhibitor was the analogue with an additional Lys residue at the *N*-terminus (inhibitor **40**), which showed a K_i of 0.21 nM.

Moreover, the selectivity of the inhibitors presented in Table 9 was evaluated. Due to fact that furin is transmembrane protease, activity toward other transmembrane proteases, MT1 and MT2, was examined. Inhibitors were tested at three concentrations (3.85, 38.5, 385 nM) against recombinant enzymes using the fluorogenic substrate Boc-Gln-Ala-Arg-AMC. All inhibitors were inactive against MT1 (Figure 62A), and displayed only negligible activity toward MT2 (Figure 62B) in all tested concentrations. These results confirmed the selectivity of the obtained inhibitors toward furin.²⁴⁸

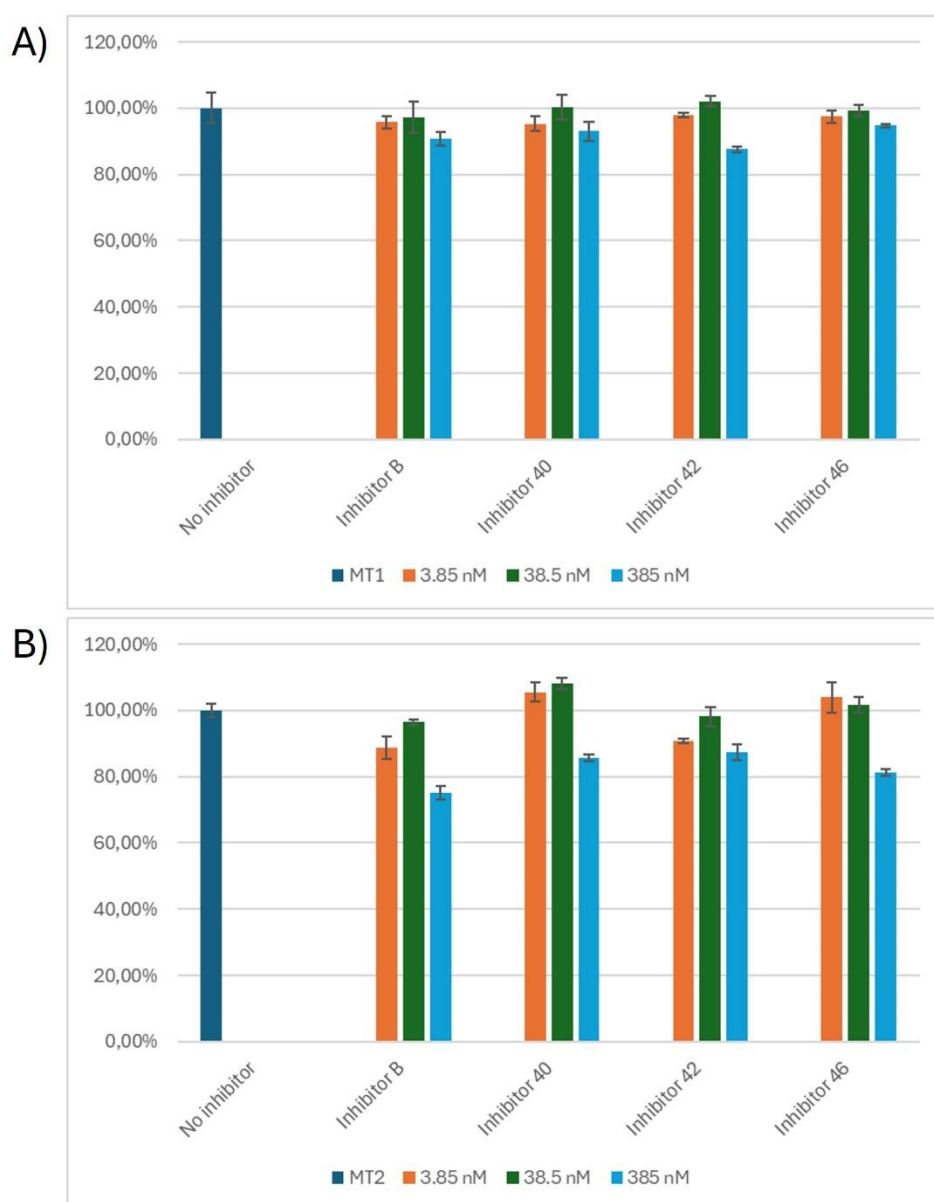


Figure 62. Results of enzymatic evaluation of furin inhibitors against MT1 (A) and MT2 (B).

Inhibitors were tested at three concentrations: 3.85, 38.5, and 385 nM.

I also performed evaluation of proteolytic stability of most potent inhibitors **40**, **42** and **46** according the method described in detail in Section 8. Results of this study are shown in Figure 63. As expected, bicyclic peptides (**40** and **46**) were less prone to degradation, compared to monocyclic peptides. A comparison of two analogues- the monocyclic inhibitor **42** and its bicyclic analogue **46**- illustrates this relationship clearly: inhibitor **42** was completely degraded after 24 h, whereas 28% of the initial concentration of inhibitor **46**, remained after 48 h.²⁴⁸

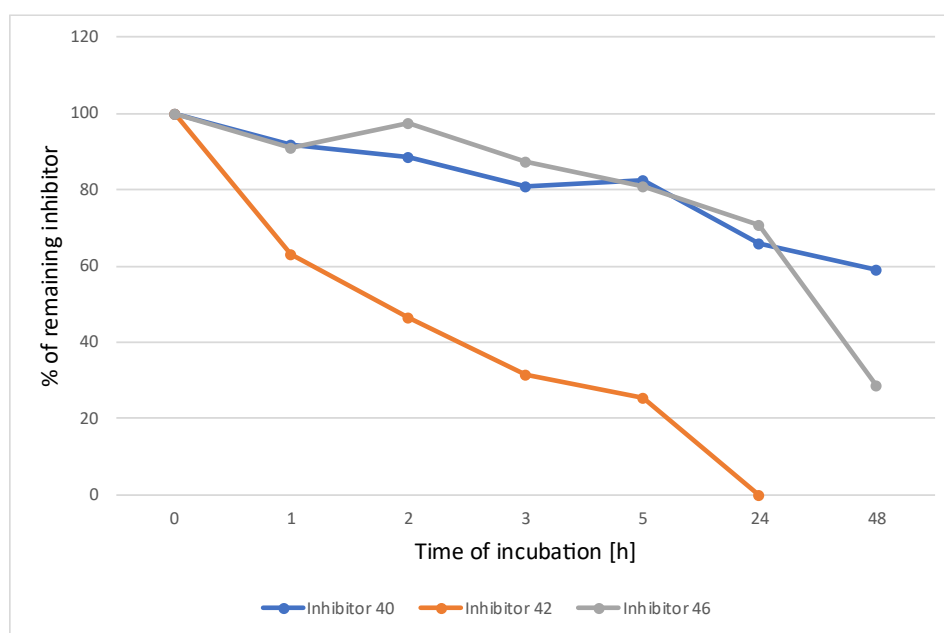


Figure 63. Stability of furin inhibitors in human serum.

11.1. Antiviral properties of furin inhibitors **40**, **42**, and **46**

As described in detail, in Section 1.1.4.1.2., furin is involved in the activation of viral glycoproteins and, consequently, contributes to viral pathogenicity. In flaviviruses, it cleaves prM protein, which facilitates the formation of mature, infectious viral particles.

Therefore, activity of the most potent furin inhibitors (**40**, **42**, and **46**) was tested against a member of the flavivirus group- ZIKV. All biological studies were conducted by dr Gabriela Brzuska and dr hab. Ewelina Król, prof. UG from the Laboratory of Recombinant Vaccines, and dr hab. Andrea Lipińska from the Laboratory of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk. This Section presents only selected, preliminary results of these studies.

As an initial step, the antiviral activity of all compounds was compared with the commercially available, non-specific furin inhibitor Dec-Arg-Val-Lys-Arg-CMK (described in detail in Section 2.4.) as a positive reference. Vero E6 cells were infected with ZIKV and treated with the inhibitors

at a concentration 50 μM for 48 h. The release of infectious virus progeny from the cells were quantified using a plaque assay, which revealed a reduction in viral titers for all tested inhibitors (Figure 64A). Furthermore, cell lysates were subjected to SDS-PAGE followed by immunoblotting with specific antibodies (Figure 64B).

The obtained results revealed that inhibitor **40** exhibited the most pronounced effect, reducing viral titers by approximately 3 log units. Inhibitors **46** and Dec-RVKR-CMK showed comparable activity, although they were slightly less effective than peptide **40**, whereas inhibitor **42** demonstrated the weakest reduction. Furthermore, inhibitor **40** significantly decreased ZIKV protein levels in cell lysates. However, no notable changes were observed in the prM to M protein ratio.

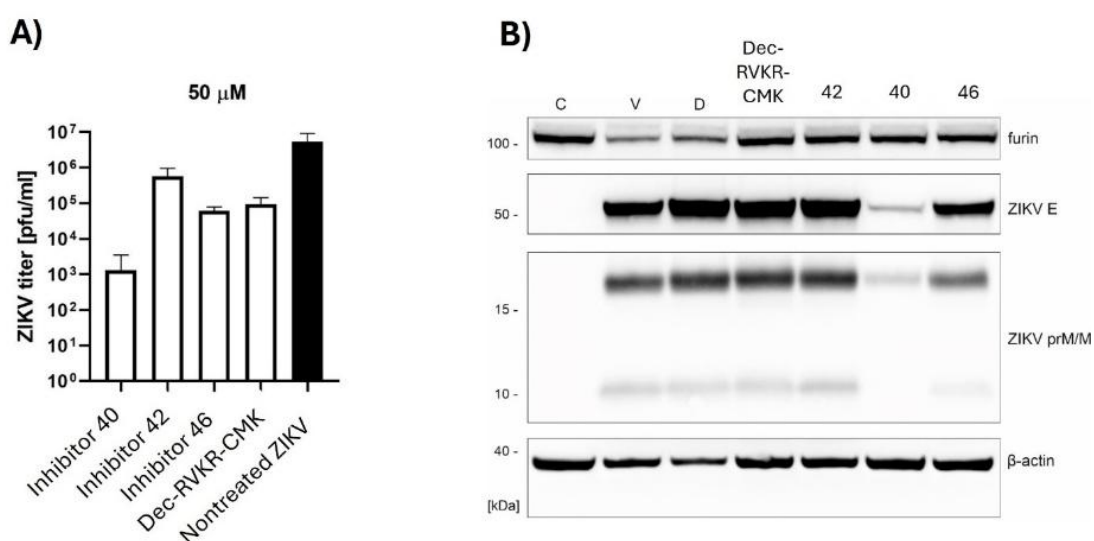


Figure 64. Comparison of antiviral activity of inhibitors 40, 42, 46 and commercially available furin inhibitor (Dec-RVKR-CMK) (A). Cell lysates were subjected to SDS-PAGE followed by immunoblotting with specific antibodies (B).

A) Vero E6 cells were infected with ZIKV (MR766 strain, MOI 0.1) and treated with inhibitors (50 μM) for 48h. The viral titers in the culture media were determined using the plaque assay.

B) C - untreated cells; V - untreated infected cells; D - DMSO-treated infected cells.

In further studies, the most potent inhibitor, inhibitor **40**, was tested. Its cytotoxicity was evaluated across a wide range of concentrations in the Vero E6 cell line. Vero E6 cells were incubated with inhibitor **40** (dissolved in PBS buffer, at concentrations 25-800 μM) for 48 h. After incubation, cell viability was assessed using the commercially available kit CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay (MTS). Figure 65 shows the viability of cells treated with inhibitor **40** compared to cells treated only with PBS buffer. Cell viability was approximately 90%, indicating that the inhibitor exhibited minimal cytotoxicity.

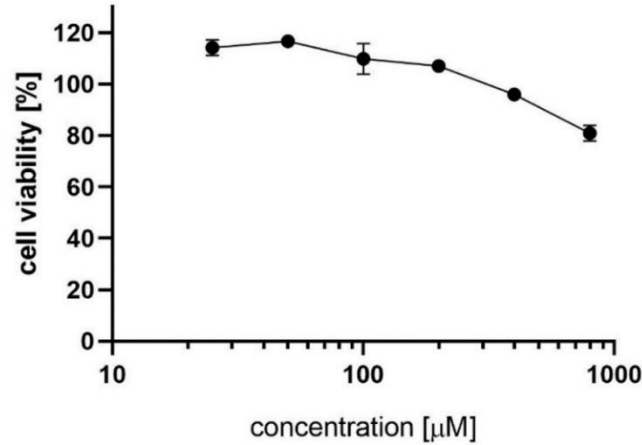


Figure 65. Cytotoxicity assessment of inhibitor 40, presented as viability of Vero E6 cell line after treatment with the peptide compared to the viability of cells treated only with PBS buffer.

Furthermore, the antiviral activity against ZIKV was assessed using two cell lines: Vero E6 and A549. Cells were infected with two viral strains (MR766 and H/PAN/2016) at a multiplicity of infection (MOI) of 0.1 MOI and subsequently incubated with inhibitor **40** (at concentrations 5 and 50 μ M in PBS buffer) for 48 h. After incubation, the viral titer released into the culture medium was determined, and the presence of viral proteins (pr, M, and E) within the cells was analyzed. In both cell lines, a reduction in the released viral titer was observed, particularly at the 50 μ M concentration (4 $\log_{10}$ in Vero E6 and 2 $\log_{10}$ in A549) (Figure 66).

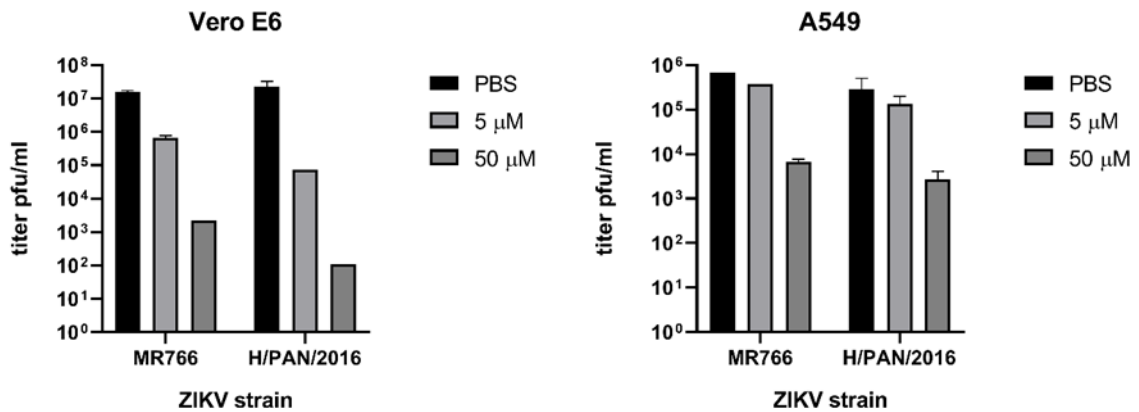


Figure 66. Antiviral assessment of inhibitor 40 against two cell lines (Vero E6 and A549) and strains of ZIKV.

Vero E6 and A549 cells were infected with ZIKV (two strains: MR766 and H/PAN/2016) at 0.1 MOI and then incubated with inhibitor 38 (50 μ M and 5 μ M) in PBS for 48 h. The virus was released into culture medium and then it was titrated on Vero E6 cells using the plaque method.

Moreover, Western blot analysis revealed that inhibitor **40** reduced the levels of viral proteins: E, pr, and M in treated cells (Figure 67).

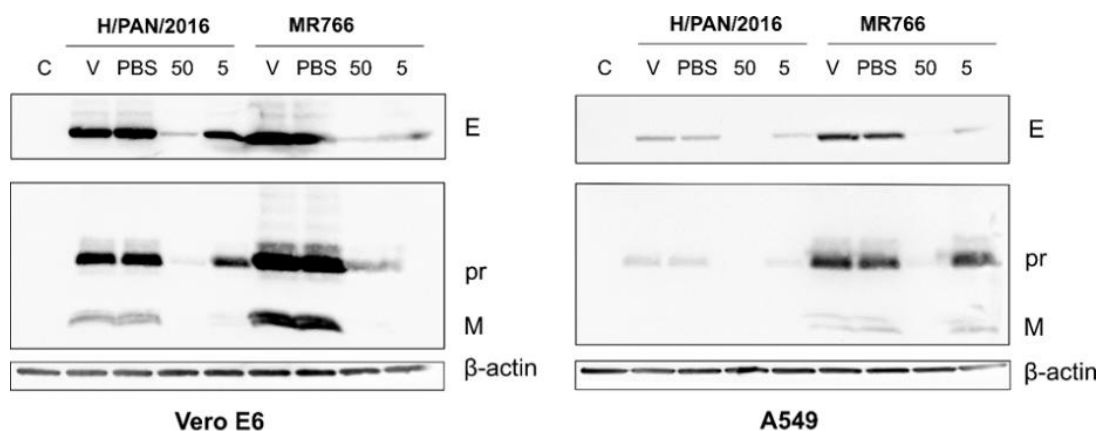


Figure 67. Western blot analysis of viral proteins (pr, M, and E) in cell lysates after infection and after treatment with inhibitor **40 (50 μ M and 5 μ M) in PBS for 48 h.** C - untreated cells; V - untreated infected cells with ZIKV (MOI 0.1)

In the next step, the activity of inhibitor **40** against ZIKV was evaluated across a wide range of concentrations (1.56 - 100 μ M). Vero E6 cells were infected with ZIKV (H/PAN/2016) and subsequently treated with the peptide for 48 h. After this time, the viral titer was determined again (Figure 68). The inhibitor demonstrated potent antiviral activity.

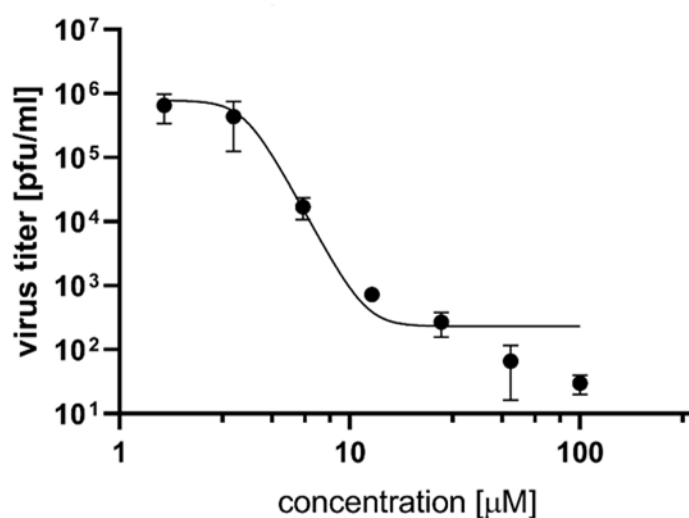


Figure 68. Antiviral activity of inhibitor **40 against ZIKV across a wide range of inhibitor concentrations.**

Vero E6 cells were infected with ZIKV (H/PAN/2016) (MOI 0.1) and then incubated with inhibitor **40** (1.56 - 100 μ M) in PBS for 48 h. Virus released into the culture medium was titrated by the plaque method.

Table 10. Antiviral activity of furin inhibitors against ZIKV.

Inhibitor	IC ₅₀ [μM]	CC ₅₀ [μM]	SI
Dec-RVKR-CMK	9.06	615.20	67.90
40	3.69	1303.00	353.12
42	22.25	707.30	31.79
46	13.45	545.80	40.58

IC₅₀ - concentration of inhibitor necessary to reduce the number of infected cells by 50%; CC₅₀ - cytotoxic concentration 50%; SI - selectivity index (SI = CC₅₀/ IC₅₀).

To compare the relative antiviral potency of each inhibitor, IC₅₀ values were calculated based on the reduction in ZIKV titers (Table 10). Inhibitor **40** demonstrated the lowest IC₅₀ value (3.69 μM), nearly three times lower than that of the reference inhibitor Dec-RVKR-CMK. Inhibitor **42** exhibited the lowest antiviral activity and its bicyclic analogue, inhibitor **46**, was slightly less active than reference inhibitor. These findings indicate that the inhibitor **40** possesses the most potent antiviral activity among the tested compounds. Studies of cytotoxicity revealed that inhibitor **40** exhibited extremely low cytotoxicity, in compare to other compounds. It was over two times less cytotoxic than Dec-RVKR-CMK. The crucial parameter in drug discovery is selectivity index (SI) which represents correlation between cytotoxicity and antiviral activity of examined compound. SI is calculated as ratio of the cytotoxic concentration 50% (CC₅₀) to the 50% inhibitory concentration (IC₅₀). Combination of a low cytotoxicity with a high antiviral properties yield in high SI. Inhibitor **40** exhibited the highest SI (353.12), nearly six times higher than SI for Dec-RVKR-CMK, and nearly eleven and eight higher than for inhibitors **42** and **46**, respectively. These very promising results prompted us to apply for patent protection.

Labeled analogues (peptides **41**, **43**, **47**) were synthesized to investigate their incorporation into cellular models. For this purpose, blood-brain barrier (BBB) permeability assays were performed using astrocytes. Prior to the experiment, transepithelial/transendothelial electrical resistance (TEER) measurements were carried out to assess BBB integrity. At the initial stage, peptide **41** was dissolved in vascular endothelial serum free media, and subsequently diluted to final concentrations 300, 100, and 10 μM. The assays were then conducted for 4, 24, 48, or 72 h. The results indicated that the incorporation of peptide **41** is strictly correlated with the duration of incubation and concentration of samples. At 10 μM peptide **41** exhibited minimal penetration, even after 72 h. At 100 μM (Figure 69) part of the cells showed positive staining after 4 h (Figure 69A). After 24, 48, and 72 h, all cells were positive, with fluorescence intensity increasing over time (Figure 69 B-D). Strong signals were also observed at the concentration of 300 μM. Peptides **43** and **46** were evaluated only at 100 μM and also exhibited the ability to permeate cells.

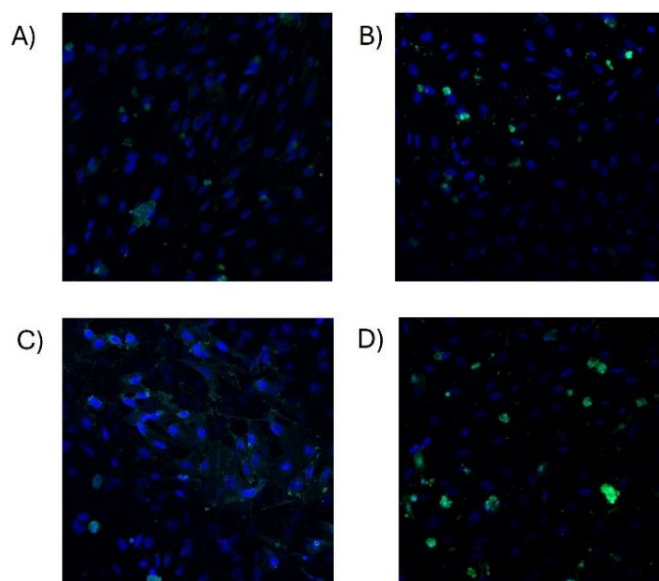


Figure 69. Fluorescence microscopy images illustrating the ability of peptide 41 (100 μ M) to permeate into astrocytes after different incubation periods: 4 h (A), 24 h (B), 48 h (C), and 72 h (D).

The blue signal corresponds to nuclei stained with 4',6-diamidino-2-phenylindole (DAPI).

Additional tests were performed, including assessment of activity inhibitor **40** against other flaviviruses (WNV and TBEV). Inhibitor **40** inhibited replication of WNV but did not exhibit activity toward TBEV.

11.2. Inhibitor **42** analogues with disulfide bridge mimetics

Similarly to HV-BBI analogue, also in case of furin inhibitors the disulfide bridge was replaced by its mimetics in order to enhance proteolytic stability. Inhibitor **42** (slightly less active than **40**) was selected for modification. The reason was purely technical. It is much easier to introduce the planned modification into the monocyclic structure than to the bicyclic one. Two analogues were obtained, however as mentioned in Section 6.7.3., peptide with diselenide bridge was synthesized by dr hab. Dawid Dębowski, prof. UG and my task was to purify crude compound. The structures of obtained analogues are presented in Figure 70.

The obtained analogues (peptides **44** and **45**) were tested against furin at three concentrations (0.077; 0.77; 7.7 nM) (Figure 71). Introduction of disulfide bridge mimetics resulted in deterioration of inhibitory activity. However, similarly to the HV-BBI analogue with triazole bridge (peptide **35**), also here substitution of disulfide bridge with triazole bridge (peptide **42**) resulted in the weakest inhibition of furin. The analogue containing a diselenide bridge (peptide **43**) had the least effect on inhibitory activity, displaying potency comparable to the parent compound (inhibitor **40**) at a concentration of 77 nM.

These peptides' potential anticancer properties were evaluated and the results are described in Section 11.2.1.

11.2.1. Anticancer properties of furin inhibitors **42**, **44**, and **45**

As described in detail, in Section 1.1.4.1.1., because furin is involved in the activation of numerous proteins in the human body, its abnormal activity can promote the development and progression of various malignancies.

Therefore, peptides **42**, **44**, and **45** were tested against cell lines known for furin overexpression and used in studies assessing furin levels. Two cell lines were utilized: U251²⁶⁶ and A549^{267,268}. U251 cells are human neuronal glioblastoma, whereas A549 cells represent human lung adenocarcinoma. In addition, the inhibitors were tested against kidney-derived cell line (293 HEK). All studies were performed by to Dr Beata Pyrzyńska from the Department of Biochemistry, Faculty of Medicine, Medical University of Warsaw. As a positive control, the well-known commercially available furin inhibitor Dec-RVKR-CMK, previously mentioned in Section 11.1., was used. Inhibitors were dissolved in PBS buffer and tested at six concentrations (0, 5, 10, 25, 50, 100 μ M). Dec-RVKR-CMK was not examined at the highest concentrations.

Cells were plated in 96-well plates with 5% fetal bovine serum (FBS) buffer and treated with the inhibitors. After three days, absorbance was measured at 450 nm using the Cell Counting Kit-8. Each concentration was tested in triplicate, and the average values were calculated. The results are presented in Figure 72.

The greatest activity against U251 and A549 cells was exhibited by inhibitor **45**. Inhibitor **44** showed slightly lower activity, while inhibitor **42** was completely inactive. This may be due to the reducing environment within cells, which can cleave the disulfide bridge, whereas the resistant analogues retained their ability to kill cancer cells. Unexpectedly, the assumed positive reference (Dec-RVKR-CMK) also did not exhibit any anticancer activity. This finding suggests that anticancer properties cannot be clearly attributed to furin inhibition. Furthermore, none of the inhibitors demonstrated cytotoxicity toward 293 HEK cells.

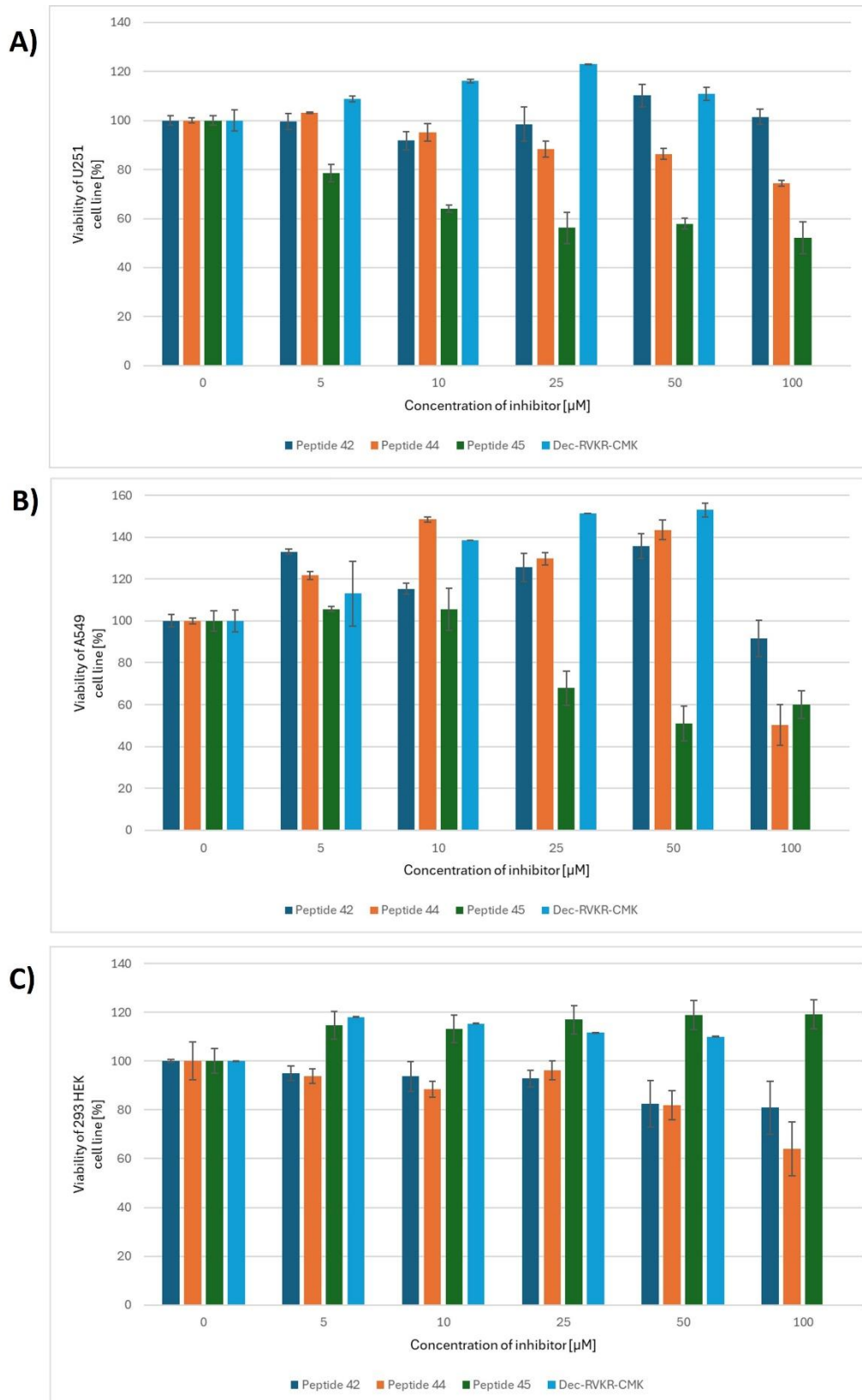


Figure 72. Viability of cells: U251 (A), A549 (B), and 293 HEK (C) after incubation with furin inhibitors.

Inhibitors were evaluated at the following concentrations: 5, 10, 25, 50, and 100 μM. Dec-RVKR-CMK was not tested at 100 μM.

11.3. Study of furin inhibitors' stability in serum

Proteolytic stability of two furin inhibitors was tested, i.e. **42** and **44** (analogue with triazole bridge). The stability of inhibitor **42** had already been evaluated and is described in Section 11 (Figure 63) as well as in our published work²⁴⁸. However, because this compound served as the starting point for the design of analogue with substituted disulfide bridge, I also examined it under the same experimental conditions as inhibitor **44**. The results of the hydrolysis of the tested analogues are shown in the Table 11 and are graphically presented in Figure 73.

Table 11. Progress of hydrolysis expressed as percentage of the area under the peak corresponding to inhibitor 42.

Time of incubation [h]	Percentage of the area under the peak corresponding to the furin inhibitor	
	Inhibitor 42 [%]	Inhibitor 44 [%]
0	100	100
1	69.9 ± 9.4	67.9 ± 1.2
2	58.2 ± 3.2	27.5 ± 2.0
3	42.4 ± 9.3	21.2 ± 2.8
24	0	0

The hydrolysis results are the average of two repetitions.

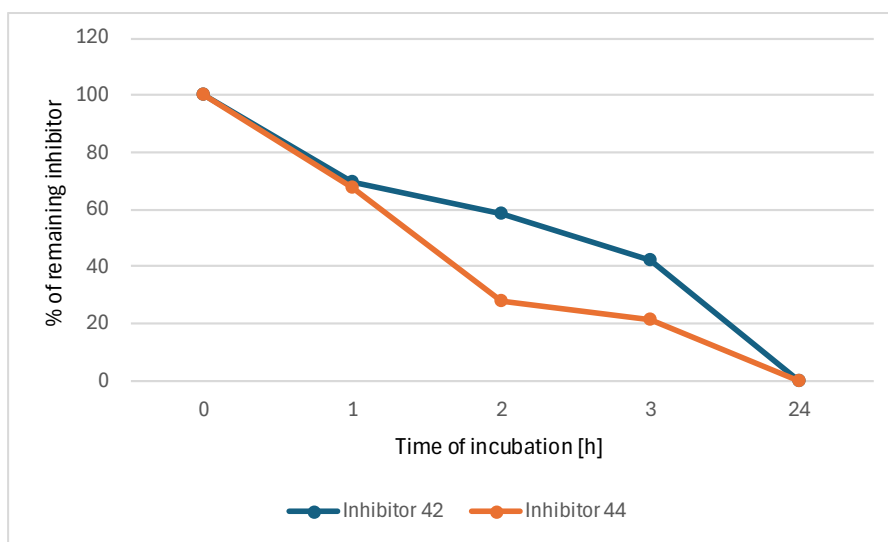


Figure 73. Stability of furin inhibitors (42 and 44) in human serum.

Repeated studies on the proteolytic resistance of inhibitor **42** yielded results nearly identical to those shown in Figure 63. In both experiments, after 1 h about 60-70% of the initial inhibitor concentration remained intact, after 3 h only 30-40% was still present, and after 24 h inhibitor **42**

was completely hydrolyzed. The MS analysis performed at the initiation of the hydrolysis reaction is presented in Figure 74A. After 2 h (Figure 74B), peptides lacking Arg1, as well as lacking both Arg1 and Arg2 were detected. No analogue missing all three Arg residues was observed.

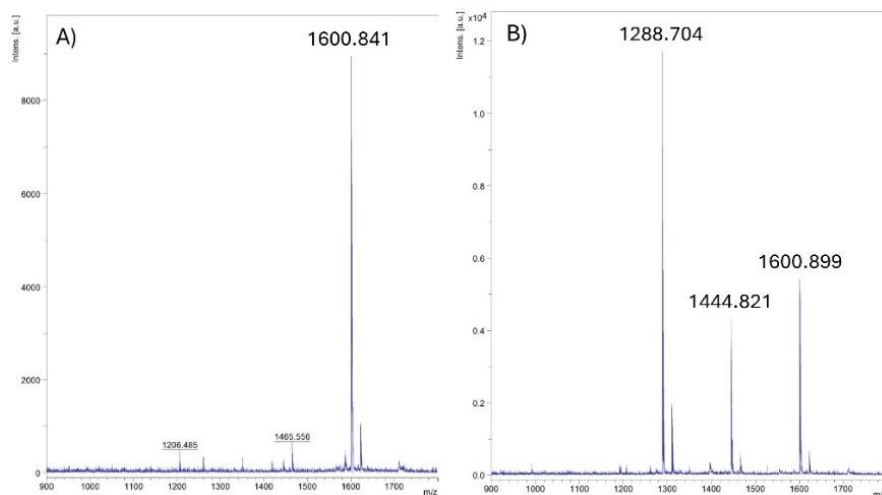


Figure 74. MS analyses of peptide 42 in serum at the initiation of the reaction (A) and after 2 h (B).

Peptide **42**: M = 1599.897; Calculated $[M+H]^+$ = 1600.904; Found $[M+H]^+$ = 1600.841;
 Peptide **42** lacking Arg1: M = 1443.796; Calculated $[M+H]^+$ = 1444.803; $[M+H]^+$ = 1444.821;
 Peptide **42** lacking Arg1 and Arg2: M = 1287.694; Calculated $[M+H]^+$ = 1288.701; Found $[M+H]^+$ = 1288.704.

Comparison of the serum stability of the disulfide-bridged analogue (inhibitor **42**) and the triazole-bridge peptide (**44**), showed that the modification of the bridge had only a minor impact on overall stability. Both inhibitors (**42** and **44**) underwent similar degrees of degradation after 1 h. However, with increasing incubation time, degradation of inhibitor **44** progressed more rapidly, with only 27.5% of inhibitor **44** remaining intact after 2 h, compared with 58.2% of inhibitor **42**. Both were completely hydrolyzed after 24 h. The MS analysis performed at the initiation of the hydrolysis reaction for peptide **44** is presented in Figure 75A. After 1 h (Figure 75B), peptides lacking Arg1 and lacking both Arg1 and Arg2 were detected, similar to the pattern observed for inhibitor **42**. As before, no analogue missing all three Arg residues was detected. Furthermore, in contrast to the trypsin assay described in Section 6.7.2.1.2., no hydrolysis within the binding loop was detected. After 24 h, inhibitor **44** was completely degraded.

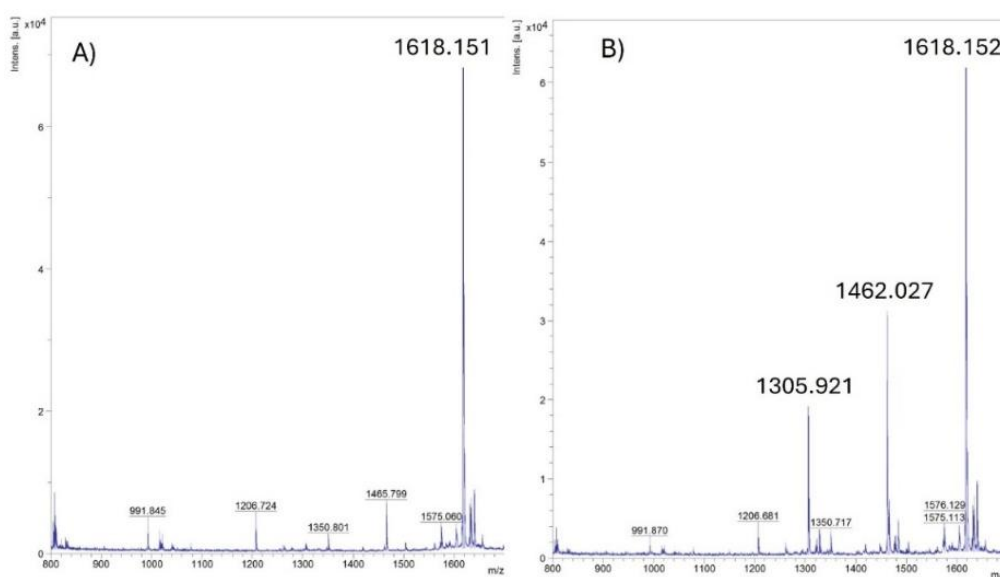


Figure 75. MS analyses of peptide 44 in serum at the initiation of the reaction (A) and after 1 h (B).

Peptide **44**: $M = 1616.958$; Calculated $[M+H]^+ = 1617.992$; Found $[M+H]^+ = 1618.151$;

Peptide **44** lacking Arg1: $M = 1460.884$; Calculated $[M+H]^+ = 1461.891$; $[M+H]^+ = 1462.027$;

Peptide **44** lacking Arg1 and Arg2: $M = 1304.783$; Calculated $[M+H]^+ = 1305.790$; Found $[M+H]^+ = 1305.921$.

11.4. Conformational analysis and structural organization of inhibitors **42** and **46**

HPLC analysis of inhibitor **42** performed at 30°C revealed two partially overlapping peaks (Figure 76A), suggesting the presence of two compounds. However, MS analysis showed a single peak corresponding to inhibitor **42**. Fittler *et al.*¹⁸² likewise reported two signals with nearly identical t_R in the HPLC chromatogram of a purified peptide, and attributed the additional signal to impurities originating from the Fmoc-Arg used during synthesis.

It contrast, the bicyclic analogue of inhibitor **42**, inhibitor **46**, exhibited only single peak signal in the HPLC chromatogram (Figure 76B). Moreover, HPLC analysis of inhibitor **42** performed at higher temperature resulted in the appearance of a single signal (Figure 76C). HPLC analysis performed in elevated temperature for inhibitor **46** is presented in Figure 76D.

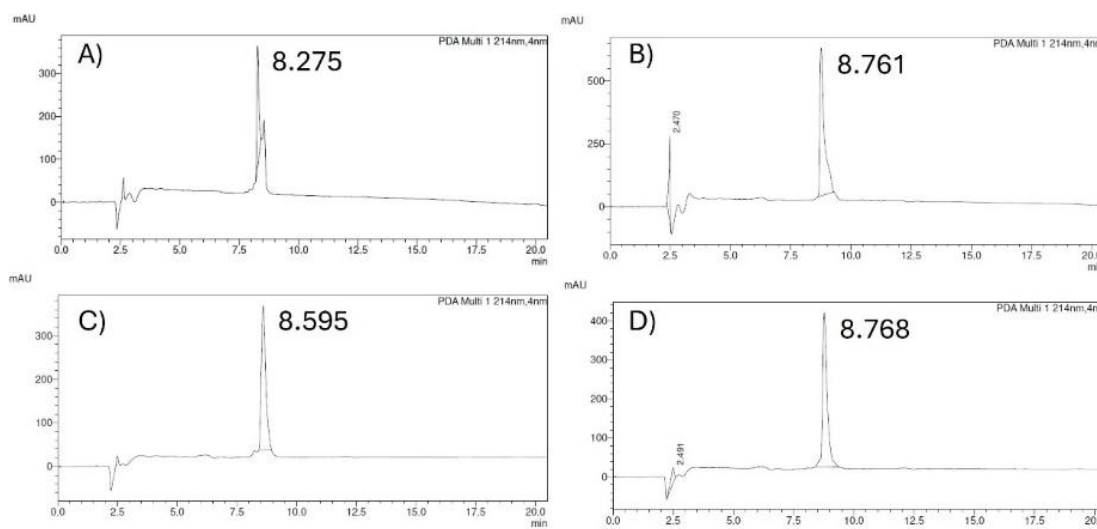


Figure 76. HPLC chromatograms recorded at 30°C for inhibitors 42 (A) and 46 (B) and at 50°C for inhibitors 42 (C) and 46 (B).

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 20 min.

These results may indicate the presence of conformational isomerization in inhibitor **42**. To verify this hypothesis, NMR analyses were required. All measurements were performed by dr Igor Zhukov from the Laboratory of Biological NMR Institute of Biochemistry and Biophysics Polish Academy of Sciences. The NMR data revealed the presence of two distinct conformers. The minor conformer corresponded to a *trans* configuration between the Ile8 and Pro9 residues, whereas the major conformer featured a *cis* configuration at this position. It was also observed that at 25°C approximately 30% of the *trans* conformer was present. This value is consistent with results reported by other groups for related BBIs analogues, in which a *cis* configuration is commonly observed.^{176,260,269} Detailed results are summarized on paper submitted to the *Journal of Molecular Biology*. The manuscript has been reviewed and is currently being revised by the authors.

11.5. Design of furin inhibitors for albumin-binding assessment

Human serum albumin (HSA) is not only a reducing agent in human body, but also a protein that effectively binds a wide range of low-molecular-weight compounds. It is the predominant protein in blood plasma, where it contributes to the regulation of osmotic pressure and participates in the metabolism and detoxification of numerous substances. Its multi-domain structure enables the binding, transport, and distribution of a broad spectrum of endogenous and exogenous ligands.²⁷⁰ Therefore, an interesting aspect was to examine the ability of HSA to bind the furin

inhibitors, as such interactions may influence the bioavailability of the peptides. Covalent and non-covalent conjugation of peptides to HSA constitutes one of the strategies used to prevent premature proteolytic degradation.^{271,272}

The group led by dr hab. Dariusz Wyrzykowski, prof. UG from the Department of General and Inorganic Chemistry, Faculty of Chemistry, University of Gdańsk, has extensive experience in studying the binding of various compounds by HSA. One of the method employed in their research is isothermal titration calorimetry (ITC). Our inhibitors (**40**, **44**, **46**) were provided to this group. Additionally, a linear analogue of inhibitor **42** (peptide **48**) and analogue of inhibitor **46** containing only backbone cyclization (peptide **49**) were synthesized to determine the relationship between the type of cyclization (or lack thereof) and the ability to bind by HSA. In peptides **48** and **49** Cys residues were replaced with Ala.

Stoichiometry, affinity, and thermodynamic parameters were determined for peptides **40**, **42**, **46**, **48**, and **49**. The studies were conducted in 10 mM cacodylate buffer at pH 7.0 and 298.15 K. The obtained thermodynamic parameters are summarized in Table 12. The first parameter determined was stoichiometry (N), which indicates the number of functional binding sites per macromolecule (HSA). The ITC data revealed significant variation in the N values for the peptide-HSA complexes: 2.40 for inhibitor **40**, 3.05 for inhibitor **42**, 2.43 for inhibitor **46**, and 1.88 for peptide **49**. In the case of peptide **48**, the heat signals were too weak to allow reliable quantification under the experiments conditions, suggesting that its interactions with HSA were extremely weak. This may reflect genuinely weak binding or could be related to methodological or experimental limitations. It is well known that ITC-derived parameters are condition-dependent and can be influenced by factors such as pH and buffer composition, due to the presence of ionizable groups in both the peptides and HSA. Therefore, meaningful comparisons of binding parameters are only valid when all measurements are performed under identical experimental conditions.

The next parameter evaluated was the binding constant ($\log K_{(ITC)}$). The highest binding constant was observed for inhibitor **42** ($\log K_{(ITC)} = 5.69$ kcal/mol), indicating that this peptide most effectively occupied HSA binding sites. Its binding affinity was considerably higher than that of the other peptides tested. The remaining peptides exhibited weaker overall interactions with HSA. The thermodynamic stabilities of the peptide-HSA complexes follows a clear trend among peptides: **42** ($\Delta G = -7.76$ kcal/mol) > **40** (-6.18 kcal/mol) > **46** (-5.40 kcal/mol) > **49** (-4.79 kcal/mol).

Binding of all peptides to HSA was an endothermic process ($\Delta H > 0$), accompanied by a large, favorable entropy change ($T\Delta S > 0$). Importantly, for each system, the magnitude of the entropy term exceeded that of enthalpy term ($|T\Delta S| > |\Delta H|$), indicating that the binding is entropy-driven.²⁷³ This thermodynamic profile suggests that the interaction is primarily governed by the hydrophobic effect.²⁷⁴ Furthermore, the unfavorable positive enthalpy values imply that the

formation of non-covalent interactions, such as hydrogen bonds or electrostatic interactions, between the peptides and HSA is likely limited.

Table 12. Thermodynamic parameters of binding interactions of the peptides with HSA.

N - stoichiometry

n/d - not determined due to too small heat signals.

Parameter	Inhibitor 40	Inhibitor 42	Inhibitor 46	Peptide 48	Peptide 49
<i>N</i>	2.40 (±0.16)	3.05 (±0.20)	2.43 (±0.22)	n/d	1.88 (±0.13)
logK_(ITC) [kcal/mol]	4.53 (±0.08)	5.69 (±0.33)	3.96 (±0.06)	n/d	3.51 (±0.12)
ΔG_(ITC) [kcal/mol]	-6.18 (±0.11)	-7.76 (±0.45)	-5.40 (±0.08)	n/d	-4.79 (±0.16)
ΔH_(ITC) [kcal/mol]	2.75 (±0.25)	0.45 (±0.05)	4.03 (±0.25)	n/d	4.94 (±0.86)
TΔS_(ITC) [kcal/mol]	8.94	8.21	9.43	n/d	9.73

These results may also indicate a relationship between cyclization and the ability to bind HSA. As demonstrated in the NMR studies, discussed in Section 11.4., inhibitor **42** undergoes *cis/trans* isomerization. Additional, backbone cyclization, effectively blocks this process, resulting in inhibitor **46** adopting exclusively the *cis* conformation. Taking this findings into account, the conformational flexibility of inhibitor **42** may contribute to its higher affinity toward HSA. In contrast, the bicyclic peptides exhibited weaker interactions, likely due to their increased structural rigidity. Peptide **49**, containing only backbone cyclization, also displayed reduced affinity for the HSA surface. The linear and unconstrained structure of peptide **48**, resulted in the weakest interactions, which is consistent with the considerations outlined above. More definitive conclusions could be drawn following MD simulations. Overall, the results demonstrate that the mode of cyclization significantly influences binding affinity to HSA. Inhibitor **42**, capable of *cis/trans* isomerization, exhibited higher affinity than structurally rigid bicyclic and backbone-cyclized analogues, as well as flexible peptide **48**.

As described in Section 11.4., the NMR analyses of inhibitor **42** revealed the presence of two conformers, which corresponded to the split signals observed in HPLC chromatograms. Similar behavior was observed for the analogue of inhibitors **42** and **46** (peptides **48** and **49**), where in the chromatograms signal splitting was also observed (Figure 77). In case of peptide **48**, a shoulder was visible at 30°C (Figure 77A), which disappeared at 50°C (Figure 77B). Peptide **49** behaved similarly to inhibitor **42**: at 30°C two separate signals were detectable (Figure 77C), whereas at 50°C only a single peak was observed (Figure 77D).

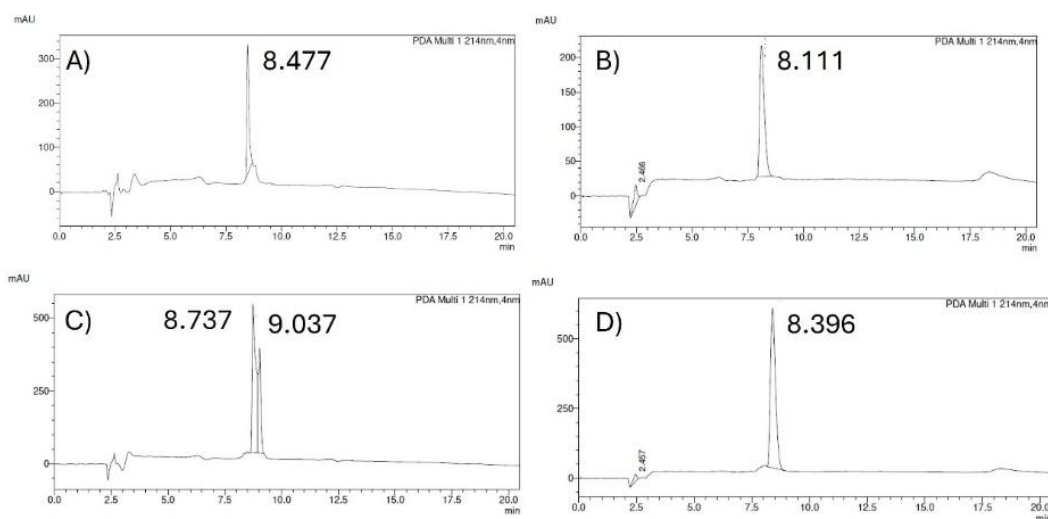


Figure 77. HPLC chromatograms recorded at 30°C for peptides 48 (A) and 49 (C) and at 50°C for peptides 48 (B) and 49 (D).

Shimadzu Prominence-I HPLC equipped with a Dr. Maisch column; 10-90% B in 20 min.

12. Optimization of the peptide *N*-methylation method

N-methylation is a common modification of biologically active peptides. Numerous studies have demonstrated its beneficial effects, including improved oral bioavailability,²⁷⁵ enhanced proteolytic resistance²⁷⁶ or increased binding affinity.^{277,278} A wide range of *N*-methylation strategies has been described, including the modification of amino acids in solution^{279,280,281,282} followed by their incorporation into the peptide sequence, as well as on-resin methylation performed directly during solid-phase peptide synthesis.^{283,284,285,253} Solid-phase *N*-methylation offers several advantages, as it is less laborious and less time-consuming than the prior synthesis of *N*-methylated amino acid derivatives.

In my dissertation, two *N*-methylated inhibitors (**1** and **15**) were planned and synthesized. The methyl group was introduced according to the procedure described in Section 6.10. Until recently, the method commonly used in our group was that described by Naoum *et al.*²⁵³ This three-step protocol requires 4 h to complete. Given the substantial acceleration of peptide synthesis achieved through ultrasonic agitation (UA), as reported by Wołczański *et al.*²³⁸, I decided to explore the possibility of applying this method instead of standard shaking during *N*-methylation. Furthermore, the efficiency of *N*-methylation was compared using a microwave-assisted peptide synthesizer (MW). The details of my work on this issue are presented in my publication (Helbik-Maciejewska *et al.*, *J. Org. Chem.*, 2025, 90, 22, 7182-7190)²⁵²). In this dissertation, I include only the most essential and relevant aspects.

For the optimization of *N*-methylation conditions, the tripeptide Arg-Trp-Gly-NH₂ was selected as a model compound, similarly to Naoum *et al.*²⁵³ Every step of the procedure was

progressively shortened, and the outcomes of this optimization are summarized in Table 13. Various reaction conditions, including standard shaking (SS), ultrasonic agitation (UA), and microwaves irradiation (MW), were evaluated to identify the most effective approach. When SS or UA were applied, the reactions were carried out at room temperature. Additionally, the effect of increasing the temperature during the methylation step from 40°C to 70°C under MW was examined. The effectiveness of each condition was assessed based on the purity of the crude product, expressed as the percentage of the area under the peak in the HPLC chromatogram. In total, 22 experimental runs on *N*-methylation peptide RWG-NH₂ were performed to determine the optimal conditions. Entries 17-19 demonstrated that satisfactory yields were achieved regardless of the equipment used (SS, UA, or MW). Overall, the reaction time was successfully reduced from 4 h to 40 min.²⁵²

Table 13. Optimization of model peptide RWG-NH₂ *N*-methylation.

Entry	Time of sulfonylation [min]	Time of methylation [min]	Time of desulfonylation [min]	Method	HPLC purity of crude product [%] ^x
1	60	15 + 15	5 + 5	UA	85
2	60	15 + 15	15 + 15	UA	n/d ^a
3	45	15 + 15	15 + 15	UA	n/d ^a
4	45	30 + 15	15 + 15	UA	88
5	30	30 + 15	15 + 15	UA	90
6	20	30 + 15	5 + 5	UA	87
7	10	30 + 15	5 + 5	UA	89
8	10	30 + 15	5 + 5	SS	90
9	10	20 + 15	5 + 5	UA	90
10	10	30 + 10	5 + 5	UA	88
11	10	15 + 15	5 + 5	UA	85
12	10	2 + 2	5 + 5	UA	58
13	5	15 + 15	5 + 5	UA	76
14	5	2 + 2	5 + 5	UA	34
15	5	15 + 5	5 + 5	UA	84
16	5	10 + 5	5 + 5	UA	75
17	5	15 + 10	5 + 5	UA	83
18	5	15 + 10	5 + 5	SS	82

19	5	15 + 10	5 + 5	MW	80
20	5	2 + 2	5 + 5	MW	75
21	5	2 + 2 (70°C)	5 + 5	MW	41
22	1	15 + 10	5 + 5	UA	56

Reaction time proposed by Naoum *et al.*²⁵³: sulfonylation (120 min), methylation (30 + 30 min), desulfonylation (30 + 30 min). The most promising conditions are marked in bold.

UA - ultrasonic agitation; SS - standard shaking, MW - microwave assisted peptide synthesizer.

^xHPLC purity of the crude product determined as the percentage of the area under the peak;

^an/d - not determined due to the presence of high amount of impurities.

After establishing the optimal reaction conditions, bolded in Table 13 (5 min for sulfonylation, 15 and 10 minutes for methylation, and 5 min, repeated twice, for desulfonylation), the method was subsequently applied to the *N*-methylation of various *N*-terminal amino in the model tripeptide in place of Arg, to assess its versatility. The amino acids tested were: His, Ser, Trp, Tyr, Asp, Glu, Cys, Phe, and Ala.

The results of these reactions are summarized in Table 14. Nearly all peptides (with the exception of Asp-Trp-Gly-NH₂) underwent *N*-methylation without difficulty and were obtained in satisfactory yields using UA, SS or MW (with crude product purities ranging from 74% to 100%). The only problematic amino acid was Asp, likely due to the prolonged exposure of the peptide to DBU, which can promote side reactions such as aspartimide formation.²⁸⁶ The solution to this issue was to abandon the second treatment with DBU and dimethyl sulfate. Although the reaction yield remained relatively low, the product was obtained with sufficient purity to allow successful purification. NMR analyses were performed for all peptides, and the results are provided in the Supplementary Materials of our publication.²⁵²

Table 14. Results of *N*-methylation reaction of various *N*-terminal amino acids carried out through: ultrasonic agitation (a); standard shaking (b); microwave assisted peptide synthesizer (c).

Peptide	HPLC purity of the crude product [%] ^x	Isolated yield [%] ^y
(N-Me)Arg-Trp-Gly-NH ₂	83 ^a	60 ^a
	82 ^b	62 ^b
	80 ^c	55 ^c
(N-Me)Arg-Trp-Gly-OH	91 ^a	54 ^a
(N-Me)His-Trp-Gly-NH ₂	96 ^a	32 ^a
	93 ^b	53 ^b
	92 ^c	72 ^c
(N-Me)Ser-Trp-Gly-NH ₂	77 ^a	39 ^a

	88 ^b	37 ^b
	90 ^c	51 ^c
(<i>N</i> -Me)Trp-Trp-Gly-NH ₂	74 ^a	35 ^a
	86 ^b	56 ^b
(<i>N</i> -Me)Tyr-Trp-Gly-NH ₂	87 ^a	45 ^a
	88 ^b	63 ^b
(<i>N</i> -Me)Asp-Trp-Gly-NH ₂	54 ^a	16 ^a
	65 ^b	16 ^b
	73 ^c	29 ^c
(<i>N</i> -Me)Glu-Trp-Gly-NH ₂	84 ^a	71 ^a
	93 ^b	42 ^b
(<i>N</i> -Me)Cys-Trp-Gly-NH ₂	78 ^a	37 ^a
	74 ^b	44 ^b
	80 ^c	49 ^c
(<i>N</i> -Me)Phe-Trp-Gly-NH ₂	98 ^a	66 ^a
	98 ^b	73 ^b
(<i>N</i> -Me)Ala-Trp-Gly-NH ₂	99 ^a	31 ^a
	100 ^b	42 ^b

^xHPLC purity of the crude product determined as the percentage of the area under the peak;^yisolated yield- the final yield of obtained peptides after purification.

Moreover, after confirming the applicability and efficiency of the optimized method, a peptide methylated at three positions was synthesized. For this purpose, we prepared a simplified analogue (Figure 78A) of the peptide originally reported by Naoum *et al.*²⁵³ (Figure 78B).

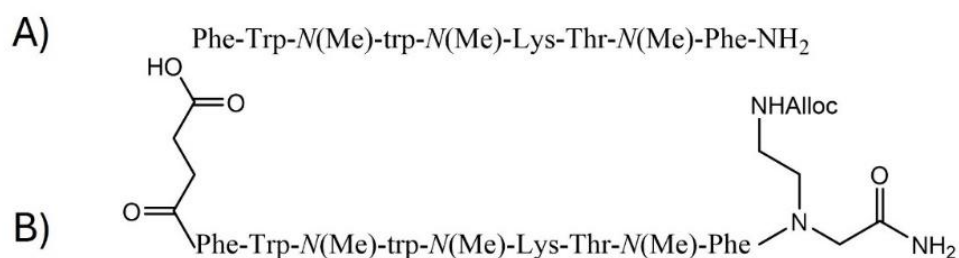


Figure 78. Peptide 1-SW1 synthesized by our group to confirm the efficiency of our method (A) and peptide 1-SW1 used by Naoum et al.²⁵³ as a model to confirm the efficiency of their method (B)

Table 15. Conditions of syntheses of peptide 1SW-1

Time of sulfonylation [min]	Time of methylation [min]	Time of desulfonylation [min]	Method	HPLC purity of crude product [%] ^x
5	15 + 10	5 + 5	UA	57
5	15 + 10	5 + 5	MW	57

UA – ultrasonic agitation, MW - microwave-assisted peptide synthesizer.

Two synthesis strategies were applied: UA and MW (Table 15). The use of the improved *N*-methylation protocol proved successful. However, it is well known that coupling to the *N*-methylated amino acid is more challenging than to a primary amine. Chatterjee *et al.*²⁸⁵ reported difficulties during the coupling of Fmoc-Thr(tBu) to *N*(Me)Phe, which required 3 h reaction repeated 3 times to achieve satisfactory efficiency. In peptide 1-SW1, a similarly problematic fragment Thr-*N*(Me)-Phe, is present. Owing to the application of UA or MW, the coupling reaction required only 15 min, repeated four times, and its progress was monitored using both Kaiser and chloranil tests. Coupling of the remaining amino acids required 15 min, repeated twice.

After cleavage of the peptide from the solid support, a minor impurity corresponding to peptide 1-SW1 lacking Thr was detected. In both synthesis approaches, the HPLC purity of the crude products reached 57%. HPLC chromatograms of the crude peptide 1-SW1 are presented in Figure 79.

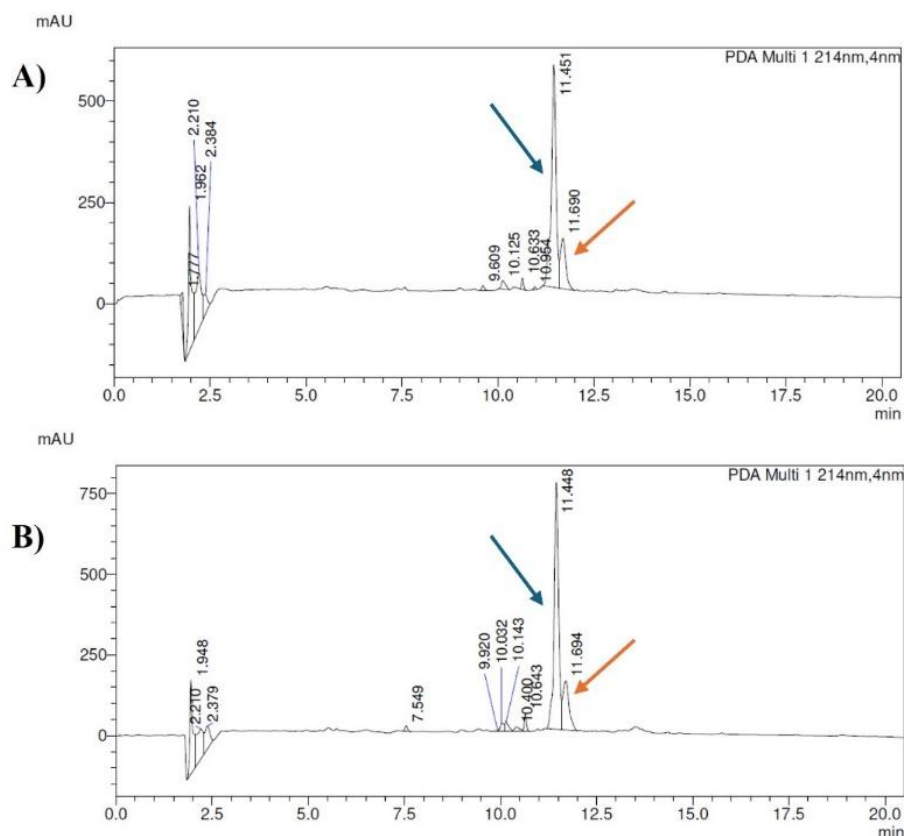


Figure 79. HPLC chromatograms of the crude peptides obtained using UA (A) and MW (B).

The blue arrow indicates peptide 1SW-1, and the orange arrow indicates peptide 1SW-1 lacking Thr.

Shimadzu Prominence-I HPLC equipped with a Kinetex column; 10-90% B in 20 min.

Successful synthesis of the various peptides presented in Table 14, as well as the final preparation of peptide 1SW-1, confirmed the effectiveness of the shortened *N*-methylation method, both in the context of methylating different amino acids and of synthesizing a multiply *N*-methylated peptide. Our studies also demonstrated that this method can be applied using diverse laboratory equipment, including a standard shaker, an ultrasonic bath, or a peptide synthesizer equipped with microwave irradiation. An additional advantage, beside the significantly reduced time of the process, was decreased exposure to highly hazardous reagents such as dimethyl sulfate.

Conclusions

The major accomplishments and results of my doctoral dissertation may be summarized in the points presented below.

- I obtained 48 peptides, designed to be proteinase inhibitors. Among them 36 were designed as MT1 inhibitors (inhibitors **1** and **2** were analogues of SFTI-1 and inhibitors **3-36** were analogues of HV-BBI); one inhibitor of MT2 (**37**); 12 peptides (**38-49**) were analogues of SFTI-1 and were designed as furin inhibitors;
One peptide included in Table 2 (peptide **45**) was synthesized by dr hab. Dawid Dębowski, prof. UG and then purified by me.
- Taking into consideration modifications applied in peptides: among obtained 48 peptides, 47 were cyclic (including 5 bicyclic peptides, 1 with backbone cyclization only, 39 with disulfide bridges and 2 with triazole bridges). Moreover, 7 peptides were labeled with 5(6)-carboxyfluorescein. 2 peptides were *N*-methylated.
- All enzymatic studies were performed in cooperation with dr Agata Gitlin-Domagalska.

MT1 inhibitors

- Based on the HV-BBI analogue, inhibitor **3**, I obtained 25 peptides designed as MT1 inhibitors. Among them, 15 proved to be promising MT1 inhibitors, exhibiting inhibitory activity comparable to that of inhibitor **3**.
- Phe13 residue in inhibitor **3** turned out to not be essential for inhibitory activity toward MT1, as its replacement did not affect its activity.
- The results of alanine scanning, performed for inhibitor **3**, confirmed significance of amino acids located within the binding loop, including crucial role of the P1 Lys5 residue in MT1 recognition, as well as other important residues such as Ser6, Ile7, Arg10. In contrast, amino acids located outside the binding loop were found to be not essential for inhibitory activity.
- Truncated analogue of inhibitor **3**, which exhibited similar activity against MT1 as the starting compound, was peptide with lacking Phe13 (inhibitor **26**).

- Among analogues with reduced amino acids located inside the binding loop (Trp3 and/or Pro11) the most active was inhibitor lacking both of these residues, however with remaining Phe13 (inhibitor **31**).
- Inhibitor **31** is an analogue with reduced molecular weight, which is advantageous for potential therapeutic applications. It exhibited the best proteolytic stability among the studied analogues. Interestingly, its shortened analogue (inhibitor **34**) was almost completely degraded after the same time period, which may indicate that the sequence of inhibitor **31** is more optimal in the context of potential therapeutic use. These findings are schematically presented in Figure 80.

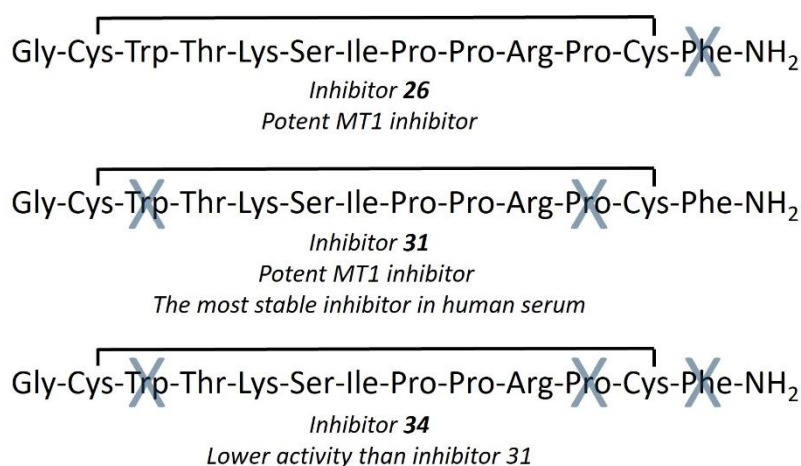


Figure 80. Schematic summary of the effects of reducing the molecular size of the MT1 inhibitors.

Removed amino acids are crossed out.

- The disulfide bridge is essential for the inhibitory activity of inhibitor **3**. Its replacement with a mimetic, triazole bridge (inhibitor **35**) resulted in a significant reduction of inhibitory potency toward MT1. This suggests that the geometry of the disulfide bridge is optimal for maintaining the spatial structure of the inhibitor binding loop.

Furin inhibitors

- Based on the SFTI-1 analogue, I obtained 10 peptides designed as furin inhibitors (peptides **38** - **47**) and 2 additional peptides (**48** and **49**), which are analogues of potent furin inhibitors and were synthesized for studies of inhibitor structural organization.

- I obtained three furin inhibitors, which exhibited nanomolar inhibitory activity against furin (inhibitors **40**, **42**, **46**).
- Inhibitors **40**, **42** and **46** led to a reduction in the ZIKV titer, as measured by the plaque assay method. Notably, the most potent furin inhibitor (**40**) turned out to exhibit extremely potent antiviral activity against ZIKV replication cycle. It exhibited higher activity than commercially available and non-selective furin inhibitor Dec-RVKR-CMK. What is important, inhibitor **40** is less cytotoxic than Dec-RVKR-CMK (it exhibited almost five-fold higher therapeutic index (SI)). The results indicates on potential therapeutic use of inhibitor **40**.

Obtained results yielded in a patent application in the Patent Office of the Republic of Poland (P.449419).

The manuscript is currently being prepared and will be submitted soon.

- Preliminary results of anticancer studies of furin inhibitors (**42**, **44**, **45**), revealed that analogues with substituted disulfide bridge exhibited anticancer activity toward U251 and A549 cell lines, while inhibitor **42** was inactive. However, the assumed positive reference (Dec-RVKR-CMK) also did not exhibit any anticancer activity. Thus, the anticancer activity of peptides **44** and **45** is rather not dependent on furin inhibition.
- Replacement of disulfide bridge in inhibitor **42** with its mimetic, triazole bridge (inhibitor **44**) resulted in slightly decreased activity. In contrast peptide **45**, which contains diselenide bridge exhibited similar activity toward furin as the parent compound and it was able to completely inhibit the enzyme at a concentration of 77 nM. This result was expected, due to the fact that substitution of disulfide bridge by diselenide bridge is consider to be isosteric, so it does have similar impact for spatial structure of binding loop in the peptide. Thus, such a modification does not affect inhibitory potency.
- Peptide **44** exhibited lower stability in a proteolytic environment compared to inhibitor **42**. The lower stability of the triazole-bridged analogue may be attributed to subtle conformational changes arising from disulfide replacement. As the triazole bridge does not fully mimic the native geometry of the disulfide bond, it may expose protease-sensitive regions and thereby accelerate peptide degradation.

Optimization of peptide *N*-methylation

- The *N*-methylation of peptides on resin can be performed using various types of laboratory equipment, such as laboratory shaker, ultrasonic bath, or a microwave-assisted peptide synthesizer. Moreover, the time of procedure may be drastically shortened (from 4 h to 40 min), while preserving satisfactory yields (74-100%).
- The optimized method was validated using a range of amino acids, including: Arg, His, Ser, Trp, Tyr, Asp, Glu, Cys, Ala, and Phe.
- Problems associated with side reactions occurring during *N*-methylation of Asp were resolved by reducing the exposure of peptide to DBU.

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List of Figures

Figure 1. The scheme of substrate (inhibitor) - protease interaction according to Schechter-Berger ⁸ model.	18
Figure 2. Catalytic mechanism of serine proteases on the example of chymotrypsin.....	20
Figure 3. Types of membrane anchored serine proteases.	21
Figure 4. Schematic representation of the structural composition of TTSPs (A) and overview of all known transmembrane serine proteases and their domain organization (B) ¹⁹	23
Figure 5. Schematic presentation of MT1 domains (A) and a ribbon stereo plot of MT1 (B)..	27
Figure 6. Schematic presentation of MT2 domains (A) and a ribbon stereo plot of MT2 (B)..	32
Figure 7. Schematic presentation of furin domains (A) and a crystal structure of a human furin (PDB: 5JXG) (B).....	36
Figure 8. The maturation process of furin (B), and a schematic representation of the biological functions of furin in the body, including its role in the development of viral and bacterial infections (A). ¹⁰⁰	38
Figure 9. ZIKV replication cycle (A) ¹⁴⁰ and structures of spiky, immature (B) and smooth, mature (C) forms of ZIKV. ¹³⁹	43
Figure 10. Front and back stereoviews of α_1 -proteinase inhibitor. ¹⁴⁸	45
Figure 11. Structures of small-molecule inhibitors: Dabigatran (A), Apixaban (B), Betrixaban (C), Rivaroxaban (D), Nafamostat (E). ¹⁵²	47
Figure 12. Structure of SFTI-1.....	50
Figure 13. Structures of HV-BBI (A); HV-BBI(3-18) (B); HV-BBI(4-16) (C).	51
Figure 14. Structure of an analogue of benzamidine, one of the first described potent small-molecule MT1 inhibitor. ¹⁹⁰	52
Figure 15. Structures of commercial available furin inhibitors: decanoyl-RVKR-chloromethylketone (Dec-RVKR-CMK, furin inhibitor I; A) and hexa-D-arginine (D6R, furin inhibitor II; B).....	55
Figure 16. Structures of activity based probes of MT1 and MT2. A) biotin-RQRR-CMK; ²³⁰ Activity based probes with phosphono bisbenzguanidines groups: B) ²³¹ ; C) ²³²	57
Figure 17. General scheme of peptide-based affinity probe.....	57
Figure 18. Starting structures used for the development of new MT1 (A) and furin (B) inhibitors.....	59
Figure 19. HPLC chromatograms of reduced (A) and oxidized (B) peptide 43. MS analysis of reduced (C) and oxidized (D) peptide 43.	69
Figure 20. HPLC chromatograms of ascorbic acid (A) and dehydroascorbic acid (B).	70
Figure 21. HPLC chromatograms of: linear (A), cyclic (B) peptide 44 and their co-injection (C).	71
Figure 22. HPLC chromatograms of reaction mixture during synthesis of peptide 44 after: 15 min (A); 1 h (B); 24 h (C) of reaction.	72
Figure 23. MS analyses of linear (A) and cyclic (B) peptide 44.	72

Figure 24. HPLC chromatogram (A) and MS analysis (B) of peptide 35.	73
Figure 25. Monitoring progress of reaction of cyclic peptide (A) and linear peptide (B) using HPLC. MS analyses of cyclic (C) and linear (D) peptide with DTT.	75
Figure 26. MS analyses of peptide 44 dissolved in buffer (A) and after addition of trypsin (B). Progress of hydrolysis showed using HPLC (C). Structure of inhibitor 44 with schematically marked observed scissile sites by red crossed lines and scissors icons (D).	77
Figure 27. HPLC chromatograms of crude (A) and pure (B) peptide 45. MS analysis of pure peptide 45 (C).	78
Figure 28. HPLC chromatogram (A) and MS analysis (B) of peptide 46.	79
Figure 29. HPLC chromatogram (A) and MS analysis (B) of peptide 10.	80
Figure 30. Structure of 5(6)-carboxyfluorescein.	81
Figure 31. HPLC chromatogram (A) and MS analysis of peptide 36 (B).	82
Figure 32. HPLC chromatogram (A) and MS analysis of Fmoc-Lys-OH (B).	83
Figure 33. HPLC analysis of Fmoc-Lys(Cf).	84
Figure 34. MS analysis of linear peptide 41.	84
Figure 35. HPLC analysis of Fmoc-Phe(4-NH-Cf) (A) and MS analysis of peak with $t_R = 17.917$ min (B). MS analysis of linear peptide 45 (C).	86
Figure 36. General N-methylation procedure described by Naoum et al. (A) ²⁵³ and method optimized by our group(B). ²⁵²	87
Figure 37. Structures of MT1 inhibitor ⁹⁷ (A) and its analogues: inhibitors 1 (B) and 2 (C).	91
Figure 38. Activity of inhibitors 1 and 2 at 0.1 μ M (A) and 10 μ M (B) against MT1 and MT2.	92
Figure 39. Structures and K_i values against MT1 and MT2 of truncated analogues of HV-BBI differing in their C-termini: amide group (A), carboxyl group (B) and backbone cyclisation (C).	93
Figure 40. Visualization of enzyme-peptide interaction maps between the analogue with a carboxylic group (A) and the analogue with an amide group at the C-terminus (B) in complex with the MT1 active site. Intramolecular hydrogen-bond networks are shown for the analogue with a carboxylic group (C) and the analogue with an amide group (D). ²⁵⁸	94
Figure 41. Inhibitory activity of HV-BBI analogues against selected proteases. Enzymatic studies performed at 10 μ M (A) and 100 nM (B) concentrations of peptide. ²⁵⁸	95
Figure 42. Structures of the Huia versabilis Bowman-Birk inhibitor (HV-BBI) and its analogues and schematic representation of the development of novel MT1 inhibitors based on HV-BBI. The modifications that are subject of my work are shown below the blue line.	96
Figure 43. Visualization of conformational changes in inhibitor 3 in complex with MT1 (PDB ID: 3P8F) (A) and MT2 (Homology model) (B).	97
Figure 44. Amino acids introduced instead of Phe13 in inhibitor 3.	98
Figure 45. Results of enzymatic evaluation of inhibitor 3 and its analogues with substituted Phe13 residue toward MT1 (A) and MT2 (B).	100
Figure 46. Results of enzymatic evaluation of inhibitor 3 and its analogues obtained through alanine scanning.	102
Figure 47. Results of enzymatic evaluation of peptide 3 and its truncated analogues 26-28.	104
Figure 48. Results of enzymatic evaluation of peptide 3 and its analogues (26 and 29-34) with deleted residues located inside the binding loop.	105
Figure 49. Scheme of synthesis of peptide 35.	106
Figure 50. Results of enzymatic evaluation of inhibitor 3 and its analogue, peptide 35, with triazole bridge.	107
Figure 51. Stability of MT1 inhibitors (3, 31, 34, and 35) in human serum.	109

Figure 52. The progress of inhibitor 3 hydrolysis in serum.	110
Figure 53. MS analyses of peptide 3 in serum at the initiation of the reaction (A) and after 24 h (B).	110
Figure 54. MS analyses of peptide 31 in serum at the initiation of the reaction (A) and after 1 h (B).	111
Figure 55. MS analyses of peptide 34 in serum at the initiation of the reaction (A) and after 1 h (B).	112
Figure 56. MS analyses of peptide 35 in serum at the initiation of the reaction (A) and after 1 h (B).	113
Figure 57. Structures of potent MT2 inhibitor ⁹⁶ (A) and its new, labeled analogue, inhibitor 37 (B).	114
Figure 58. Preliminary inhibition assay of inhibitor 37 at 0.1 μ M (A) and 10 μ M (B).	114
Figure 59. Fluorescence microscopy image showing peptide 37 attachment to the membrane of MT2-rich MCF7 cells.	115
Figure 60. Schematic representation of the development of novel furin inhibitors. Structures of sequentially modified peptides: SFTI-1 (A); furin inhibitor obtained by Fittler et al. ¹⁸² (B); general formula of the peptide amide library with presented amino acids which were introduced in positions P1, P2, P4, P5 (C); peptide selected through combinatorial chemistry (D); peptides I-V with an additional basic amino acid at P6 position and/ or backbone cyclization (E); labeled analogues for biological studies (F).	117
Figure 61. Results of enzymatic evaluation of furin inhibitors.	118
Figure 62. Results of enzymatic evaluation of furin inhibitors against MT1 (A) and MT2 (B).	119
Figure 63. Stability of furin inhibitors in human serum.	120
Figure 64. Comparison of antiviral activity of inhibitors 40, 42, 46 and commercially available furin inhibitor (Dec-RVKR-CMK) (A). Cell lysates were subjected to SDS-PAGE followed by immunoblotting with specific antibodies (B).	121
Figure 65. Cytotoxicity assessment of inhibitor 40, presented as viability of Vero E6 cell line after treatment with the peptide compared to the viability of cells treated only with PBS buffer.	122
Figure 66. Antiviral assessment of inhibitor 40 against two cell lines (Vero E6 and A549) and strains of ZIKV.	122
Figure 67. Western blot analysis of viral proteins (prM/M and E) in cell lysates after infection and after treatment with inhibitor 40 (50 μ M and 5 μ M) in PBS for 48 h.	123
Figure 68. Antiviral activity of inhibitor 40 against ZIKV across a wide range of inhibitor concentrations.	123
Figure 69. Fluorescence microscopy images illustrating the ability of peptide 41 (100 μ M) to permeate into astrocytes after different incubation periods: 4 h (A), 24 h (B), 48 h (C), and 72 h (D).	125
Figure 70. Structures of inhibitor 42 analogues: peptide 44 (with triazole bridge) and peptide 45 (with diselenide bridge).	126
Figure 71. Results of enzymatic evaluation of peptide 42 analogues, peptides with triazole bridge (44) and with diselenide bridge (45).	126
Figure 72. Viability of cells: U251 (A), A549 (B), and 293 HEK (C) after incubation with furin inhibitors.	128
Figure 73. Stability of furin inhibitors (42 and 44) in human serum.	129
Figure 74. MS analyses of peptide 42 in serum at the initiation of the reaction (A) and after 2 h (B).	130

Figure 75. MS analyses of peptide 44 in serum at the initiation of the reaction (A) and after 1 h (B).....	131
Figure 76. HPLC chromatograms recorded at 30°C for inhibitors 42 (A) and 46 (B) and at 50°C for inhibitors 42 (C) and 46 (B).....	132
Figure 77. HPLC chromatograms recorded at 30°C for peptides 48 (A) and 49 (C) and at 50°C for peptides 48 (B) and 49 (D).....	135
Figure 78. Peptide 1-SW1 synthesized by our group to confirm the efficiency of our method (A) and peptide 1-SW1 used by Naoum et al. ²⁵³ as a model to confirm the efficiency of their method (B).....	138
Figure 79. HPLC chromatograms of the crude peptides obtained using UA (A) and MW (B).	140
Figure 80. Schematic summary of the effects of reducing the molecular size of the MT1 inhibitors.....	142

List of Tables

Table 1. Functional overview of PCs members.....	34
Table 2. Primary structures, calculated and founded masses, and final yields of obtained peptides.....	60
Table 3. Amino acid derivatives which were used for syntheses.....	63
Table 4. Sequences of peptide 3 analogues with substituted Phe13. The introduced modifications indicated in bold.	99
Table 5. Sequences of peptide 3 analogues obtained through alanine scanning. The introduced Ala residue is indicated in bold.	101
Table 6. Sequences of truncated peptide 3 analogues.....	103
Table 7. Sequences of peptide 3 and its analogues with deleted residues located inside the binding loop.....	105
Table 8. Progress of proteolysis expressed as the percentage of the area under the peak corresponding to selected inhibitors.	108
Table 9. Inhibitory constants (K_i) calculated for selected furin inhibitors.	118
Table 10. Antiviral activity of furin inhibitors against ZIKV.	124
Table 11. Progress of hydrolysis expressed as percentage of the area under the peak corresponding to inhibitor 42.	129
Table 12. Thermodynamic parameters of binding interactions of the peptides with HSA. ...	134
Table 13. Optimization of model peptide RWG-NH ₂ N-methylation.	136
Table 14. Results of N-methylation reaction of various N-terminal amino acids carried out through: ultrasonic agitation (a); standard shaking (b); microwave assisted peptide synthesizer (c).....	137
Table 15. Conditions of syntheses of peptide 1SW-1	139

Academic achievements

Publications:

- 1) Gitlin-Domagalska A., Hassanzadeh M., Helbik-Maciejewska A., Lepage M., Joushomme A., Désilets A., Dębowski D., Boudreault PL, Leduc R., Rolka K., „Structural insights into frog skin-derived cyclic peptides as selective matriptase inhibitors”, *European Journal of Medicinal Chemistry*, 302(Pt 2), **2026**, 118331, DOI: 10.1016/j.ejmech.2025.118331
- 2) Helbik-Maciejewska A., Gitlin-Domagalska A., Glavaš M., Ptaszyńska N., Dębowski D., Łęgowska A., Rolka K., „Time-reduced N-methylation: exploring a faster synthesis technique”, *Journal of Organic Chemistry*, 90(22), **2025**, 7182-7190, DOI: 10.1021/acs.joc.5c00083
- 3) Glavaš M., Gitlin-Domagalska A., Ptaszyńska N., Starego D., Freza S., Dębowski D., Helbik-Maciejewska A., Łęgowska A., Gilon C., Rolka K., „Synthesis of novel arginine building blocks with increased lipophilicity compatible with solid-phase peptide synthesis”, *Molecules*, 28(23), **2023**, 7780, DOI: 10.3390/molecules28237780
- 4) Gitlin-Domagalska A., Dębowski D., Maciejewska A., Samsonov S., Maszota-Zieleniak M., Ptaszyńska N., Łęgowska A., Rolka K., „Cyclic peptidic furin inhibitors developed by combinatorial chemistry”, *ACS Medicinal Chemistry Letters*, 14, **2023**, 458–465. DOI: 10.1021/acsmedchemlett.3c00008
- 5) Gitlin-Domagalska A., Maciejewska A., Dębowski D., „Bowman-Birk inhibitors: insights into family of multifunctional proteins and peptides with potential therapeutical applications”, *Pharmaceutics*, 13(12), **2020**, 1-40, DOI: 10.3390/ph13120421

Submitted manuscript:

Dębowski D., Helbik-Maciejewska A., Gitlin-Domagalska A., Ptaszyńska N., Łęgowska A., Maszota-Zieleniak M., Samsonov S. A., Zhukov I., Rolka K., „Conformational Analysis and Structural Characterization of Furin Inhibitors and Their Enzyme Complexes: Structural Insights into the Role of Cis/Trans Isomerization of the P2'–P3' Peptide Bond”

Conferences:

- 1) Helbik-Maciejewska A., Gitlin-Domagalska A., Dębowskim D., Łęgowska A., Zerrouqi A., Pyrzyńska B., Rolka K., „Cyclic furin inhibitors with disulfide bridge mimetics with anticancer activity against glioblastoma and lung cells”, 27th Polish Peptide Symposium, 07-11.09.2025, Szklarska Poręba, Poland, Oral presentation
- 2) Helbik-Maciejewska A., Gitlin-Domagalska A., Glavaš M., Ptaszyńska N., Dębowski D., Łęgowska A., Rolka K., „Time-efficient peptide N-methylation”, 27th Polish Peptide Symposium, 07-11.09.2025, Szklarska Poręba, Poland, Poster presentation
- 3) Helbik-Maciejewska A., Brzuska G., Dębowski D., Gitlin-Domagalska A., Lipińska A., Król E., Rolka K., „Novel, non-covalent peptide furin inhibitors suppressing Zika virus

replication”, 37th European Peptide Symposium and 14th International Peptide Symposium, 25-29.05.2024, Florence, Italy, Poster presentation

- 4) Helbik-Maciejewska A., Gitlin-Domagalska A., Dębowski D., Maszota-Zieleniak M., Samsonov S., Désilets A., Boudreault PL., Rolka K., „Selektywne peptydowe inhibitory matryptazy-1”, XX Ogólnopolskie Seminarium Doktorantów i Studentów "Na pograniczu chemii, biologii i farmacji" (XX NPCIBiF)), 26-29.05.2024, Jarnołtówek, Poland
- 5) Maciejewska A., Gitlin-Domagalska A., Dębowski D., Łęgowska A., Rolka K., „Novel, cyclic peptidic furin inhibitors”, 26th Polish Peptide Symposium, 03-07.09.2023, Stare Jabłonki, Poland, Oral presentation
- 6) Maciejewska A., Gitlin-Domagalska A., Dębowski D., Rolka K., „Chemiczna synteza i ocena aktywności inhibitorowej analogów inhibitora trypsyny SFTI-1 wobec wybranych transmembranowych proteinaz serynowych”, XVI Kopernikańskie Seminarium Doktoranckie (XVI KSD), 29-30.06.2023, Toruń, Poland, Poster presentation
- 7) Maciejewska A., Gitlin-Domagalska A., Dębowski D., Łęgowska A., Rolka K., „Nowe bicykliczne peptydowe inhibitory furyny”, XIX Ogólnopolskie Seminarium Doktorantów i Studentów "Na pograniczu chemii i biologii" (XIX NPCIB), 04-07.06.2023, Trzebieiszowice, Poland, Oral presentation
- 8) Maciejewska A., Gitlin-Domagalska A., Dębowski D., Rolka K., „Synteza chemiczna koniugatów złożonych z inhibitora proteasomu oraz peptydów o różnych właściwościach biologicznych”, Konferencja naukowa ChemBiŚ, 24-25.06.2022, Gdańsk, Poland, Poster presentation

Research experience:

2025 – present	Principal Investigator in NCN Preludium 23 Title of project: Frog skin derived cyclic peptide as a molecular scaffold to create a novel tool for combining visualization and inhibition of cancer-associated enzyme (matriptase-1) (UMO-2024/53/N/ST5/01298)
2024	Investigator (phd student) SONATA15 2019/35/D/NZ7/00174 Principal Investigator: dr Agata Gitlin-Domagalska
2024 – 2025	Principal Investigator in UGrants-start 4 (1220/158/2024) – a grant program supporting PhD students and young scientists from University of Gdańsk in publishing in prestigious journals
2023 – 2024	Principal Investigator in Grant for Young Scientists from Faculty of Chemistry, University of Gdańsk BMN 2023 (539-T060-B051-23) – a grant program supporting PhD students and young scientists
2022 – 2023	Principal Investigator in UGrants-start 2 (1220/54/2022) – a grant program supporting PhD students and young scientists from University of Gdańsk in publishing in prestigious journals

2022 – 2023 **Principal Investigator** in Grant for Young Scientists from Faculty of Chemistry, University of Gdańsk BMN 2022 (539-T060-B023-22) – a grant program supporting PhD students and young scientists

2020 – 2021 **Investigator (student)**
Title of project: Lipophilic Prodrug Charge Masking (LPCM) as a novel strategy to enhance oral bioavailability of charged active peptides. Optimization and generalization of the method (SONATA15 2019/35/D/NZ7/00174)
Principal Investigator: dr Agata Gitlin-Domagalska

Other:

1. 29.05.2024, Jarnołtówek, Poland, XX National Seminar for Ungraduated Ph.D. Students and Students „On the border of biology, chemistry and pharmacy” (XX Ogólnopolskie Seminarium Doktorantów i Studentów "Na pograniczu chemii, biologii i farmacji" (XX NPCIBiF)) - Award for the best presentation in the field of pharmaceutical sciences; title of presentation: Selective, peptidic matriptase-1 inhibitors (Selektywne peptydowe inhibitory matryptazy-1)
2. Member of the Organizing Committee of 26th Polish Peptide Symposium in Stare Jabłonki, Poland, 03-07.09.2023 – member of Organizing Committee
3. Conducting workshops for primary and secondary school students during "Open Days" at Faculty of Chemistry, University of Gdańsk, 2023-2026