2. Appendix to the application

AUTHOR'S PRESENTATION

Presenting the description of an output and scientific achievements, especially specified in art. 16 of the Act of 14 March 2003

Magdalena Wysocka, PhD

Laboratory of Bioorganic Chemistry

Department of Molecular Biochemistry

Faculty of Chemistry

University of Gdansk

Gdańsk, 30–03–2016

I. NAME AND SURNAME:

Magdalena Wysocka

II. OWNED DIPLOMAS, SCIENTIFIC /ARTISTIC DEGREES – NAMES, PLACE AND YEAR OF OBTAINING AND DOCTORAL DISSERTATION TITLE.

1. Diploma of chemical sciences doctor in the field of chemistry, Faculty of Chemistry, University of Gdansk, **2008**.
   Title of doctoral dissertation: *Application of combinatorial chemistry methods in the synthesis of chromogenic substrates of selected proteinases.*
   Promoter: professor *Krzysztof Rolka*
2. Diploma of master's in chemistry, Faculty of Chemistry, University of Gdansk, **2003**.
   Title of master's thesis: *Chemical synthesis of 91–120 fragment of human prion protein.*
   Supervisor: *Hanna Miecznikowska, PhD*

III. INFORMATION OF SO FAR EMPLOYMENT IN SCIENTIFIC/ ARTISTIC INSTITUTIONS:

1. **2009-current**, adjunct, Faculty of Chemistry, University of Gdansk.
2. **2008–2009**, assistant, Faculty of Chemistry, University of Gdansk.

IV. PRESENTATION OF SCIENTIFIC AND RESEARCH ACHIEVEMENTS:

Table 1: List of the most important scientific and research achievements.

No.	Achievements	Before scientific doctor degree obtaining	After scientific doctor degree obtaining	In total
1.	Scientific publications in the journals from *Journal Citation Reports (JCR)* database	5	32	37
2.	Head of internal projects University of Gdansk	–	4	4
3.	Head of external projects (MSHE)	–	3	3
4.	Participation in research projects (contractor)	1	3	4
5.	Patent applications	–	1	1
6.	Authorship or co-authorship of monographs, scientific publications in international or national journals other that listed in *Journal Citation Reports* database (including peer-reviewed conference materials)	1	17	18
7.	Number of citations *(without self-citations)*	17	140	157
8.	*Impact factor* of publications	12.44	95.31	107.75
9.	Number of MSHE points	154	854	1008
10.	Hirsh index	1	10	10

V. SCIENTIFIC ACHIEVEMENT ACCORDING TO ART. 16, PARAGRAPH 2 OF THE ACT OF 14.03.2003 ON ACADEMIC DEGREES AND SCIENTIFIC TITLE (OJ 65, ITEM 595 AS AMENDED):

My scientific achievement, obtained after doctoral degree obtaining, which constitutes an important contribution to the development of scientific discipline of chemistry, referred to in Article. 16 paragraph. 2. of the Act, is monothematic series of publications entitled:

PEPTIDES AND PEPTIDOMIMETICS AS A TOOL FOR PROTEOLYTIC ENZYMES EXAMINATION

*This cycle is formed by **12** scientific publications with consistent themes, specified in the list below in section Va. All publications are listed in the Journal Citation Reports (JCR) database, and their total impact factor is **40.58** (**MSHE points 366**). The list of publications is presented in chronological order, and the full texts of publications and statements of co-authors are included in the appendices.*

A) List of publications (author/authors, title, publisher, year of publishing):

H.1. **Wysocka M.**, Lesner A., Majkowska G., Łęgowska A., Guzow K., Rolka K., Wiczk W., *The new fluorogenic substrates of neutrophil proteinase 3 optimized in prime site region*, Anal. Biochem., 399, 196-201, (**2010**). [**IF 3.24**], MSHE points 32

H.2. **Wysocka M**., Lesner A., Guzow K., Kulczycka J., Łęgowska A., Wiczk W., Rolka K., *Highly specific substrates of proteinase 3 containing 3-(2-benzoxazol-5-yl)-L-alanine and their application for detection of this enzyme in human serum*, Anal Chem., 82, 3883-3889, (**2010**). [**IF 5,87**], MSHE points 32

H.3. **Wysocka M.,** Spichalska B., Lesner A., Jaros M., Brzozowski K., Łęgowska A., Rolka K., *Substrate specificity and inhibitory study of human airways trypsin-like proteinase*, Bioorg. Med. Chem., 18, 5504-5509, (**2010**). [**IF 2.98**], MSHE points 32

H.4. **Wysocka M**., Lesner A., Popow J., Łęgowska M., Rolka K., *Pegylated FRET peptides as substrates of proteolytical enzymes*, Prot. Pept Lett., 19, 1237-1244, (**2012**). [**IF 1.99**], MSHE points 15

H.5. **Wysocka M.,** Lesner A., Gruba N., Korkmaz B., Gauthier F., Kitamatsu M., Łęgowska A., Rolka K., *Three wavelength substrate system of neutrophil serine proteinases*, Anal. Chem., 84, 7241-7248, (**2012**). [**IF 5.69**], MSHE points 45

H.6. **Wysocka M**., Lesner A., *Future of protease assay*, Curr Pharm Des., 19, 1062-1067, (**2013**). [**IF 3.29**], MSHE points 35

H.7. Popow-Stellmaszyk J., **Wysocka M**., Lesner A., Korkmaz B., Rolka K., *A new proteinase 3 substrate with improved selectivity over human neutrophil elastase*, Anal. Biochem., 442, 75–82, (**2013**). [**IF 2.31**], MSHE points 30

H.8. **Wysocka M.,** Gruba N., Miecznikowska A., Popow-Stellmaszyk J., Gütschow M., Stirnberg M., Furtmann N., Bajorath J., Lesner A., Rolka K., *Substrate specificity of human matriptase-2*, Biochimie, 97, 121-127, (**2014**). [**IF 2.96**], MSHE points 30

H.9. Lęgowska M., **Wysocka M.**, Burster T., Pikuła M., Rolka K., Lesner A., *Ultrasensitive internally quenched substrates of human cathepsin L*, Anal Biochem., 466, 30-37, (**2014**). [**IF 2.22**], MSHE points 25

H.10. **Wysocka M.,** Wojtysiak A., Okońska M., Gruba N., Jarząb M., Wenta T., Lipińska B., Grzywa R., Sieńczyk M., Rolka K., Lesner A., *Design and synthesis of new substrates of HtrA2 protease*, Anal Biochem., 475, 44-52, (**2015**). [**IF 2.22**], MSHE points 25

H.11. Gruba N., **Wysocka M.,** Brzezińska M., Dębowski D., Rolka K., Martin N., Lesner A., *Novel internally quenched substrate of the trypsin-like subunit of 20S eukaryotic proteasome*, Anal Biochem., (**2015**) Aug 24. Pii: S0003-2697(15)00395-4. Doi: 10.1016/j.ab.2015.08.019. [**IF 2.22**], MSHE points 25

H.12. **Wysocka M.**, Gruba N., Grzywa R., Giełdoń A., Bąchor R., Brzozowski K., Sieńczyk M., Jenne D., Szewczuk Z., Rolka K., Lesner A., *PEGylated substrates of NSP4 protease: A tool to study protease specificity*, Sci. Rep. 6, 22856; doi: 10.1038/srep22856 (**2016**). [**IF 5.58**], MSHE points 40

Statements of all co-authors determining the individual contribution of each of them in the creation of particular works are included in Appendix 5.

B) Presentation of scientific/artistic aim of mentioned above publications and their results, together with the description of their possible application.

PREFACE. Presented cycle of 12 monothematic scientific publications [**H.1—H.12**] in the range of chemistry concerns the design, synthesis and analysis of substrate activity of selected proteases: proteinase 3 (PR3), human neutrophil elastase (HNE), human airway trypsin-like protease (HAT), cathepsin G (CG), matriptase-2 (MT-2), cathepsin L (Cat L), HtrA2, proteasome 20S and fourth neutrophil serine proteinase (NSP4).

The results of the experimental works carried out after doctoral degree obtaining, constituting the basis of this elaboration, have been published in 12 peer-reviewed articles in journals contained in Journal Citation Reports (JCR) database.

INTRODUCTION. The proteolytic enzymes, also known as proteinases, belong to the group of hydrolytic enzymes which primary function is hydrolysis of a peptide bond in the molecules of substrates such as proteins and peptides [1]. The aforementioned phenomenon is referred to as proteolysis, and it is one of the key processes in any organism. The reactions catalyzed by the proteinases occur with the emergence of life until its end. They include process of fertilization, cell cycle control, tissues differentiation, signal transduction, protein digestion, early immune response or apoptosis [2–4]. Proteolytic enzymes account for about 2% of the human genome [5].

Seven different groups-families of proteases are distinguished due to the mechanism of peptide bond hydrolysis and construction of catalytic apparatus, and they include: serine proteases, cysteine proteases, aspartic proteases, metalloproteinases, threonine proteases, and those with still unknown catalytic mechanism [6]. Despite these differences, each of these groups of enzymes interact with substrate molecule in its active site (**Fig. 1**). Proteolysis mechanism consists of the process of substrate molecule binding, its hydrolysis and formed products release. In 1967, Schechter and Berger proposed the nomenclature for active sites of enzyme and interacting with it substrate, which determines the residue of the substrate with symbol P, and the corresponding residues of the enzyme with symbol S [7].

[1]. Neurath H., *Evolution of proteolytic enzymes*, Science, (**1984**), 224, 350–357.
[2]. Barrett A. J., *Cellular proteolysis. An overview*, Ann N Y Acad Sci., (**1992**), 674, 1–15.
[3]. Wyllie A.H., *Apoptosis: an overview*, Br Med Bull., (**1997**); 53, 451–465.
[4]. Laflamme B.A., Wolfner M.F., *Identification and function of proteolysis regulators in seminal fluid*, Mol Reprod Dev., (**2013**), 80, 80–101.
[5]. Puente X.S., Sánchez L.M., Overall C.M., López-Otín C., *Human and mouse proteases: a comparative genomic approach*, Nat Rev Genet., (**2003**), 4, 544–558.
[6]. *Handbook of Proteolytic enzymes*, 3rd edition, Editor in Chief: N. D. Rawlings and G. S. Salvensen, Academic Press, 2013, ISBN: 978–0–12–382219–2.
[7]. Schechter I., Berger A., *On the size of the active site of proteases. I. Papain*, Biochem. Biophys. Res. Commun., (**1967**), 27, 157–162.

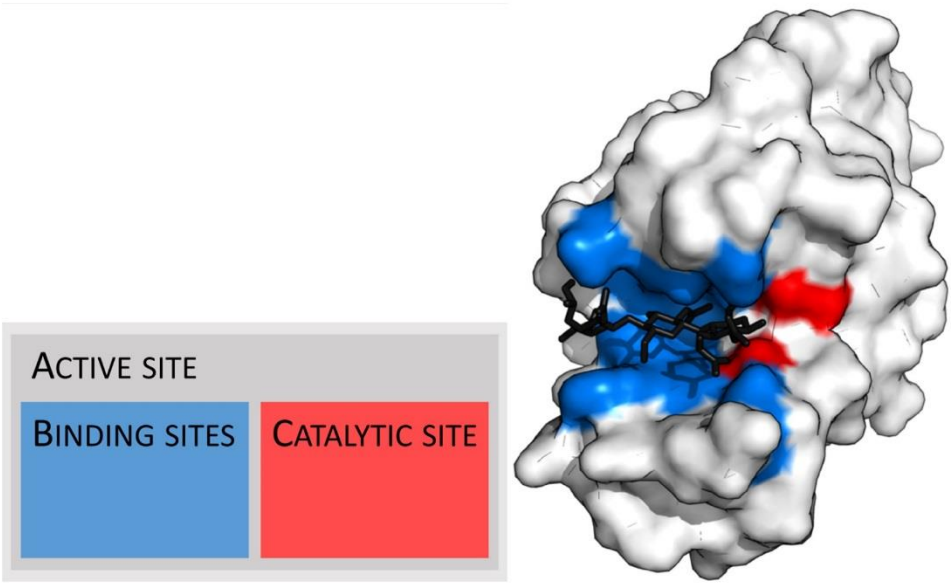


FIGURE 1: Spatial construction of enzyme active site.

Figure 2 presents a diagram of active site of the enzyme interacting with the substrate. The bond between residues P_1-P_1' is subject to degradation during the process of peptide bond hydrolysis. Another residues which constitute *N*-terminal fragment are referred to as P_2, P_3, P_4, P_n. In contrast, those present in *C*-terminal side of the hydrolyzed chain are referred to as P_2', P_3', P_4', P_n'.

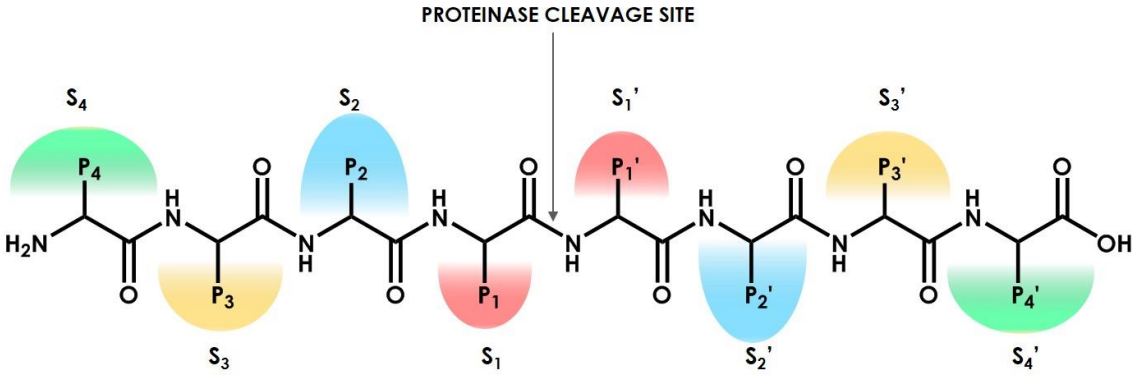


FIGURE 2: Nomenclature of enzyme–substrate interactions. Figure based on [7].

Proteolysis process in every living organism is strictly monitored both in time and space. The control mechanisms include compartmentalization, presence of inhibitors, synthesis of inactive proenzymes or proteinases autodegradation [8,9]. However, faulty or inefficient verification of an activity of one or several enzymes may lead to their excessive or delocalized performance, which results in organism homeostasis disturbances. There are many reports in the literature linking an excessive and uncontrolled activity of proteolytic enzymes with disease (pathophysiological) processes [10,11]. **Table 1** shows some examples of this phenomenon.

TABLE 1: Examples of proteolytic enzymes as disease markers.

ENZYME	PATHOLOGICAL STATE	LITERATURE REFERENCE
KLK3 and other representatives of kallikreins	prostate and craniofacial cancers	[12–14]
Viral proteases	viral infections	[15,16]
Bacterial proteases	bacterial infections	[17,18]
Urinary tissue plasminogen activator (utPA)	cancers	[19,20]
Furin and other proprotein convertases	bacterial infections, prostate cancer	[21,22]
Matryptaza-2	breast cancer	[23,24]
HtrA2	ovarian cancers	[25,26]
Caspases	decreased activity of cancer marker	[27,28]
Extracellular matrix metalloproteinases	cancer	[29,30]
Neutrophil proteinases	inflammation processes, lung cancer	[31–33]
Cathepsins	breast, skin cancer	[34,35]

[8]. Turk B., Turk D., Salvesen G.S., *Regulating cysteine protease activity: essential role of protease inhibitors as guardians and regulators*, Curr Pharm Des., (**2002**), 8, 1623-1637.
[9]. Khokha R., Murthy A., Weiss A., *Metalloproteinases and their natural inhibitors in inflammation and immunity*, Nat Rev Immunol., (**2013**), 13, 649-665.
[10]. Strowig T., Henao-Mejia J., Elinav E., Flavell R., *Inflammasomes in health and disease*, Nature, (**2012**), 481, 278-286.
[11]. Hadler-Olsen E., Fadnes B., Sylte I., Uhlin-Hansen L., Winberg J.O., *Regulation of matrix metalloproteinase activity in health and disease*, FEBS J., (**2011**), 278, 28-45.
[12]. Pampalakis G., Sotiropoulou G., *Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer*, Biochim Biophys Acta., (**2007**), 1776, 22-31.
[13]. Clements J.A., Willemsen N.M., Myers S.A., Dong Y., *The tissue kallikrein family of serine proteases: functional roles in human disease and potential as clinical biomarkers*, Crit Rev Clin Lab Sci., (**2004**), 41, 265-312.
[14]. LeBeau A.M., Kostova M., Craik C.S., Denmeade S.R., *Prostate-specific antigen: an overlooked candidate for the targeted treatment and selective imaging of prostate cancer*, Biol Chem., (**2010**) 391, 333-43.
[15]. Lee S.K., Potempa M., Swanstrom R., *The choreography of HIV-1 proteolytic processing and virion assembly*, J Biol Chem., (**2012**), 287, 40867-40874.
[16]. Polacek C., Gullberg M., Li J., Belsham G.J., *Low levels of foot-and-mouth disease virus 3C protease expression are required to achieve optimal capsid protein expression and processing in mammalian cells*, J Gen Virol., (**2013**), 94, 1249-1258.
[17]. Broz P., Monack D.M., *Molecular mechanisms of inflammasome activation during microbial infections*, Immunol Rev., (**2011**), 243, 174-190.
[18]. Caulfield A.J., Walker M.E., Gielda L.M., Lathem W.W., *The Pla protease of Yersinia pestis degrades fas ligand to manipulate host cell death and inflammation*, Cell Host Microbe., (**2014**), 15, 424-434.
[19]. Nielsen V.G., Matika R.W., Ley M.L., Waer A.L., Gharagozloo F., Kim S., Nfonsam V.N., Ong E.S., Jie T., Warneke J.A., Steinbrenner E.B., *Tissue-type plasminogen activator-induced fibrinolysis is enhanced in patients with breast, lung, pancreas and colon cancer*, Blood Coagul Fibrinolysis., (**2014**), 25, 248-253.
[20]. McMahon B., Kwaan H.C., *The plasminogen activator system and cancer*, Pathophysiol Haemost Thromb., (**2008**), 36, 184-94.
[21]. Yao Z., Sun B., Hong Q., Yan J., Mu D., Li J., Sheng H., Guo H., *PACE4 regulates apoptosis in human prostate cancer cells via endoplasmic reticulum stress and mitochondrial signaling pathways*, Drug Des Devel Ther., (**2015**), 9, 5911-5923.
[22]. Couture F., D'Anjou F., Desjardins R., Boudreau F., Day R., *Role of proprotein convertases in prostate cancer progression*, Neoplasia, (**2012**), 14, 1032-1042.
[23]. Wang C.Y., Meynard D., Lin H.Y., *The role of TMPRSS6/matriptase-2 in iron regulation and anemia*, Front Pharmacol., (**2014**), 5, 1-6.
[24]. Sanders A.J., Webb S.L., Parr C., Mason M.D., Jiang W.G., *The type II transmembrane serine protease, matriptase-2: Possible links to cancer?*, Anticancer Agents Med Chem., (**2010**), 10, 64-69.
[25]. Xu Z., Chen Y., Xu G., Peng C., Liu E., Li Y., Niu J., Li C., *Omi/HtrA2 pro-apoptotic marker differs in various hepatocellular carcinoma cell lines owing to ped/pea-15 expression level*, Oncol Rep., (**2015**), 905-912.
[26]. Li S., Wan M., Cao X., Ren Y., *Expression of AIF and HtrA2/Omi in small lymphocytic lymphoma and diffuse large B-cell lymphoma*, Arch Pathol Lab Med., (**2011**), 135, 903-908.
[27]. Olsson M., Zhivotovsky B., *Caspases and cancer*, Cell Death Differ., (**2011**), 18, 1441–1449.
[28]. Shalini S., Dorstyn L., Dawar S., Kumar S., *Old, new and emerging functions of caspases*, Cell Death Differ., (**2015**), 22, 526-39.
[29]. Deryugina E.I., Quigley J.P., *Tumor angiogenesis: MMP-mediated induction of intravasation- and metastasis-sustaining neovasculature*, Matrix Biol., (**2015**), 44-46, 94-112.
[30]. Houghton A.M., *Matrix metalloproteinases in destructive lung disease*, Matrix Biol., (**2015**), 44-46, 167-174.
[31]. Heutinck K.M., ten Berge I.J., Hack C.E., Hamann J., Rowshani A.T., *Serine proteases of the human immune system in health and disease*, Mol Immunol., (**2010**), 47, 1943-1955.
[32]. Gregory A.D., Kliment C.R., Metz H.E., Kim K.H., Kargl J., Agostini B.A., Crum L.T., Oczypok E.A., Oury T.A., Houghton A.M., *Neutrophil elastase promotes myofibroblast differentiation in lung fibrosis*, J Leukoc Biol., (**2015**), 98, 143-52.
[33]. Cools-Lartigue J., Spicer J., Najmeh S., Ferri L., *Neutrophil extracellular traps in cancer progression*, Cell Mol Life Sci., (**2014**), 71, 4179-4194.
[34]. Löser R., Pietzsch J., *Cysteine cathepsins: their role in tumor progression and recent trends in the development of imaging probes*, Front Chem., (**2015**), Jun 23;3:37. doi: 10.3389/fchem.2015.00037. eCollection 2015.
[35]. Turk V., Stoka V., Vasiljeva O., Renko M., Sun T., Turk B., Turk D., *Cysteine cathepsins: from structure, function and regulation to new frontiers*, Biochim Biophys Acta., (**2012**), 1824, 68-88.

Thus, when undesired proteolytic activity can lead to pathologic conditions and even death of the organism, the detection of active proteolytic enzyme may be part of such disorders diagnosis in the organism.

Numerous methods have been developed so far to facilitate the determination of proteolytic enzymes activity. **Table 2** presents some of them [36–42].

TABLE 2: Selected methods of proteolytic activity determination.

METHOD	SAMPLE ANALYSIS	LITERATURE REFERENCE
chromogenic substrates	blood coagulation enzymes	[36]
fluorescent substrates	caspases	[37]
luminescent substrates	proteases panel	[38]
surface plasmon resonance (SPR)	caspase 3	[39]
electrochemical	caspase 3	[40]
immunoenzymatic test (ELISA)	TNF converting enzyme (tumor necrosis factor)	[41]
molecular probes	proteases panel	[42]

The area of my interests includes peptides labeled with fluorescent moieties which hydrolysis catalyzed by a specified proteolytic enzyme leads to such a peptidomimetic (substrate) breakdown, and, consequently, to an increase in moiety fluorescence. One of the desired features of such a compound, except a high degree of affinity for examined enzyme, or a significant hydrolysis rate, is its selectivity, i.e. susceptibility to proteolysis only by selected proteolytic enzyme.

One of the two streams of this elaboration was preparation of selective substrates of selected proteolytic enzymes by chemical synthesis, enabling generation of tools for preliminary diagnosis of selected human diseases. The second stream of the research was characteristics of substrate specificity of certain proteolytic enzymes. Both directions of my research have points in common.

[36]. Rosén S., *Chromogenic methods in coagulation diagnostics*, (**2005**), 25, 259-266.
[37]. Packard B.Z., Komoriya A., *Intracellular protease activation in apoptosis and cell-mediated cytotoxicity characterized by cell-permeable fluorogenic protease substrates*, Cell Res., (**2008**), 18, 238-247.
[38]. Leippe D.M., Nguyen D., Zhou M., Good T., Kirkland T.A., Scurria M., Bernad L., Ugo T., Vidugiriene J., Cali J.J., Klaubert D.H., O'Brien M.A., *A bioluminescent assay for the sensitive detection of proteases*, Biotechniques. (**2011**), 51, 105-110.
[39]. Chen H., Mei Q., Hou Y., Zhu X., Koh K., Li X., Li G., *Fabrication of a protease sensor for caspase-3 activity detection based on surface plasmon resonance*, Analyst., (**2013**), 138, 5757-5771.
[40]. Xiao H., Liu L., Meng F., Huang J., Li G., *Electrochemical approach to detect apoptosis*, Anal Chem., (**2008**), 80, 5272-5275.
[41]. Wang Y.W., Ren H.L., Wang H.F., Li F.D., Li H.H., Zheng Y.H., *Combining detection of Notch1 and tumor necrosis factor-a converting enzyme is a reliable biomarker for the diagnosis of abdominal aortic aneurysms*, Life Sci., (**2015**), 127, 39-45.
[42]. Serim S., Haedke U., Verhelst S.H., *Activity-based probes for the study of proteases: recent advances and developments*, ChemMedChem., (**2012**), 7, 1146-1159.

Most of the works is an effect of cooperation with: Laboratory of Photobiophysics, Faculty of Chemistry, University of Gdansk; Laboratory of Immunology and Clinical Transplantation, University Clinical Center in Gdansk; University of Tours; University of Okayama; University of Bonn; Department of Medical Chemistry and Microbiology, Faculty of Chemistry, Wroclaw University of Technology; Department of Biochemistry, Faculty of Biology, University of Gdansk; Helmholtz Center in Munich; Department of Endocrinology and Diabetes, Medical University of Ulm; Institute of Pharmaceutical Sciences, University of Utrecht; Department of Electrochemistry, Faculty of Chemistry, University of Bialystok.

SCIENTIFIC AIM OF THE WORK. The studies aimed at an elaboration of synthetic methods allowing to obtain a set of chemical compounds used to monitor the proteolytic activity of selected enzymes. I focused in my research on the analysis of substrate specificity of enzymes such as elastase, proteinase 3, proteinase HAT, cathepsin G, matriptase-2 of human mast cells, mitochondrial protease HtrA2, human proteasome 20S, cathepsin L and fourth neutrophil serine proteinase. As a result of these methods elaboration, I obtained **67** fluorogenic substrates and **2** inhibitors.

As part of my research, I elaborated and used an innovative method of an examination of substrate specificity of enzymes in *C*-terminal area (prime subsites) [43].

In the case of HAT, matriptase-2, cathepsin L, HtrA2, human proteasome 20S subunits and NSP4, the works resulted in determination of substrate specificity of so far uncharacterized enzymes [44–49]. In one case (HAT), obtained substrates become the structure leading to the design and synthesis of efficient inhibitors of this enzyme [44].

Another group of compounds that we obtained as part of the works were selective substrates enabling detection of specific proteolytic enzyme in the presence of other proteases, often with a similar selectivity. At this stage of the study, I focused on a group of neutrophil serine proteases, which level of activity in biological material is of a significant diagnostic value.

43 [**H.7**]. Popow-Stellmaszyk J., **Wysocka M**., Lesner A., Korkmaz B., Rolka K., *A new proteinase 3 substrate with improved selectivity over human neutrophil elastase*, Anal. Biochem., (**2013**), 442, 75–82.

44 [**H.3**]. **Wysocka M.,** Spichalska B., Lesner A., Jaros M., Brzozowski K., Łęgowska A., Rolka K., *Substrate specificity and inhibitory study of human airways trypsin-like proteinase*, Bioorg. Med. Chem., (**2010**),18, 5504-5509.

45 [**H.8**]. **Wysocka M.,** Gruba N., Miecznikowska A., Popow-Stellmaszyk J., Gütschow M., Stirnberg M., Furtmann N., Bajorath J., Lesner A., Rolka K., *Substrate specificity of human matriptase-2*, Biochimie, (**2014**), 97, 121-127.

46 [**H.9**]. Łęgowska M., **Wysocka M.**, Burster T., Pikuła M., Rolka K., Lesner A., *Ultrasensitive internally quenched substrates of human cathepsin L*, Anal Biochem., (**2014**), 466, 30-37.

47 [**H.10**]. **Wysocka M.,** Wojtysiak A., Okońska M., Gruba N., Jarząb M., Wenta T., Lipińska B., Grzywa R., Sieńczyk M., Rolka K., Lesner A., *Design and synthesis of new substrates of HtrA2 protease*, Anal Biochem., (**2015**), 475, 44-52.

48 [**H.11**]. Gruba N., **Wysocka M.,** Brzezińska M., Dębowski D., Rolka K., Martin N., Lesner A., *Novel internally quenched substrate of the trypsin-like subunit of 20S eukaryotic proteasome*, Anal Biochem., (**2015**) Aug 24. Pii: S0003-2697(15)00395-4. Doi: 10.1016/j.ab.2015.08.019.

49 [**H.12**]. **Wysocka M.**, Gruba N., Grzywa R., Giełdoń A., Bąchor R., Brzozowski K., Sieńczyk M., Jenne D., Szewczuk Z., Rolka K., Lesner A., *PEGylated substrates of NSP4 protease: A tool to study protease specificity*, Sci. Rep. 6, (**2016**), 22856; doi: 10.1038/srep22856.

We elaborated a highly selective substrates characterized by high specificity constant k_{cat}/K_M, enabling pM detection of examined enzymes concentrations. Particular attention was devoted to human neutrophil enzyme, i.e. proteinase 3, which increased activity correlates with a positive diagnosis of granulomatosis with polyangiitis, formerly known as Wegener's disease [50]. This particular form of vasculitis is a rare and often incurable disease. Substrate obtained in the present work is one of the elements of that disease diagnostics.

A general scheme of proceeding during the procedure for obtaining efficient and selective substrates of selected proteases is presented in **Figure 3**.

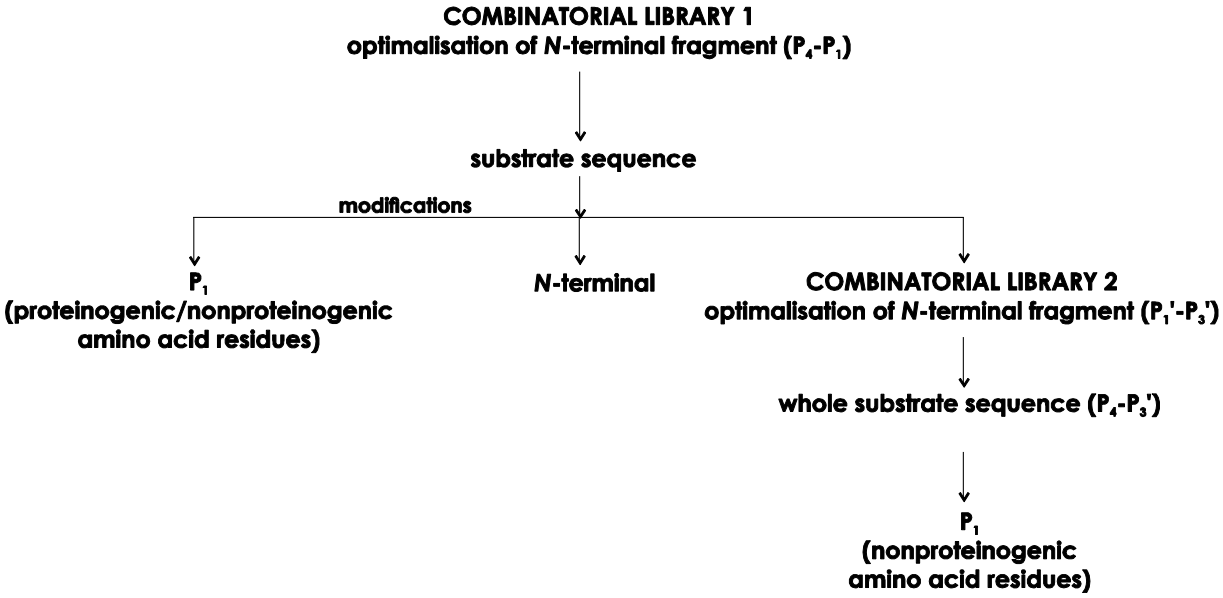


FIGURE 3: General strategy of the study.

RESULTS. The group of compounds that are substrates containing fluorescent group in their molecules, is important in two areas of research on proteolytic enzymes. The first is the use of fluorescently-labeled peptide mixtures (sublibraries) for the characterization of substrate-binding sites (substrate mapping). The second is labeled peptides obtaining and their application as diagnostic tools to monitor selected protease activity.

An analysis of substrate specificity was conducted using the solid phase synthesis method with Fmoc/tBu procedure, using the method of cutting and bonding (in case of combinatorial libraries). The general formula of synthesized libraries containing fluorescent substrates is presented below:

50 [**H.2**]. **Wysocka M**., Lesner A., Guzow K., Kulczycka J., Łęgowska A., Wiczk W., Rolka K., *Highly specific substrates of proteinase 3 containing 3-(2-benzoxazol-5-yl)-L-alanine and their application for detection of this enzyme in human serum*, Anal Chem., (**2010**), 82, 3883-3889.

ABZ-X_4-X_3-X_2-X_1-ANB-NH_2

where:
ABZ – *2-aminobenzoic acid (fluorescence donor)*
X_4, X_3, X_2 – *set of 19 proteinogenic amino acid residues, except cysteine*
X_1 – *specified amino acid residues determining specificity of the examined enzyme*
ANB-NH_2 – *5-amino-2-nitrobenzoic acid amide (fluorescence acceptor)*

Due to the presence of donor-acceptor pair in peptide molecule, it was possible to observe donor fluorescence quenching as a result of fluorescence resonance energy transfer (FRET). In the case of hydrolysis of peptide bond located between residue P_1, and a molecule 5-amino-2-nitrobenzoic acid amide [51] or 3-nitro-L-tyrosine [52] of such labeled peptide, an increase in fluorescence is proportional to the activity (indirectly to the concentration) of the enzyme [43–50, 53–55].

The procedure of appropriate specific sequence determination, called library deconvolution, was conducted using an iterative method, analyzing each position X in the presence of examined proteolytic enzyme. The process mentioned above allowed to obtain the sequences of substrates undergoing proteolysis the most efficiently, and we also established the substrate specificity of the examined protease.

The second area of research concerned determination of enzymatic activity of specific protease as a marker of pathological condition. For this purpose, we used the modified substrates (selected during specificity examination), so that to increase the selectivity of their interactions only with one selected proteolytic enzyme. In order to achieve this objective, we made the exchange of relevant amino acid residues of initial peptide with their non-protein analogs. Also fluorescent marker was subject to the modification in this process [50,54,55].

[51]. Hojo K., Maeda M., Iguchi S., Smith T., Okomata H., Kawasaki K., *Amino acids and peptides. XXXV. Facile preparation of p-nitroanilide analogs by the solid-phase method*, Chem. Pharm. Bull., (**2000**), 48, 1740-1744.

[52]. Meldal M., Breddam K., *Anthralanilamide and nitrotyrosine as a donor-acceptor pair in internally quenched fluorescent substrates for endopeptidases: multicolumn peptide synthesis of enzyme substrates for subtilisin Carlberg and pepsin*, Anal Biochem, (**1979**),195, 228-235.

53 [**H.1**]. **Wysocka M.**, Lesner A., Majkowska G., Łęgowska A., Guzow K., Rolka K., Wiczk W., *The new fluorogenic substrates of neutrophil proteinase 3 optimized in prime site region*, Anal. Biochem., (**2010**), 399, 196-201.

54 [**H.4**]. **Wysocka M**., Lesner A., Popow J., Łęgowska M., Rolka K., *Pegylated FRET peptides as substrates of proteolytical enzymes*, Prot. Pept Lett., (**2012**), 19, 1237-1244.

55 [**H.5**]. **Wysocka M.,** Lesner A., Gruba N., Korkmaz B., Gauthier F., Kitamatsu M., Łęgowska A., Rolka K., *Three wavelength substrate system of neutrophil serine proteinases*, Anal. Chem., (**2012**), 84, 7241-7248.

SUBSTRATES WITH *C*-TERMINAL LABEL (ANB)

***Human airway trypsin-like protease* (HAT)**

Human airway trypsin-like protease (HAT) belongs to the transmembrane enzymes [56], and its function in the human organism includes the degradation of fibrinogen and activation of receptor PAR2 (protease-activated receptor 2) [57].

It is worth to emphasize, that as the first group in the world we have determined the substrate specificity of HAT. Only the primary specificity of that enzyme has been known up to now.

Substrates sequences selected using combinatorial chemistry methods for protease HAT include: ABZ-Arg-Gln-Asp-Arg-ANB-NH_2 and ABZ-Arg-Gln-Asp-Lys-ANB-NH_2. The substrates demonstrated high values of specificity constant (k_{cat}/K_M), 454.4 $\times 10^3$ $M^{-1}\times s^{-1}$ and 195.2$\times 10^3$ $M^{-1}\times s^{-1}$, respectively. I would like to emphasize that one of the obtained compounds demonstrates 8-fold greater specificity constant compared to the value previously determined for a substrate commonly used for proteinase HAT (Boc-Phe-Ser-Arg-4-MCA, k_{cat}/K_M = 58.2$\times 10^3$ $M^{-1}\times s^{-1}$). Then, based on the sequences of obtained substrates, we synthesized two peptidomimetics containing an aldehyde group at *C*-terminal position. The inhibition constants, which we determined for both compounds, indicate that they are efficient inhibitors of this enzyme (inhibition constants K_i are as follows: ABZ-Arg-Gln-Asp-Arg-H 54.3 nM, ABZ-Arg-Gln-Asp-Lys-H 112.3 nM) (**Table 3**). Moreover, the presence of aspartic acid residue at position X_2 causes, that they demonstrate high selectivity for the examined proteases (human: matriptase and tryptase, and bovine β-trypsin) [44].

TABLE 3: Braking power and selectivity of HAT inhibitors.

PEPTIDE	ENZYME	K_i [nM]
ABZ-Arg-Gln-Asp-Arg-H	HAT	54.3±2.1
	Matriptase	972.9±82.2
	Tryptase	2127.5±171.9
	Bovine β-trypsin	3925.1±94.8
ABZ-Arg-Gln-Asp-Arg-H	HAT	112.3±7.5
	Matriptase	1710.7±110.1
	Tryptase	3753.3±282.5
	Bovine β-trypsin	7232.2±252.9

[56]. Yasuoka S., Ohnishi T., Kawano S., Tsuchihashi S., Ogawara M., Masuda K., Yamaoka K., Takahashi M., Sano T, *Purification, characterization, and localization of a novel trypsin-like protease found in the human airway*, Am J Respir Cell Mol Biol., **(1997)**, 16, 300-308.

[57]. Chokki M., Eguchi H., Hamamura I., Mitsuhashi H., Kamimura T., *Human airway trypsin-like protease induces amphiregulin release through a mechanism involving protease-activated receptor-2-mediated ERK activation and TNF alpha-converting enzyme activity in airway epithelial cells*, FEBS J., **(2005)**, 272, 6387-6399.

Substrates of neutrophil proteinase 3

The sequence of proteinase 3 substrate was modified in a subsequent process by the addition to *C*-terminal carboxyl group ANB, located on the aromatic ring, of 19 proteinogenic amino acids. The general formula of synthesized compounds is presented below:

ABZ-Tyr-Tyr-Abu-ANB-X-NH_2

where:
ABZ – *2-aminobenzoic acid (fluorescence donor)*
X – *set of 19 proteinogenic amino acid residues, except cysteine*
ANB-NH_2 – *5-amino-2-nitrobenzoic acid amide (fluorescence acceptor)*

Obtained substrates were incubated with two enzymes with similar primary specificity, isolated from human neutrophils: elastase and proteinase 3 [58]. Compared to the initial substrate of proteinase 3: ABZ-Tyr-Tyr-Abu-ANB-NH_2 (k_{cat}/K_M = 189×10^3 $M^{-1}\times s^{-1}$), only two of the compounds were characterized by higher kinetics parameters obtained for the examined enzyme [53].

This concerns two substrates: ABZ-Tyr-Tyr-Abu-ANB-Asn-NH_2 (k_{cat}/K_M = 201×10^3 $M^{-1}\times s^{-1}$) and ABZ-Tyr-Tyr-Abu-ANB-Gln-NH_2 (k_{cat}/K_M = 275×10^3 $M^{-1}\times s^{-1}$). It is worth to emphasize, that substrates obtained by us, in addition to the aforementioned specificity, exhibited extremely high selectivity for human neutrophil elastase and did not undergo hydrolysis in its presence. It can be concluded based on the results obtained that an appropriate modification of *C*-terminal carboxylic function with amino acid residues can result in obtaining of selective and specific proteinase substrates. Moreover, an effect of primed positions modification on kinetic parameters of obtained compounds motivated me to design peptide libraries containing variable positions just in that area of enzyme-substrate interactions.

[58]. Pham C.T., *Neutrophil serine proteases fine-tune the inflammatory response*, Int J Biochem Cell Biol., **(2008)**, 40, 1317–1333.

SUBSTRATES WITH *C*-TERMINAL LABEL (Tyr(3-NO_2))

Substrates of neutrophil proteinase 3

In order to perform a detailed analysis of the above mentioned enzyme-substrate interactions, I was forced to replace previously used donor-acceptor pair (ABZ — ANB-NH_2) to one that was characterized by a higher efficiency of energy quenching for longer peptide chains. Based on the literature data, I selected L-tyrosine derivative modified with nitro group at position 3 [52]. Designed substrate containing ABZ— Tyr(3-NO_2)-NH_2 pair exhibited similar spectral properties as the substrate containing ANB acid molecule.

The next stage was synthesis of the library of a general formula:

ABZ-peptide-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2

where:
ABZ – *2-aminobenzoic acid (fluorescence donor)*
peptide – *Tyr-Tyr-Abu*
X_1', X_2', X_3' – *set of 19 proteinogenic amino acid residues, except cysteine*
Tyr(3-NO_2)-NH_2 – *3-nitro-L-tyrosine amide (fluorescence acceptor)*

which parallel deconvolution in the presence of two enzymes isolated from human neutrophils: proteinase 3 and elastase, resulted in obtaining of the compound ABZ-Tyr-Tyr-Abu-Asn-Glu-Pro-Tyr(3-NO_2)-NH_2, which specificity constant was ($k_{cat}/K_M = 1596\times10^3\ M^{-1}\times s^{-1}$), i.e. over 8-fold more than for the substrate obtained by me so far [43]. Moreover, this study contains a description of the method allowing to determine the substrate specificity of any proteolytic enzyme in both primed positions, as well as in *N*-terminal fragment (non-primed). A key stage in determination of specific sublibraries or individual peptides susceptibility to proteolysis is their precise analysis using mass spectrometry techniques and reverse phase chromatography with fluorescence detector. Analysis of this type can confirm the presence of highly fluorescent product formed as a result of hydrolysis of the appropriate library, and thus to determine which peptide bond was subjected to hydrolysis. This is particularly important in the first two stages of deconvolution process. An example of such elaborated procedure is presented in **Figure 4**.

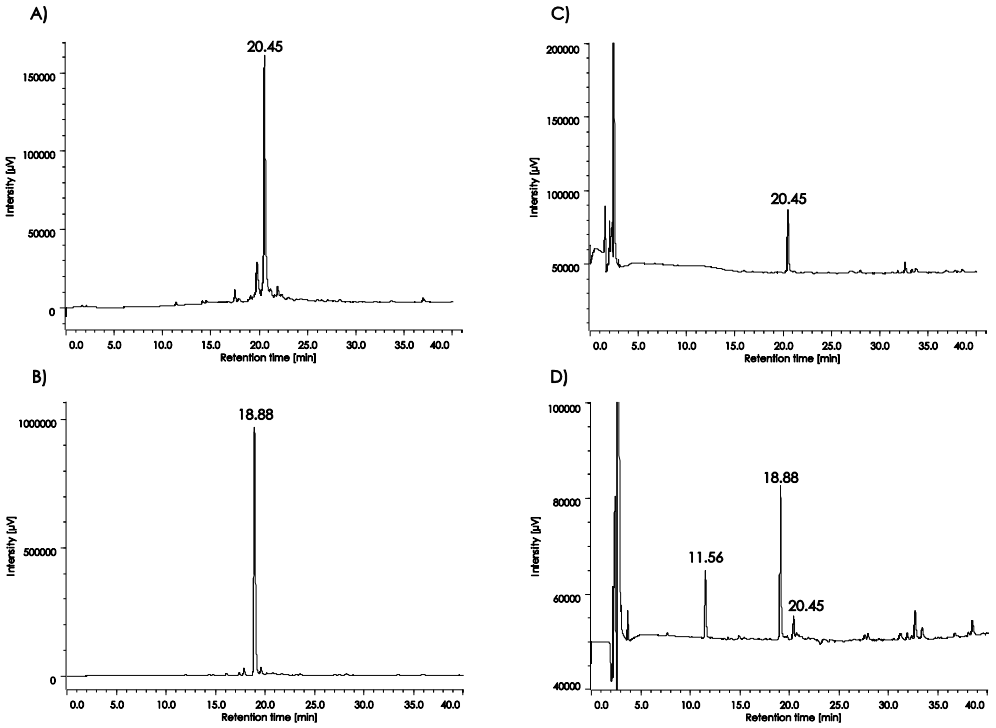


FIGURE 4: HPLC analysis ABZ-Tyr-Tyr-Abu-Asn-Glu-Pro-Tyr(3-NO_2)-NH_2: A) and B) fluorescent detection λ_{ex} = 320 nm, λ_{em} = 450 nm, C) and D) UV_{226} detection. A) and C) chromatograms of substrate, B) and D) chromatograms of substrate after 15 min incubation with PR3. Signals: t_R = 18.88 min, *N*-terminal fragment: ABZ-Tyr-Tyr-Abu-OH; t_R = 20.45 min, ABZ-Tyr-Tyr-Abu-Asn-Glu-Pro-Tyr(3-NO_2)-NH_2; t_R = 11.56, Asn-Pro-Glu-Tyr(3-NO_2)-NH_2.

The studies with similar specificity aimed to determine the specificity of particular proteolytic enzyme were also conducted for matriptase-2, cathepsin L, proteinase HtrA2, human proteasome and proteinase NSP4. The results obtained for these enzymes are presented below.

Matriptase-2

This enzyme is a representative of transmembrane proteases, and two structural domains: catalytic domain and transmembrane domain embedding enzyme in the cell membrane, can be distinguished within its spatial structure. In the light of recent studies, it is believed that matriptase-2 is involved in iron ions metabolism in the organism [59,60]. Furthermore, the overexpression of this enzyme in humans correlates with breast cancer development. Within the collaboration of professor Krzysztof Rolka with professor Michael Gütschow of the Rhine Friedrich Wilhelm University in Bonn, we obtained the preparation containing catalytic domain of matriptase-2 and determined the full substrate specificity for this enzyme [45]. As a result of activities involving deconvolution of **combinatorial library 1**, **and then combinatorial library 2** (**Fig. 3**), we selected specific and selective substrates allowing to monitor this enzyme activity (**Table 4**).

[59]. Szabo R., Bugge T.H., *Type II transmembrane serine proteases in development and disease*, Int J Biochem Cell Biol., **(2008)**, 40, 1297-1316.

[60]. Finberg K.E., Heeney M.M., Campagna D.R., Aydinok Y., Pearson H.A., Hartman K.R., Mayo M.M., Samuel S.M., Strouse J.J., Markianos K., Andrews N.C., Fleming M.D., *Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA)*, Nat Genet., **(2008)**, 40, 569-571.

TABLE 4: Kinetic parameters of matriptase-2 substrates.

SEQUENCE	K_M [M]×10⁻⁶	k_{cat} [s⁻¹]	k_{cat}/K_M [s⁻¹×M⁻¹]×10⁴
ABZ-Ile-Arg-Ala-Arg-Ser-Ala-**Gly**-Tyr(3-NO_2)-NH_2	8.35±1.21	3.79±0.38	45.42±1.82
ABZ-Ile-Arg-Ala-Arg-Ser-Ala-**Ala**-Tyr(3-NO_2)-NH_2	35.5±8.81	2.24±0.28	6.32±0.78
ABZ-Ile-Arg-Ala-Arg-Ser-Ala-**Ser**-Tyr(3-NO_2)-NH_2	24.5±3.92	2.66±0.17	10.98±1.05

Furthermore, we demonstrated that the amino acid sequence of *C*-terminal fragment of peptide chain which is subject to degradation catalyzed by matriptase-2, should contain small aliphatic amino acid residues such as Ala, Ser or Gly. Obtained thus data could form the basis for the design of new selective inhibitors of this very interesting enzyme.

Cathepsin L

We also conducted an analysis of substrate specificity for the enzyme belonging to the family of cysteine proteinases—cathepsin L. This proteinase is mainly observed in lysosomes, however its presence was also detected in cell nucleus, where it is involved in proteolysis of histones [61,62]. The aim of our study was to obtain selective fluorogenic substrate, which would be concurrently efficiently hydrolyzed by cathepsin L. For this purpose, we designed three series of compounds:

ABZ-Bip-X_1-Tyr(3-NO_2)-NH_2

ABZ-Bip-X_1-X_1'-Tyr(3-NO_2)-NH_2

ABZ-Bip-X_1-X_1'-X_2'-Tyr(3-NO_2)-NH_2

where:

ABZ	*– 2-aminobenzoic acid (fluorescence donor)*
Bip	*– diphenyl-L-alanine*
X_1, X_1', X_2',	*– set of 19 proteinogenic amino acid residues, except cysteine*
Tyr(3-NO_2)-NH_2	*– 3-nitro-L-tyrosine amide (fluorescence acceptor)*

from which we finally selected the substrate with extremely high specificity constant (k_{cat}/K_M = 2.6×10⁷ $M^{-1}\times s^{-1}$) having the sequence ABZ-Bip-Arg-Ala-Gln-Tyr(NO_2)-NH_2 [46]. Using this compound, we managed successfully determine cathepsin L activity of a concentration of about 10 pM. The resulting substrate also exhibited significant selectivity for the enzymes of lysosomal cathepsins family. This allowed to apply the selected compound in order to determine cathepsin L activity in biological material. For this purpose, we incubated obtained substrate with normal cells lysate (keratinocytes and fibroblasts), as well as with tumor cells (melanoma-line A375).

[61]. Guncar G., Pungercic G., Klemencic I., Turk V., Turk D., *Crystal structure of MHC class II-associated p41 Ii fragment bound to cathepsin L reveals the structural basis for differentiation between cathepsins L and S*, EMBO J., **(1999)**, 15, 18, 793-803.

[62]. Goulet B., Baruch A., Moon N.S., Poirier M., Sansregret L.L., Erickson A., Bogyo M., Nepveu A., *A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor*, Mol Cell., **(2004)**, 14, 207-219.

The results obtained indicate 40–50% increased activity of cathepsin L in skin cancer cells (**Fig. 5**). This result can be the basis for a quick diagnostic test which would enable skin cancer pre-diagnosis.

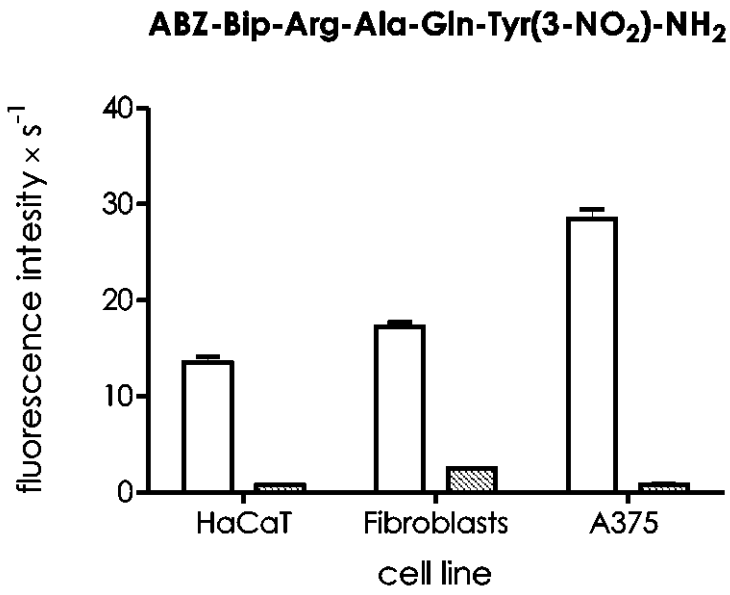


FIGURE 5: Activity of cathepsin L in cell lines lysates: keratinocytes (HaCaT), fibroblasts and melanoma (A375) — white bars. Stripped bars — control, where cell lysates were previously incubated with cathepsin L inhibitor.

HtrA2

Within the study on proteolytic enzymes we started the cooperation with a group of professor Barbara Lipińska from the Department of Biochemistry, Faculty of Biology of the University of Gdansk. The subject of common research were eukaryotic proteins of HtrA family (high temperature requirement A) which demonstrate chaperone and proteolytic activity [63]. The first enzyme for which we determined the substrate specificity was HtrA2. In the human organism, this proteinase is present in the mitochondrial intermembrane space. HtrA2 is involved in the process of life cycle of cells regulation, including apoptosis. Its increased activity is observed in case of cancer processes. Using the methods of combinatorial chemistry, we obtained a peptidomimetic, which can serve as a tool to monitor the activity of this enzyme in biological systems. The compound of a sequence ABZ-Ile-Met-Thr-Abu-Tyr-Met-Phe-Tyr(3-NO_2)-NH_2 exhibited a specificity constant k_{cat}/K_M = 14 535 $M^{-1}\times s^{-1}$ [47] and a very high selectivity to other enzymes of HtrA family (**Fig. 6**).

[63]. Gray C.W., Ward R.V., Karran E., Turconi S., Rowles A., Viglienghi D., Southan C., Barton A., Fantom K.G., West A., Savopoulos J., Hassan N.J., Clinkenbeard H., Hanning C., Amegadzie B., Davis J.B., Dingwall C., Livi G.P., Creasy C.L., *Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response*, Eur J Biochem., **(2000)**, 267, 5699-5710.

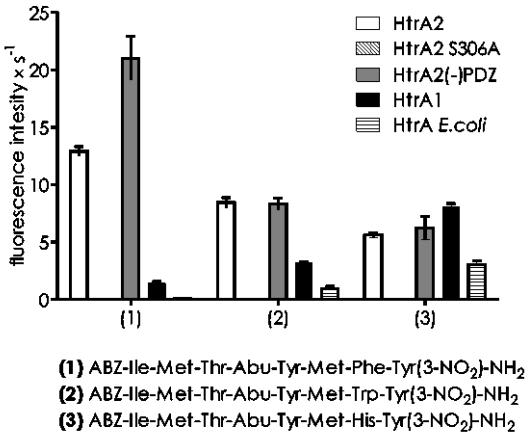


FIGURE 6: Fluorescence intensity increase over the time for substrates 1, 2 and 3 in the presence of HtrA family proteinase panel.

Eukaryotic proteasome 20S

The aim of the proposed study was to obtain a fluorescent peptide derivatives being the substrates of human proteasome 20S: multi-catalytic protein complex present in every living cell [64,65]. The proteolytic activity of the proteasome is noted within subunit 20S in domains: β1 and β1′, β2 and β2′ as well as β5 and β5′ (caspase-, trypsin and chymotrypsin-like, respectively). These compounds were designed in such a way that they are subject to a selective hydrolysis, i.e. are susceptible to an activity of only one subunit of human proteasome of a particular substrate specificity. In the next stage of the study we evaluated the level of selected subunit activity in healthy individuals, and in the patients with diagnosed course of specific cancer disease. For this purpose, we synthesized three libraries of the general formula:

ABZ-peptide-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2

peptide:	**Val-Val-Ser-Tyr**	**– chymotrypsin subunit,**
	Val-Val-Ser-Arg	**– trypsin subunit,**
	Ile-Leu-Met-Asp	**– caspase subunit,**

where:

ABZ	*– 2-aminobenzoic acid (fluorescence donor)*
X_1', X_2', X_3'	*– set of 19 proteinogenic amino acid residues, except cysteine*
Tyr(3-NO_2)-NH_2	*– 3-nitro-L-tyrosine amide (fluorescence acceptor)*

Peptide sequences for the three catalytic subunits of proteasome (Val-Val-Ser-Tyr, Val-Val-Ser-Arg and Ile-Leu-Met-Asp) were selected using the methods of combinatorial chemistry in the context of works carried out with Dawid Dębowski, PhD, from the Department of Molecular Biochemistry, Faculty of Chemistry of the University of Gdansk.

[64]. Kopp F., Hendil K.B., Dahlmann B., Kristensen P., Sobek A., Uerkvitz W., *Subunit arrangement in the human 20S proteasome*, Proc Natl Acad Sci U S A, **(1997)**, 94, 2939-2944.

[65]. Groll M., Heinemeyer W., Jäger S., Ullrich T., Bochtler M., Wolf D.H., Huber R., *The catalytic sites of 20S proteasomes and their role in subunit maturation: a mutational and crystallographic study*, Proc Natl Acad Sci U S A, **(1999)**, 96, 10976-10983.

The deconvolution process of these libraries, optimized in prim positions (according to the notation of Schechter Berger), allowed us to select three substrates, which were very efficiently subject to hydrolysis in the presence of a specific subunit of the human proteasome (**Table 5**).

TABLE 5: Sequences of substrates selected for proteasome 20S.

SUBSTRATE SEQUENCE	PROTEASOME SUBUNIT	LITERATURE REFERENCE
ABZ-Val-Val-Ser-Tyr↓-Ala-Met-Gly-Tyr(3-NO_2)-NH_2	– chymotrypsin	[66]
ABZ-Val-Val-Ser-Arg↓-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2	– trypsin	[48]
ABZ-Ile-Leu-Met-Asp↓-Ala-Met-Leu-Tyr(3-NO_2)-NH_2	– caspase-like	[67]
where: ↓-site of enzymatic hydrolysis (P_1-P_1')		

In the presence of proteasome the obtained substrates were subject to selective hydrolysis by selected proteasome subunit to two fragments resulting from decomposition of peptide bond between residues P_1 and P_1'. Using these three fluorogenic substrates, we were able to determine proteasome activity in selected biological material. Unfortunately, an attempt to modify the *N*-terminus of fluorophore molecule in order to obtain the compounds differing in the wavelength of emitted radiation, was unsuccessful. Any modifications to this position resulted in a loss of the desired substrate specificity, and consequently to the inhibition of its proteolysis.

Within the cooperation with professor Joanna Chorostowska-Wynimko from the National Institute of Tuberculosis and Lung Diseases in Warsaw and Marcin Matuszewski, PhD, from the Department of Urology, Medical University of Gdansk, we collected research material (human plasma and urine) in order to determine the profile of proteasome activity in healthy subjects and patients with diagnosed cancer and immune system disorders.

The study allowed to conclude that the substrate ABZ-Val-Val-Ser-Tyr-Ala-Met-Gly-Tyr(3-NO_2)-NH_2 in buffered urine of patients with bladder cancer is subject to decomposition characteristic for chymotrypsin-like subunit of human proteasome. In order to conduct the experiments in the presence of specific and selective proteasome inhibitors, we made contact with Alexei Kisselev, PhD, from Dartmouth Medical School, USA, from whom we received the above compounds. Hydrolysis of the substrate was inhibited in the presence of proteasome 20S inhibitors. This phenomenon was not observed for urine samples from healthy volunteers. The results have been included in the patent application [68].

[66]. Gruba N., **Wysocka M.**, Brzezinska M., Dębowski D., Gorodkiewicz E., Lesner A., Rolka K., *New internally quenched substrates of human 20S proteasome*, Peptides 2014, Proceedings of 33rd European Peptide Symposium, Sofia, European Peptide Society, Sofia **2014**, (E. Naydenova, T. Pajpanova, D. Danalev, eds.), str. 48-50.

[67]. Gruba N., **Wysocka M.**, Dębowski D., Lesner A., Rolka K., *Characteristics of caspase-like specificity of human 20S proteasome using combinatorial chemistry methods*, Peptides 2014, Proceedings of 33rd European Peptide Symposium, Sofia, European Peptide Society, Sofia **2014**, (E. Naydenova, T. Pajpanova, D. Danalev, eds.), str. 344-345.

[68]. Dębowski D., Gruba N., Lesner A., **Wysocka M.**, *Nowy związek, sposób jego otrzymywania, roztwór farmaceutyczny zawierający nowy związek, sposób określania obecności choroby nowotworowej, zestaw do wykrywania nowotworów oraz zastosowanie hydrolizy nowego związku do wykrywania nowotworów*. Zgłoszenie patentowe nr P.408905, (**2014**).

The study conducted did not allow an unequivocal confirmation of the usefulness of other two substrates hydrolyzed by trypsin-and caspase-like subunit in the diagnosis of bladder cancer. In both cases, we found no simple ill-healthy relationship.

The results strongly suggest that we obtained highly selective fluorogenic substrates of human proteasome 20S and 26S, characterized by high values of specificity constant (k_{cat}/K_M). Modifications to the position P_1 of the substrates of chymotrypsin (**Fig. 7**) and trypsin specificity (**Fig. 8**) demonstrated that the proteasome efficiently hydrolyzes substrates containing protein amino acid residues in that position of the (Tyr, Phe, Trp) for chymotrypsin, or Arg for trypsin specificity.

The substrate of a sequence ABZ-Val-Val-Ser-Phe(4-guanidine)-Ala-Met-Gly-Tyr(3-NO_2)-NH_2 appeared to be a compound with interesting properties. This peptide underwent efficient proteolysis catalyzed by trypsin-like subunit, not chymotrypsin-like as originally expected.

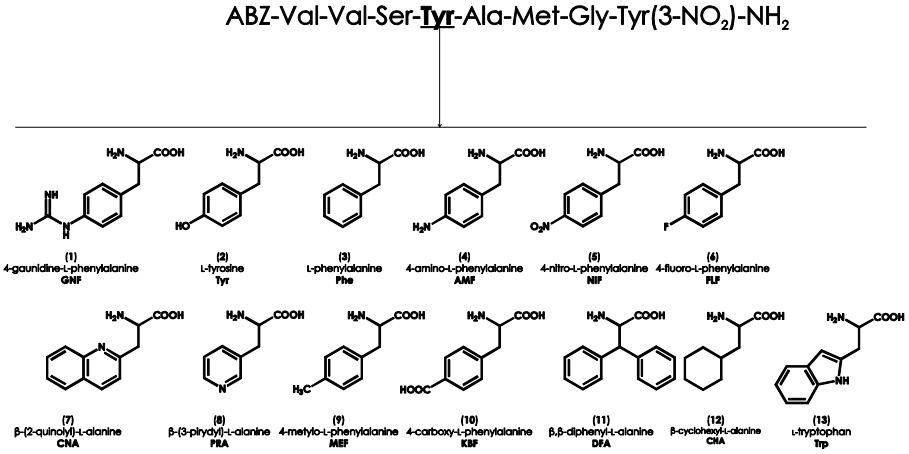
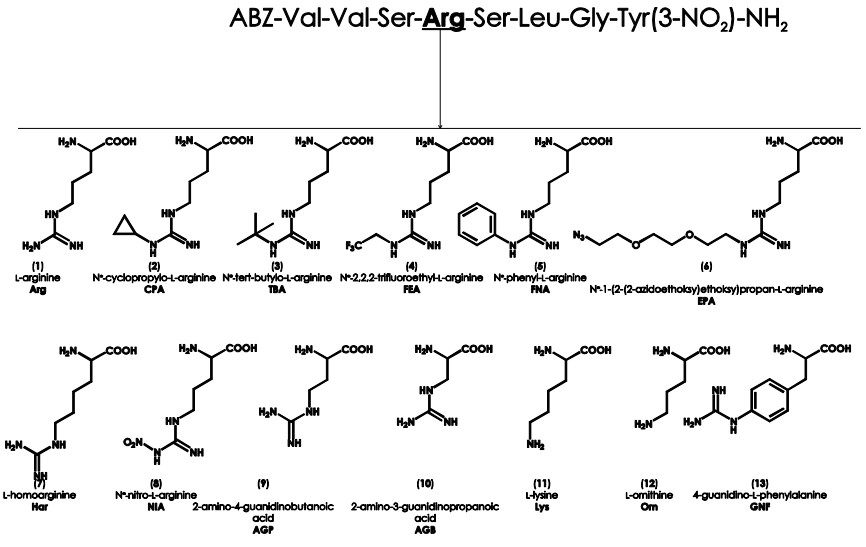


FIGURE 7: Structural formulas of a series of amino acid derivatives introduced in position P_1 of substrate ABZ-Val-Val-Ser-**Tyr**-Ala-Met-Gly-Tyr(3-NO_2)-NH_2.



RYSUNEK 8: Structural formulas of a series of arginine derivatives introduced in position P_1 of substrate ABZ-Val-Val-Ser-**Arg**-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2.

Selected substrates:

ABZ-Val-Val-Ser-Tyr-Ala-Met-Gly-Tyr(3-NO_2)-NH_2

ABZ-Val-Val-Ser-Arg-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2

ABZ-Ile-Leu-Met-Asp-Ala-Met-Leu-Tyr(3-NO_2)-NH_2

ABZ-Val-Val-Ser-Phe(4-guanidine)-Ala-Met-Gly-Tyr(3-NO_2)-NH_2

were subject to detailed examination in the presence of inhibitors, and proteasome 20S (SDS) and 26S activator. The limits of detection for proteasome 20S are presented in **Table 6**.

TABLE 6: Limits of obtained substrates quantification in the presence of proteasome 20S.

SUBSTRATE SEQUENCE	DETECTION LIMIT [pM]
ABZ-Val-Val-Ser-Tyr-Ala-Met-Gly-Tyr(3-NO_2)-NH_2	5
ABZ-Val-Val-Ser-Arg-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2	5
ABZ-Ile-Leu-Met-Asp-Ala-Met-Leu-Tyr(3-NO_2)-NH_2	58
ABZ-Val-Val-Ser-Phe(4-guanidine)-Ala-Met-Gly-Tyr(3-NO_2)-NH_2	12

The values obtained for selected substrates were characterized by higher detection limit with respect to commercially available immunoassays (ELISA), the use of which is expensive and the procedure is time-consuming.

Compounds: ABZ-Val-Val-Ser-Phe(4-guanidine)-Ala-Met-Gly-Tyr(3-NO_2)-NH_2, ABZ-Val-Val-Ser-Arg-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2 and ABZ-Val-Val-Ser-Tyr-Ala-Met-Gly-Tyr(3-NO_2)-NH_2 were subject to preliminary tests in biological material. In the case of plasma, we observed high variability due to the methodology of material collection (activation of plasma coagulation factors). In turn, urine of patients with bladder cancer is a diagnostics material for which we were able to obtain a clear ill-healthy correlation. Fluorescence increase is inhibited by commercially available proteasome inhibitors, and substrate ABZ-Val-Val-Ser-Tyr-Ala-Met-Gly-Tyr(3-NO_2)-NH_2 was subject to proteolysis in the same way as the isolated proteasome.

MODIFICATIONS OF SUBSTRATES *N*-and *C*-TERMINUS

Modifications of substrates with C-terminal marker (ANB)

The study on the series of substrates selected for: proteinase 3 [69], cathepsin G [70], neutrophil elastase [71] and protease HAT [44], were a continuation of works on the effect of substrate molecule extending on its strength and selectivity of interactions with enzyme.

[69]. **Wysocka M.**, Lesner A., Guzow K., Mackiewicz L., Łęgowska A., Wiczk W., Rolka K., *Design of selective substrates of proteinase 3 using combinatorial chemistry methods*, Anal. Biochem., (**2008**), 378, 208-215.

[70]. Lesner A., **Wysocka M.**, Guzow K., Wiczk W., Łęgowska A., Rolka K., *Development of sensitive cathepsin G fluorogenic substrate using combinatorial chemistry methods*, Anal. Biochem., (**2008**), 375, 306-312.

[71]. **Wysocka M.**, Kwiatkowska B., Rzadkiewicz M., Lesner A., Rolka K., *Selection of new chromogenic substrates of serine proteinases using combinatorial chemistry methods*, Comb. Chem. High T. Scr., (**2007**), 10, 171-180.

Modifications of this series of compounds involved an introduction of functionalized polyethylene glycol-PEG (8-amino-3,6-dioxaoctanoic acid) to the molecule sequence at the *C*-or *N*-terminus of the substrate molecule, or to both groups at the same time.

Such modification significantly increased the solubility of the resulting compounds in aqueous solutions. Chemical synthesis allowed to obtain 16 substrates for which kinetic parameters (k_{cat}, K_M and k_{cat}/K_M) were determined. Analyzing the data obtained, we could conclude unequivocally that the presence of polyethylene chain in *N*-terminal fragment of the substrate significantly increased (3–4 fold) its kinetic parameters, while the opposite effect was noted for the modification of the *C*-terminal carboxyl group, leading to a significant (2–3 fold) decrease in specificity constant compared to the initial compound, mainly due a decreased rate of proteolysis of thus obtained compounds (**Fig. 9**). Parallel introduction of glycol derivatives at the *N*-and *C*-terminus to a little degree affected the specificity constant, causing concurrently that the resulting peptidomimetics were perfectly soluble in aqueous solutions [54].

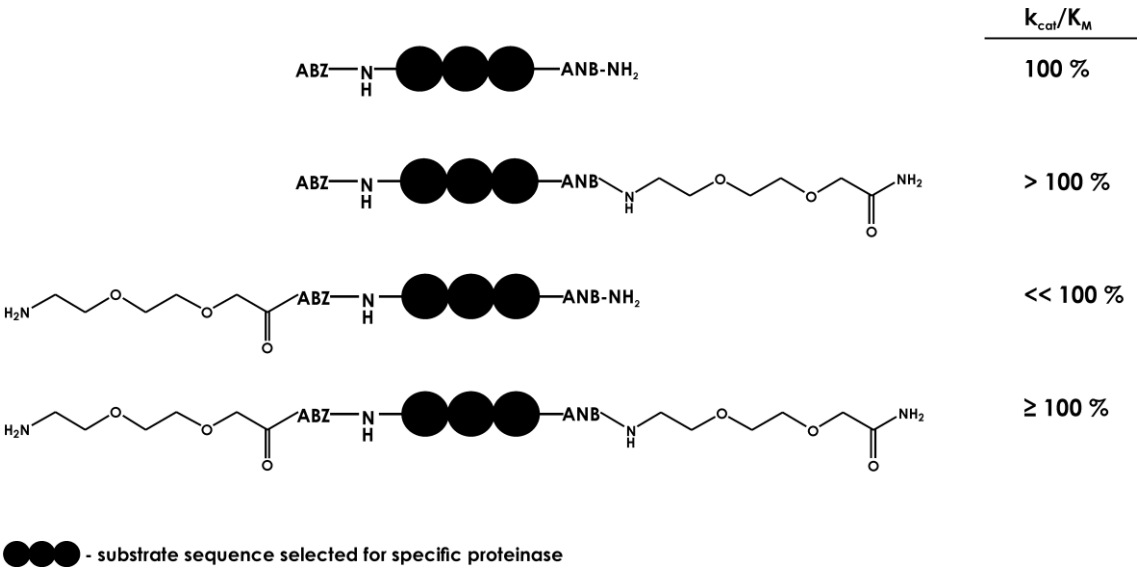


FIGURE 9: Scheme of substrate sequence modifications with functionalized polyethylene glycol molecule.

I independently evaluated the selected substrates with the highest specificity constants in terms of their biological properties in tissue culture laboratory of the Department of Immunology at the Medical University of Gdansk led by professor Piotr Trzonkowski. This study involved an evaluation of their ability to migrate across a cell membrane of tumor lines and normal cells. Unfortunately, none of the examined substrates effectively penetrated the cell membrane of tumor lines (HELA, Jurkat), as well as non-transformed ones (human neutrophils and fibroblasts).

Substrates modified in an area of fluorescence donor and acceptor

Another modification of substrates of neutrophil serine proteases involved replacement of already existing fluorescence donor-acceptor pairs (ABZ-ANB-NH_2), and the introduction of a set of amino acid Lys, Orn, and Ala derivatives in that site. These amino acids had fluorescent moieties in the side chain, which spectral features were selected so that after the excitation, the donor emitted a radiation in characteristic region of the spectrum, different for each of the examined enzymes (**Fig. 10**).

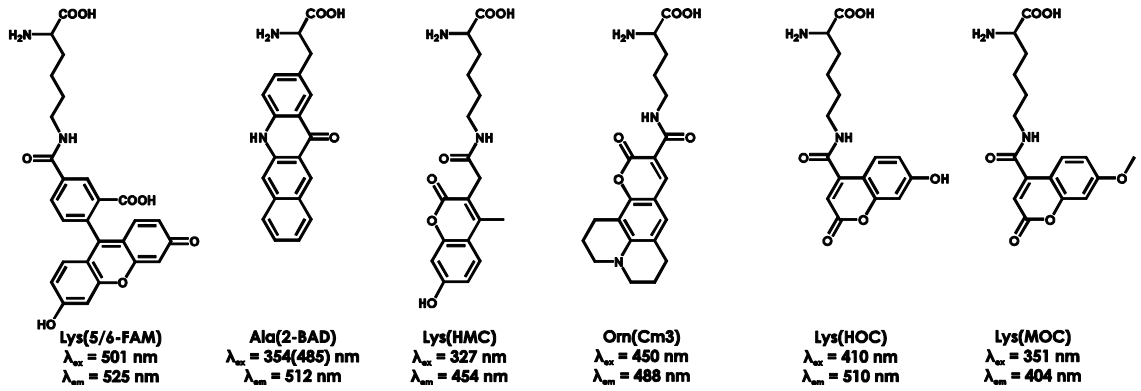


FIGURE 10: Structural formulas of selected fluorescent amino acid derivatives of Lys, Orn, and Ala.

Then, based on our earlier works and literature data, we selected three peptide sequences which are subject to selective enzymatic hydrolysis by a single enzyme belonging to the group of neutrophil serine proteinases (cathepsin G, human neutrophil elastase, and proteinase 3). I introduced each of selected fluorescent donor-acceptor pairs (**pair 1**: Lys(5/6-FAM) / Ala(2-BAD), **pair 2**: Lys(HMC) / Orn(Cm3), **pair 3**: Lys(HOC) / Lys(MOC)) to the *N*-and *C*-terminus of three selected substrate sequences. It is worth to emphasize that at this stage we obtained three combinations of donor-acceptor pair unpublished so far, which values of the fluorescence emission maxima are significantly different. The values of specificity constants determined for 9 substrates were a factor determining the selection of the right combination of fluorescent markers (**Table 7**) [55].

TABLE 7: Kinetic parameters determined for neutrophil serine proteases.

SEQUENCE	ENZYME	K_M [M]×10⁻⁶	k_{cat} [s⁻¹]	k_{cat}/K_M [s⁻¹×M⁻¹]×10⁴
(1) PEG-HOC-Tyr-Tyr-Abu-MOC	**PR3**	**18.5±0.4**	**9.7±1.5**	**52.41±3.21**
(2) PEG-HOC-Val-Thr-Gnf-Ser-Asp-MOC	CG	27.6±5.3	3.1±0.4	11.21±0.95
(3) PEG-HOC-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-MOC	HNE	125.1±8.3	5.1±1.3	4.08±1.05
(4) PEG-FAM-Tyr-Tyr-Abu-BAD	PR3	31.4±2.2	3.8±1.2	12.22±5.27
(5) PEG-FAM-Val-Thr-Gnf-Ser-Asp-BAD	CG	9.9±1.1	2.7±0.3	27.27±1.51
(6) PEG-FAM-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-BAD	**HNE**	**24.2±3.1**	**6.2±0.9**	**25.60±1.64**
(7) PEG-HMC-Tyr-Tyr-Abu-CM3	PR3	24.3±1.2	7.8±0.7	32.15±2.83
(8) PEG-HMC-Val-Thr-Gnf-Ser-Asp-CM3	**CG**	**19.0±1.1**	**7.2±2.1**	**37.90±1.89**
(9) PEG-HMC-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-CM3	HNE	74.5±3.7	1.3±0.1	7.41±0.36

where:
PEG = 8-amino-3,6-dioxaoctanoic acid; HOC = Lys(HOC)-OH, where HOC = 7-hydroxy-2-coumarin; MOC = Lys-(Moc)-OH, where Moc = 7-methoxy-coumarin; FAM = Lys(Fam)-OH, where Fam = 5(6)-fluorescein, BAD = Ala(BAD)-OH, where Bad = [benzo[β]acridine-12(5H)-on-2-yl; HMC = Lys(Hmc)-OH, where Hmc = 7-hydroxy-4-methylcoumarin and CM3 = Orn(Cm3)-OH, where Cm3 = 3-coumarin 343.

Finally, three peptides characterized by the highest specificity constants for the examined enzymes were selected for further studies:

(**1**)	PEG-HOC-Tyr-Tyr-Abu-MOC	PR3
(**6**)	PEG-FAM-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-BAD	HNE
(**8**)	PEG-HMC-Val-Thr-Gnf-Ser-Asp-CM3	CG

and except the kinematic parameters, we determined the minimum concentration of enzymes resulting in a noticeable fluorescence increase. The data obtained are presented in **Table 8**.

TABLE 8: Quantification limits determined for the selected substrates.

	SUBSTRATE SEQUENCE	ENZYME	DETECTION LIMIT [nM]
(**1**)	PEG-HOC-Tyr-Tyr-Abu-MOC	PR3	1.8
(**6**)	PEG-FAM-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-BAD	HNE	112.8
(**8**)	PEG-HMC-Val-Thr-Gnf-Ser-Asp-CM3	CG	37.3

In addition, we determined the selectivity against these enzymes for each of them. The results obtained clearly indicated that the increase in peptides fluorescence is the result of enzymatic hydrolysis of peptide bond P_1-P_1'. Then, we repeated the selected experiments (detection limit determination) for the mixture of three substrates (PR3, CG, HNE) *in vitro* and *in vivo*. The results of the experiments are presented in **Figure 11**.

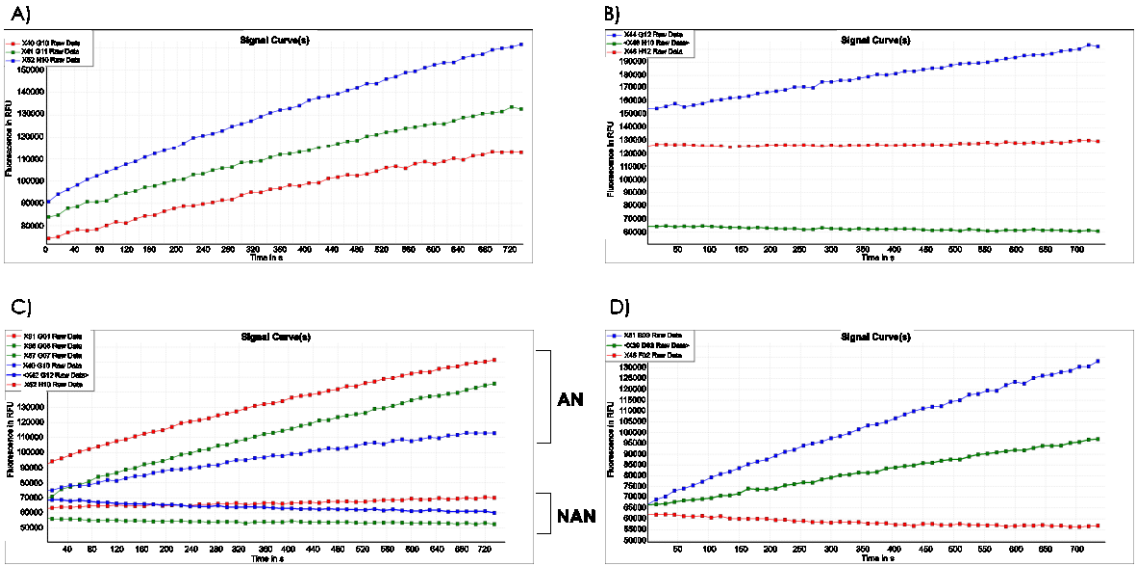


FIGURE 11: Fluorescence increase over the time measured for: A) incubation of single substrate with suitable enzyme (substrate **1** with PR3, substrate **6** with HNE, substrate **8** with CG) presented in one figure, B) equimolar mixture of substrates (**1**, **6** and **8**) incubated with HNE, C) equimolar mixture of substrates incubated with non-activated (NAN–lower three lines) and activated neutrophils (AN–upper three lines), D) equimolar mixture of substrates incubated with activated neutrophils with an addition of selective inhibitor PR3. Line colors: red-λ_{em} = 404 nm (substrate PR3: PEG-HOC-Tyr-Tyr-Abu-MOC), blue-λ_{em} = 512 nm (substrate HNE: PEG-FAM-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-BAD), green-λ_{em} = 454 nm (substrate CG: PEG-HMC-Val-Thr-Gnf-Ser-Asp-CM3).

In order to perform the experiments in the presence of human neutrophils, we made contact with the research group of professor Francis Gauthier, of the French Academy of Sciences in Tours (France)-a world expert in the field of research on neutrophils and their fluorescent substrates. This cooperation allowed to conduct a series of experiments using three pre-selected fluorescent substrates. Incubation of the examined substrates with activated neutrophils corresponded to a specific enzyme concentration range from 72 nM to 584 nM. In the case of non-activated neutrophils application, the level of enzyme concentrations was below the limit of quantification (0.32 nM). The minimum number of cells allowing to observe a noticeable fluorescence increase was: 35 000 cells for Pr3, 60 000 cells for CG, and 180 000 cells for HNE, these values are burdened with 15% error.

Moreover, using the elastase substrate PEG-FAM-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-BAD (concentration of 45 nM), we proved that it is able to penetrate the cell membrane, and demonstrates a detectable FRET within the cell, that is subject to a decrease with time (an increase in donor and decrease in acceptor fluorescence are observed), however the mechanism of this process is still unknown. The degree of cell membrane penetration is high, however time dependent. The flow cytometry data (**Fig. 12**) point that the highest fluorescence is observed after 20 minutes incubation of the cells with the substrate, while 60 min incubation results in one-third decrease in cells fluorescence.

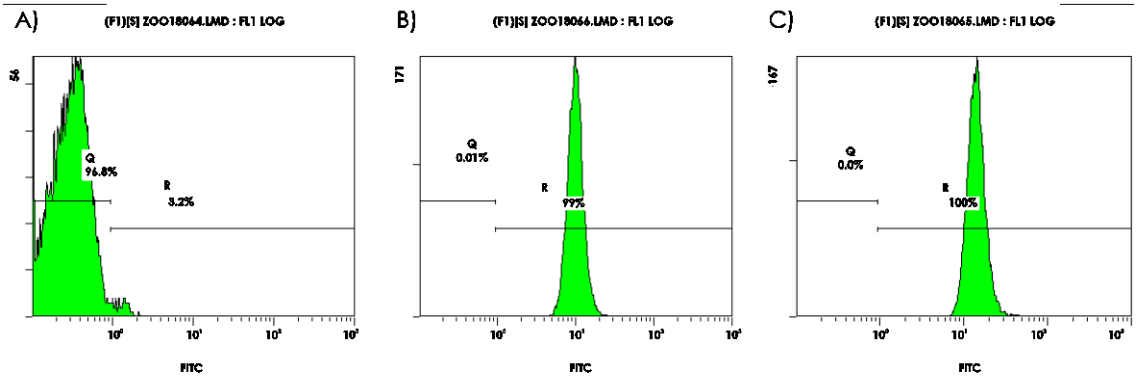
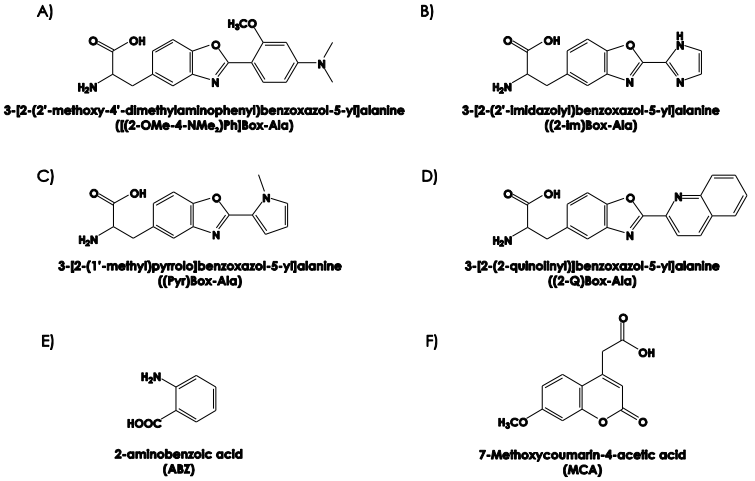


FIGURE 12: Flow cytometry analysis of activated human neutrophils with the substrate (**6**) (HNE); A) control sample, B) 5 minutes incubation, C) 20 minutes incubation.

Substrates modified in an area of fluorescence donor

Another strategy involved the selection and examination of the activity of one of the proteolytic enzymes of human neutrophils—proteinase 3. The starting sequence was the substrate: ABZ-Tyr-Tyr-Abu-ANB-NH_2, in which we decided to modify the *N*-terminal group of the fluorophore with series of benzoxazole-L-alanine derivatives (**Fig. 13**). This study allowed to obtain the substrate of a sequence (Pyr)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2 with specificity constant $k_{cat}/K_M = 1.5\times10^6\ M^{-1}\times s^{-1}$, more than 8-fold higher value obtained for the original sequence [50]. Moreover, the selected compound exhibited extremely high selectivity, and in human serum was subject to hydrolysis only in that enzyme presence.



RYSUNEK 13: Chemical structures of 3-(2-benzoxazol-5-yl) alanine derivatives (A-D), ABZ (E) and MCA (F) introduced to peptide *N*-terminus.

Such obtained compound was used in order to determine an activity of the examined enzyme in human serum. Preliminary studies carried out in cooperation with Julia Kulczycka, PhD, from the Department of Immunology, Medical University of Gdansk, led by professor Piotr Trzonkowski on a sample of 12 patients demonstrated the correlation between this enzyme activity and the concentration of antibodies (c-ANCA), which are one of the markers of

granulomatosis with polyangiitis (formerly known as Wegener's disease), a serious autoimmune disease which without the treatment leads to patient's death (**Fig. 14**) [72].

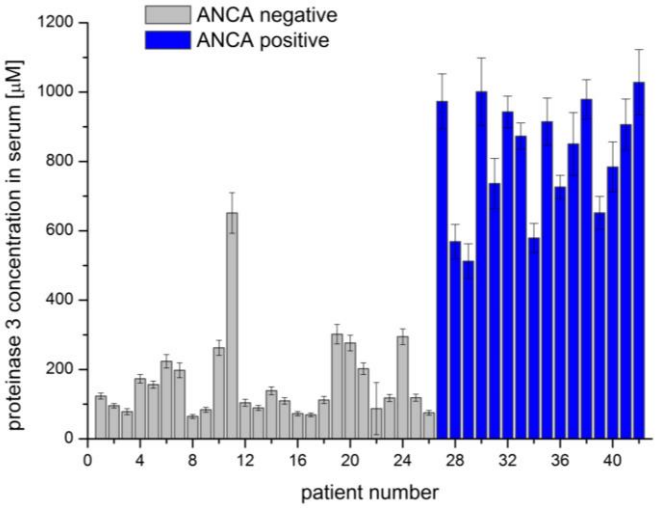


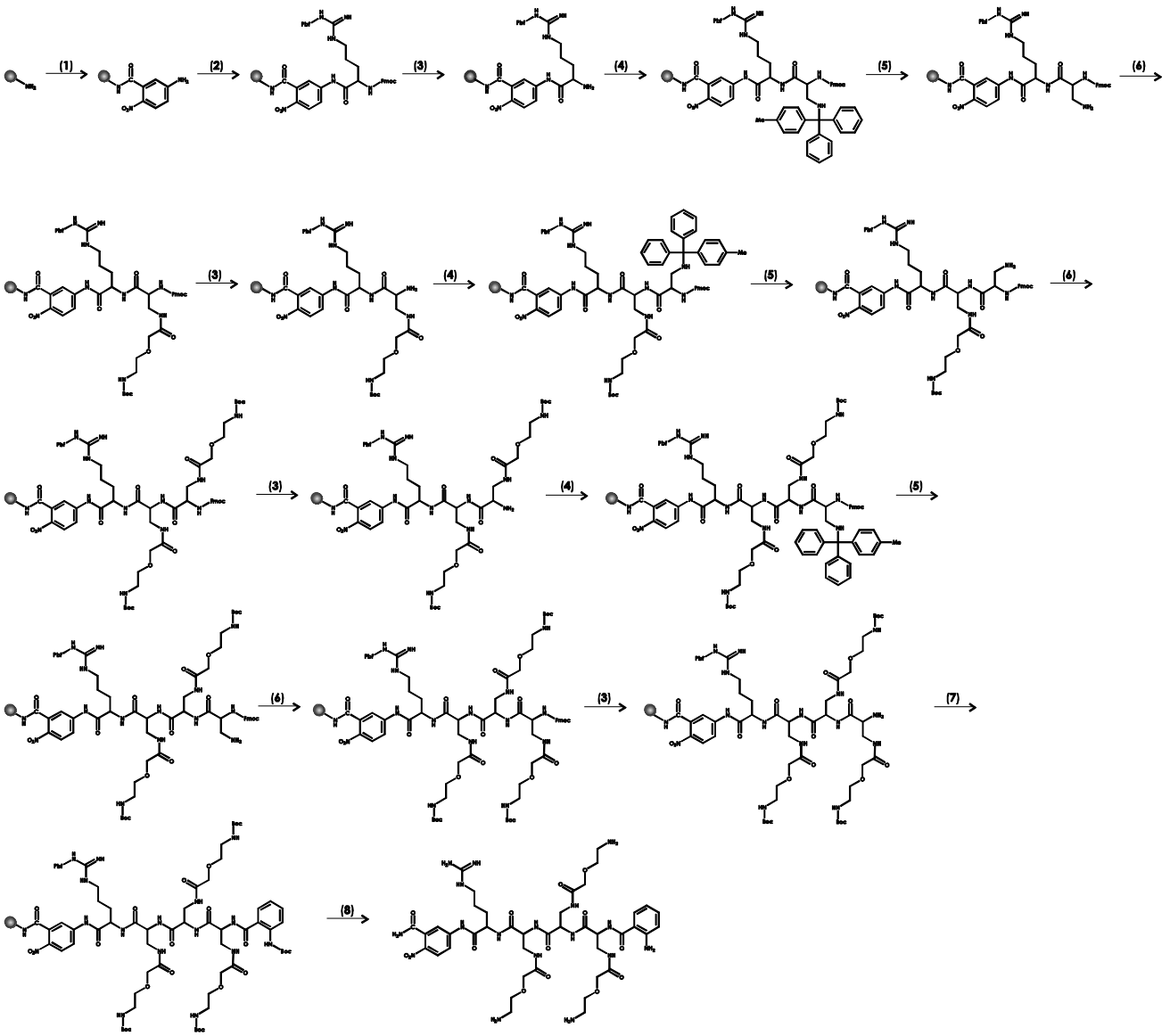
FIGURE 14: Graphical presentation of the results obtained on serum sample from 42 (30 samples c-ANCA–negative, and 12 samples c-ANCA–positive).

We expect that the method elaborated by us, will allow in the future for better and quicker diagnosis of this serious disease. Currently, in cooperation with professor Zbigniew Zdrojewski, the head of the Department of Connective Tissue Diseases and Geriatrics, Medical University of Gdansk, we conduct in-depth research on a larger group of patients, whose levels of proteinase 3 and antibodies c-ANCA will be correlated with the clinical course of the disease.

COMBINATORIAL LIBRARY OF PEPTIDOMIMETICS DAPEG

In the last period I have focused my research interests on the obtaining and examination of physicochemical and enzymatic properties of a class innovative peptidomimetics designed me [49]. I synthesized the newly obtained substrates using submonometric method according to the scheme presented in **Figure 15**. Cyclically repeated steps: attachment of L-2,3-diaminopropionic acid residues in the form of a derivative (Fmoc-Dap (Mtt)), removing the protective moiety of the β-amino group ((Mtt-*p*-methyltrityl) semiortogonal to Boc and Cbz cover), then the process of attaching of the appropriate PEG derivative with a variable number of replicates of oxy-ethylene group, and additionally differing with terminal functional group. The above scheme of the synthesis resulted in obtaining new molecules of peptidomimetics that were called DAPEG (as a hybrid of the amino acid DAP residue, and a derivative of polyethylene glycol-PEG). In my opinion, the potential of this new class of compounds is unlimited.

[72]. Kantari C., Pederzoli-Ribeil M., Amir-Moazami O., Gausson-Dorey V., Moura I.C., Lecomte M.C., Benhamou M., Witko-Sarsat V., *Proteinase 3, the Wegener autoantigen, is externalized during neutrophil apoptosis: evidence for a functional association with phospholipid scramblase 1 and interference with macrophage phagocytosis*, Blood, **(2007)**, 110, 4086–4095.



(1) **a** ANB (3 eq.), TBTU (3 eq.), DMAP (2 eq.), DIPEA (6 eq.)
b ANB (3 eq.), HBTU (3 eq.), DMAP (2 eq.), DIPEA (6 eq.)
c ANB (3 eq.), HATU (3 eq.), DMAP (2 eq.), DIPEA (6 eq.)
(2) Fmoc-Arg(Pbf) (9 eq.), $POCl_3$ (9 eq.) in pyridine
-15°C (20'), 20°C (30'), 40°C (6h)
(3) 20% piperidine in NMP
(4) Fmoc-Dap(Mtt) (3 eq.), TBTU (3 eq.), DIPEA (6 eq.)
(5) **a** TFA/TIS/DCM (1:2:97 v:v:v) (30')
b 1% DIPEA in DMF (2×3')
(6) O1(Boc) (2 eq.), TBTU (2 eq.), DIPEA (4 eq.)
(7) Boc-ABZ (2 eq.), TBTU (2 eq.), DIPEA (4 eq.)
(8) cleaved from the resin: TFA/phenol/TIS/H_2O (88:5:2:5 v/v)

FIGURE 15: Scheme of peptidomimetics DAPEG synthesis using submonometric method.

Due to great flexibility of polyethylene glycol derivatives and a wide range of functional groups, such kind of compounds together with a certain combination of oxy-ethylene group replicates and mentioned chemical moieties, have the ability to adjust to a target ligand molecule.

I conducted the selection of the substrate sequences with the highest susceptibility to proteolysis in the presence of newly discovered human neutrophil proteinase NSP4 [73] with trypsin specificity, using the methods of combinatorial chemistry synthesizing the library presented in **Figure 16**.

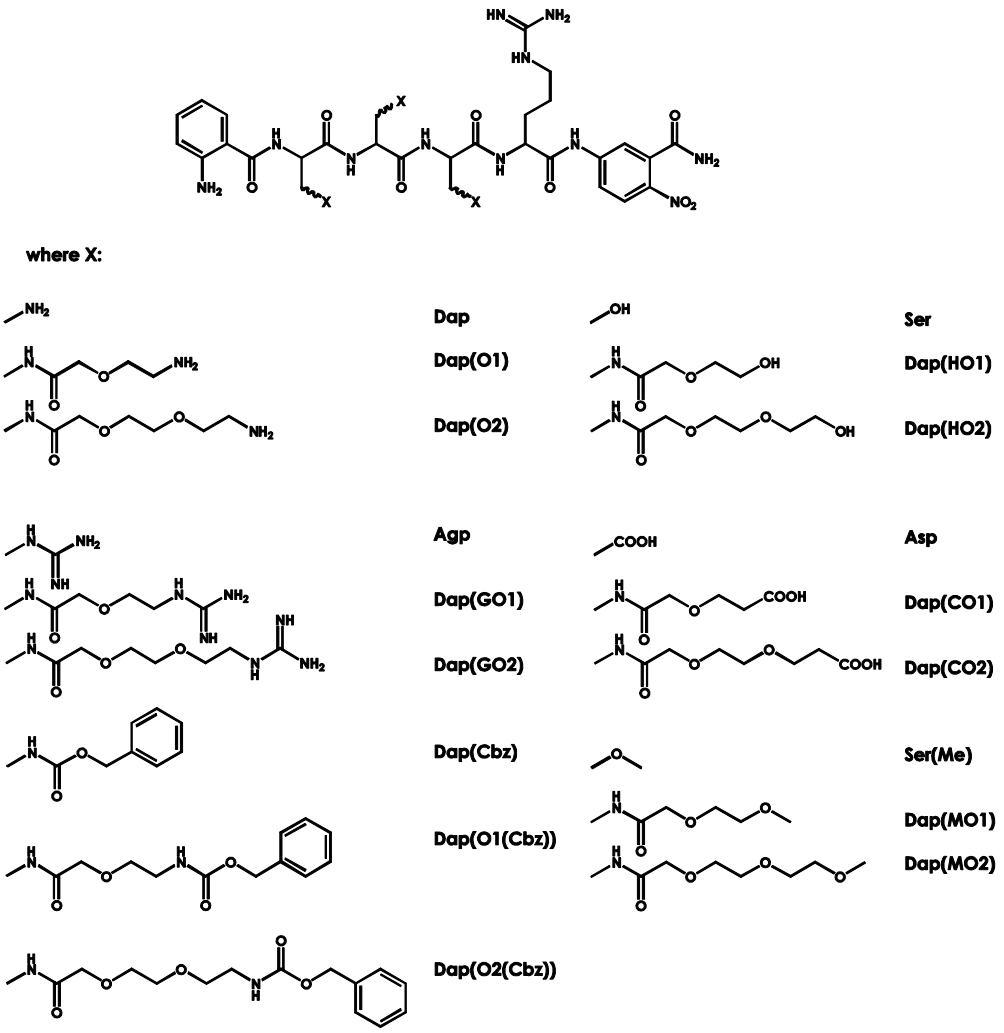


FIGURE 16: General formula of DAPEG combinatorial library.

The substrate obtained as a result of the above library deconvolution:
ABZ-Dap(O2(Cbz))-Dap(Cbz)-Dap(GO1)-Arg-ANB-NH_2 is a brand new peptidomimetic interacting, according to the results of molecular modeling, with the active site of the enzyme outside the conventional binding sites, specifically in the areas on enzyme surface defined as the secondary binding sites away from the active site (exosites) (**Fig. 17**).

[73]. Hu H.Y., Gehrig S., Reither G., Subramanian D., Mall M.A., Plettenburg O., Schultz C, *FRET-based and other fluorescent proteinase probes*, Biotechnol J., **(2014)**, 9, 266–281.

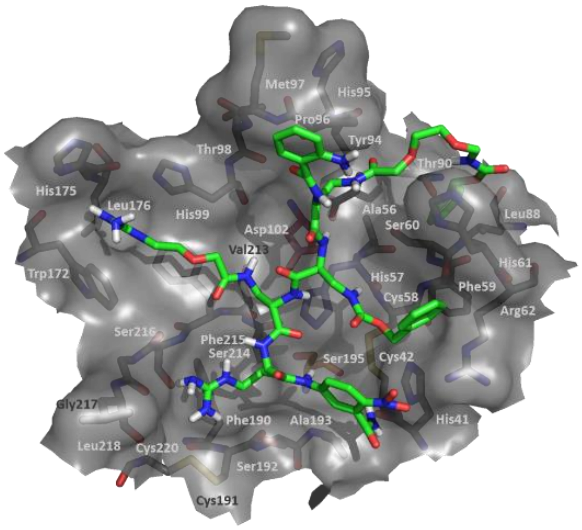


FIGURE 17: Model of interactions of proteinase NSP4 with obtained substrate: ABZ-Dap(O2(Cbz))-Dap(Cbz)-Dap(GO1)-Arg-ANB-NH_2.

Within the cooperation with professor Zbigniew Szewczuk from the Group of Peptides and Proteins Chemistry and Stereochemistry, Faculty of Chemistry, University of Wroclaw, we made the fragmentation analysis of MS/MS of the new substrate, and the results obtained fully present its structure. We also conducted an analysis of two-dimensional NMR, which confirmed the structure of its molecule. The resulting substrate interacts with NSP4 in an extremely efficient (specificity constant k_{cat}/K_M over 10×10^4 $M^{-1}\times s^{-1}$) and highly selective manner (out of 16 experimental enzymes, only one-KLK 14 in a slight way hydrolyzed the compound obtained by me) (**Fig. 18**).

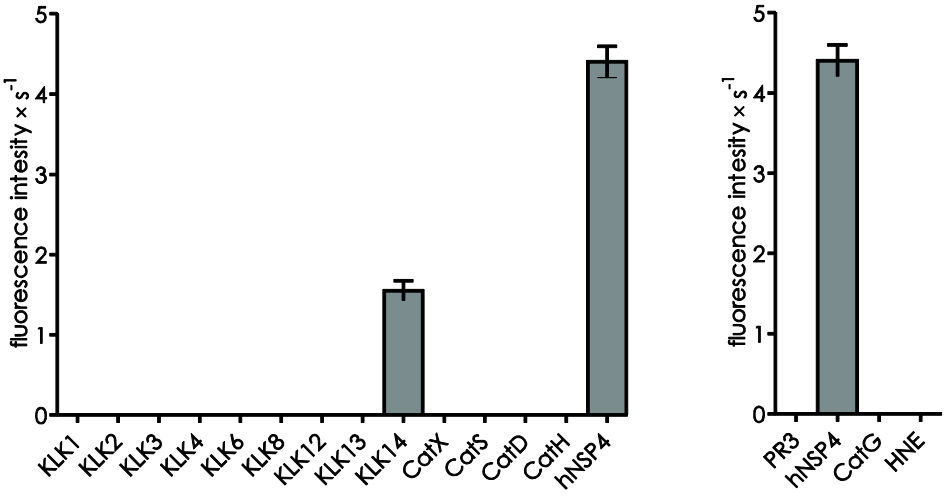


FIGURE 18: Selectivity of substrate ABZ-Dap(O2(Cbz))-Dap(Cbz)-Dap(GO1)-Arg-ANB-NH_2.

SUMMARY OF THE MOST IMPORTANT ACHIEVEMENTS:

1. The studies conducted (using the techniques of combinatorial chemistry and parallel synthesis) allowed to obtain **67** fluorogenic substrates and **2** inhibitors presented in **the summary table 9**.
2. Together with my colleagues, I made the characteristics of substrate specificity of **9** proteinases (**8** serine and **1** cysteine).
3. We elaborated new synthetic methods and the strategy allowing to obtain peptide libraries containing fluorogenic substrates.
4. We synthesized **10** combinatorial libraries (containing a total of **177 307** compounds) for **7** selected enzymes, which deconvolution resulted in obtaining of highly selective and highly specific substrates.
5. We elaborated a method of proteinase 3 analysis in biological material, which enables a preliminary diagnosis of granulomatosis with polyangiitis.
6. We obtained the system of substrates allowing for the simultaneous analysis of three enzymes belonging to the group of serine neutrophil proteases.
7. We elaborated the method for the synthesis of new peptidomimetics, which building elements are residues of L-2,3-diaminopropionic acid modified in side chain with functionalized mono- or di-oxy-ethylene glycol chains. The above mentioned derivatives were used as components of the tetrapeptide substrate library – peptidomimetics, in which three variable positions (contained an equimolar mixture of the compounds used), and one was not subject to modifications. The process of such library deconvolution allowed to obtain efficient and highly selective substrates of trypsin-like proteinases, such as NSP4 or kallikrein 14.
8. We proposed an application of the obtained substrates of chymotryptic subunit of proteasome20S, as a tools for bladder cancer diagnostics.

TABLE 9: Primary structures of obtained compounds.

	SEQUENCE	ENZYME	ANALOG NUMBER IN PUBLICATION	PUBLICATION NUMBER
1.	ABZ-Tyr-Tyr-Abu-ANB-Gln-NH_2	PR3/ HNE	14	H.1.
2.	ABZ-Tyr-Tyr-Abu-ANB-Asn-NH_2		16	
3.	[(2-OMe-4-NMe)$_2$Ph]Box-Ala-Tyr-Tyr-Abu-ANB-NH_2	PR3	1	H.2.
4.	(2-im)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2		2	
5.	(Pyr)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2		3	
6.	(2-Q)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2		4	
7.	MCA-Ala-Tyr-Tyr-Abu-ANB-NH_2			

8.	ABZ-Arg-Gln-Asp-Arg-ANB-NH_2	HAT	1	H.3.
9.	ABZ-Arg-Gln-Asp-Lys-ANB-NH_2		2	
10.	ABZ-Arg-Gln-Asp-Arg-H		1a	
11.	ABZ-Arg-Gln-Asp-Lys-H		2a	
12.	ABZ-Tyr-Tyr-Abu-ANB-PEG-NH_2	PR3	1A	H.4.
13.	PEG-ABZ-Tyr-Tyr-Abu-ANB-NH_2		1B	
14.	PEG-ABZ-Tyr-Tyr-Abu-ANB-PEG-NH_2		1C	
15.	ABZ-Phe-Val-Thr-Gnf-ANB-NH_2	CG	2	
16.	ABZ-Phe-Val-Thr-Gnf-ANB-PEG-NH_2		2A	
17.	PEG-ABZ-Phe-Val-Thr-Gnf-ANB-NH_2		2B	
18.	PEG-ABZ-Phe-Val-Thr-Gnf-ANB-PEG-NH_2		2C	
19.	ABZ-Phe-Phe-Pro-Val-ANB-NH_2	HNE	3	
20.	ABZ-Phe-Phe-Pro-Val-ANB-PEG-NH_2		3A	
21.	PEG-ABZ-Phe-Phe-Pro-Val-ANB-NH_2		3B	
22.	PEG-ABZ-Phe-Phe-Pro-Val-ANB-PEG-NH_2		3C	
23.	ABZ-Arg-Gln-Asp-Arg-ANB-PEG-NH_2	HAT	4A	
24.	PEG-ABZ-Arg-Gln-Asp-Arg-ANB-NH_2		4B	
25.	PEG-ABZ-Arg-Gln-Asp-Arg-ANB-PEG-NH_2		4C	
26.	PEG_2-ABZ-Arg-Gln-Asp-Arg-ANB-NH_2		4D	
27.	PEG_3-ABZ-Arg-Gln-Asp-Arg-ANB-NH_2		4E	
28.	PEG-HOC-Tyr-Tyr-Abu-MOC	PR3	1	H.5.
29.	PEG-HOC-Val-Thr-Gnf-Ser-Asp-MOC	CG	2	
30.	PEG-HOC-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-MOC	HNE	3	
31.	PEG-FAM-Tyr-Tyr-Abu-BAD	PR3	4	
32.	PEG-FAM-Val-Thr-Gnf-Ser-Asp-BAD	CG	5	
33.	PEG-FAM-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-BAD	HNE	6	
34.	PEG-HMC-Tyr-Tyr-Abu-CM3	PR3	7	
35.	PEG-HMC-Val-Thr-Gnf-Ser-Asp-CM3	CG	8	
36.	PEG-HMC-Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-CM3	HNE	9	
37.	ABZ-Tyr-Tyr-Abu-Tyr(3-NO_2)-NH_2	PR3	2	H.7.
38.	ABZ-Tyr-Tyr-Abu-Asn-Tyr(3-NO_2)-NH_2		3	
39.	ABZ-Tyr-Tyr-Abu-Asn-Glu-Tyr(3-NO_2)-NH_2		4	
40.	ABZ-Tyr-Tyr-Abu-Asn-Glu-Pro-Tyr(3-NO_2)-NH_2		5	
41.	ABZ-Ile-Arg-Ala-Arg-Ser-Ala-Gly-Tyr(3-NO_2)-NH_2	MT-2	I	H.8.
42.	ABZ-Ile-Arg-Ala-Arg-Ser-Ala-Ala-Tyr(3-NO_2)-NH_2		II	
43.	ABZ-Ile-Arg-Ala-Arg-Ser-Ala-Ser-Tyr(3-NO_2)-NH_2		III	
44.	ABZ-Bip-Arg-Ala-Ser-Tyr(3-NO_2)-NH_2	Cat L		H.9.
45.	ABZ-Bip-Arg-Ala-Gln-Tyr(3-NO_2)-NH_2			
46.	ABZ-Bip-Arg-Ala-Ala-Tyr(3-NO_2)-NH_2			

47.	ABZ-Ile-Met-Thr-Abu-Tyr-Met-Phe-Tyr(3-NO_2)-NH_2	HtrA2	1	H.10.
48.	ABZ-Ile-Met-Thr-Abu-Tyr-Met-Trp-Tyr(3-NO_2)-NH_2		2	
49.	ABZ-Ile-Met-Thr-Abu-Tyr-Met-His-Tyr(3-NO_2)-NH_2		3	
50.	ABZ-Ile-Met-Thr-Ser-Tyr-Met-Phe-Tyr(3-NO_2)-NH_2		1a	
51.	ABZ-Ile-Met-Val-Ser-Tyr-Met-Phe-Tyr(3-NO_2)-NH_2		1b	
52.	ABZ-Ile-Met-Val-Abu-Tyr-Met-Phe-Tyr(3-NO_2)-NH_2		1c	
53.	ABZ-Val-Val-Ser-Arg-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2	20S	1	H.11.
54.	ABZ-Val-Val-Ser-CPA-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		2	
55.	ABZ-Val-Val-Ser-TBA-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		3	
56.	ABZ-Val-Val-Ser-FEA-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		4	
57.	ABZ-Val-Val-Ser-FNA-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		5	
58.	ABZ-Val-Val-Ser-EPA-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		6	
59.	ABZ-Val-Val-Ser-Har-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		7	
60.	ABZ-Val-Val-Ser-NIA-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		8	
61.	ABZ-Val-Val-Ser-AGP-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		9	
62.	ABZ-Val-Val-Ser-AGB-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2		10	
63.	ABZ-Val-Val-Ser-Lys-Ala-Ser-Leu-Tyr(3-NO_2)-NH_2		11	
64.	ABZ-Val-Val-Ser-Orn-Ala-Ser-Leu-Tyr(3-NO_2)-NH_2		12	
65.	ABZ-Val-Val-Ser-GNF-Ala-Ser-Leu-Tyr(3-NO_2)-NH_2		13	
66.	ABZ-Val-Val-Ser-Arg-Ala-Met-Gly-Tyr(3-NO_2)-NH_2		14	
67.	ABZ-Val-Val-Ser-GNF-Ala-Met-Gly-Tyr(3-NO_2)-NH_2		15	
68.	ABZ-Val-Val-Ser-Arg-ANB-NH_2		16	
69.	ABZ-Dap(O2(Cbz))-Dap(Cbz)-Dap(GO1)-Arg-ANB-NH_2	NSP4	3	H.12.

VI. DESCRIPTION OF OTHER SCIENTIFIC AND RESEARCH (ARTISTIC) ACHIEVEMENTS:

Period 1998—2008 (before scientific doctor degree obtaining)

In 1998–2003 I studied at the Faculty of Chemistry, University of Gdansk. The research work conducted during master's studies concerned the synthesis of the fragment of human protein of prion 91–120. I performed the subject of master's thesis within the cooperation with professor Krzysztof Rolka with professor Henryk Kozłowski from the Faculty of Chemistry, University of Wroclaw. I performed my master's thesis entitled: *Chemical synthesis of fragment 91–120 of human prion protein* under the supervision of Hanna Miecznikowska, PhD (Faculty of Chemistry, University of Gdansk), and in the year 2003 I obtained the master's degree.

In October 2003, I began doctoral studies in the Department of Bioorganic Chemistry (now the Department of Molecular Biochemistry), Faculty of Chemistry, University of Gdansk. I performed my doctoral thesis: *Application of combinatorial chemistry methods in the synthesis of*

chromogenic substrates of selected proteinases under the supervision of professor Krzysztof Rolka. I defended the doctoral thesis in June 2008, obtaining a doctoral degree in chemical sciences in the range of chemistry.

The main issues of my study was design and synthesis of peptide libraries of chromogenic substrates containing 5-amino-2-nitrobenzoic acid (ANB) residue in *C*-terminus. In the course of the study I described four serine proteinases (bovine α-chymotrypsin, and three neutrophil proteinases: human elastase, cathepsin G and proteinase 3). As a result of enzymatic examinations of the obtained libraries, I selected 13 peptide sequences selected the most efficiently hydrolysable under the influence of the examined proteinases. Resulting substrates were subject to further modifications obtaining their 59 analogs.

Results of all research conducted in the period 2003–2008 were presented on three conferences (including one on an international scale), and published in 5 scientific articles (Appendix 3) listed in the *Journal Citation Reports* database (total IF 12.44).

In the course of doctoral studies, I conducted laboratory of Biochemistry for students of Chemistry and Environmental Protection of the Faculty of Chemistry, University of Gdansk, as well as laboratory of Organic Chemistry for students of the Faculty of Biology, University of Gdansk.

Period 2008—2016 (after scientific doctor degree obtaining)

In September 2008, I was employed as an assistant in the Department of Bioorganic Chemistry (now the Department of Molecular Biochemistry), Faculty of Chemistry, University of Gdansk, and since February 2009 I have been working as an adjunct.

Main scientific and research issues after doctor degree obtaining include:

1. Determination of the substrate specificity of selected proteolytic enzymes through the design and synthesis using combinatorial chemistry methods of peptide libraries, and their biochemical analysis. Selected proteinases include: four neutrophil serine proteinases (HNE, CG, PR3, and NSP4), human: tryptase, matriptase-2, HtrA2, Cat L, proteasome 20S and a number of bacterial enzymes (SufA, SplD, Stp A, B and C).
2. Design, synthesis and biological examination of selective and efficiently hydrolyzed substrates, as molecular probes of selected enzymes of major importance in medical diagnostics — (proteinase 3, neutrophil elastase, proteasome 20S).
3. Elaboration of a strategy for determination of substrate specificity of enzymes in an area of *C*-terminal residues.
4. Design of new structural units of the peptides constituting the substituted residues of L-2,3-diaminopropionic acid (DAP) modified in the side chain with polyethylene glycol

(PEG) derivatives, and libraries composed of them in order to obtain new molecular probes of selected proteolytic enzymes.

5. Participation in the research on determination of antimicrobiotic properties of selected peptides (OGTI and HV-BBI) and their peptidomimetic, as well as an evaluation of their potential for pathogenic bacteria growth inhibiting.
6. Search for new types of low-molecular inhibitors of proteinase 3 and cathepsin G: aldehyde and phosphonic peptide derivatives.
7. Participation in the works concerning obtaining of inhibitor molecules of selected serine proteases, which initial sequence was SFTI-1 molecule.
8. Biological studies of fluorescent SFTI-1 derivatives.

The relatively wide range of scientific and research works carried out on the borderline of chemistry and biochemistry required a multidisciplinary approach to the analysis of the problems and establishing scientific cooperation with several renowned research centers:

1. Professor Timo Burster, *Department of Neurosurgery, Ulm University Medical Center, Ulm Germany*; cooperation involves cellular examination of selected substrates and inhibitors.
2. Professor Wiesław Wiczk, *Laboratory of Photobiophysics, Faculty of Chemistry, University of Gdansk*; cooperation concerns obtaining of new fluorescent compounds and their use as a fluorescence donors in the design of new efficient substrates for proteolytic enzymes.
3. Julia Kulczycka, PhD, *Laboratory of Clinical Immunology and Transplantology, University Clinical Center in Gdansk*; cooperation concerns the determination of proteinase 3 activity in human plasma.
4. Professor Francis Gauthier and Brice Korkmaz, PhD, *Faculté de Médecine de Tours, Université François Rabelais in Tours, France*; cooperation concerns cellular studies performed on human neutrophils.
5. Professor Józef Oleksyszyn and Marcin Sieńczyk, PhD, *Department of Medical Chemistry and Microbiology, Faculty of Chemistry, University of Wroclaw*; cooperation is focused on the design and chemical synthesis of irreversible inhibitors (phosphonic peptidomimetic) of selected proteinases.
6. Professor Barbara Lipińska, *Department of Biochemistry, Faculty of Biology, University of Gdansk*; cooperation concerns the design, chemical synthesis and enzymatic examination of substrates and modulators of an activity of family HtrA enzymes.
7. Professor Dieter Jenne, *Helmholtz Centrum in Munich*; cooperation concerns the determination of specificity and synthesis of molecular probes of neutrophil proteases 4.

8. Professor Nathaniel Martin, *Faculty of Science, Utrecht University, The Netherlands*; cooperation concerns the design and synthesis of the novel substrates of human proteasome.
9. Ewa Gorodkiewicz, PhD, *Department of Electrochemistry, Faculty of Biology and Chemistry, University of Bialystok*; cooperation concerns the study on human proteasome substrates.
10. Mizuki Kitamatsu, PhD, *Department of Bioscience and Biotechnology, Okayama University, Japan*; cooperation concerns the synthesis of fluorescent derivatives of Lys, Orn, and Al, and their application for the synthesis of human neutrophil proteinases substrates.

The results of all research conducted in the years 2008—2016 (after scientific doctor degree obtaining) were presented in a form of: **32** articles in journals from *Journal Citation Reports* database*: (Analytical Biochemistry, Analytical Chemistry, Biochimica et Biophysica Acta - Proteins and Proteomics, Biochimie, Bioorganic and Medicinal Chemistry, Biopolymers, Chembiochem, Current Pharmaceutical Design, Journal of Biological Chemistry, Journal of Peptide Science, Molecular Diversity, Peptides, PLoS ONE, Protein and Peptide Letters, Scientific Reports),* **13** papers published in peer-reviewed conference materials, **1** patent application, and were also presented in a form of **55** oral lectures or poster presentations on 16 scientific conferences (national and international).

My full research and teaching output was discussed in detail in Appendix 3.

- → **My Total scientific output consists of 37 publications in journals from JCR database,**
  1 patent application.
  Before scientific doctor degree obtaining I was a co-author of 5 publications in journals from JCR database.
- → **Total Impact Factor (according to the year of publication) is 107.75 (12.44 before scientific doctor degree obtaining).**
- → **Number of citations in the years 2008—2016 (without self-citations) according to WoS is 157**
  (17 before scientific doctor degree obtaining)
- → **Hirsh index — 10 (according to WoS).**

The scientific and research works conducted by me were/are funded by: **Committee for Scientific Research** (doctoral grant), **Ministry of Science and Higher Education** (3 Iuventus Plus grants), **Faculty of Chemistry, University of Gdańsk** (4 grants). Currently, I play the role of scientific supervisor of National Science Center grant NCN (Preludium 5) of Jadwiga Popow-Stellmaszyk: *Fluorescent substrates of neutrophil proteinases: design, synthesis, selection using combinatorial chemistry methods, and their application in the characteristics of selected proteolytic enzymes*.

In the range of teaching activity, I carry out the following classes for students of the Faculty of Chemistry, University of Gdansk: Biochemistry (laboratory), Biochemistry (auditorium), laboratories: diploma, specialization and master's for students of the Department of Molecular Biochemistry, Faculty of Chemistry, University of Gdansk. So far, I promoted **7 graduates and 10 masters**. I am currently the supervisor of two master's theses and an auxiliary promoter of two doctoral dissertations: *Study of substrate specificity of human proteasome using combinatorial chemistry methods* (Natalia Gruba, MSc, Faculty of Chemistry, University of Gdansk) and *Substrates and low molecular inhibitors of selected proteolytic enzymes from kallikreins family. Chemical synthesis and enzymatic studies* (Anna Wojtysiak, MSc, Faculty of Chemistry, University of Gdansk).

Further scientific and research plans:

My scientific and research plans for the coming years can be divided into a few major trends :

1. Design, synthesis and characterization of compounds which building elements will constitute DAPEG residues. In the near future I plan to obtain libraries for proteolytic enzymes with chymotryptic and elastase specificity.
2. Using selective sequences of the substrates, I plan to obtain conjugates of these compounds with the quantum dots, using chemical synthesis methods. I plan to describe the and subject to biological tests the new substrates obtained this way.
3. Characteristics of the kallikreins family enzymes (substrates, inhibitors).
4. I plan to attach the selected selective compounds to the electrodes with specific properties, in order to obtain a new innovative system of proteolytic enzymes detection. I intend to perform these studies in cooperation with the research group of professor Tadeusz Ossowski from the faculty of Chemistry, University of Gdansk.
5. Another area of research, which can be considered a continuation of previously conducted studies, will be the diagnosis of selected pathological conditions in biological material (inter alia granulomatosis with polyangiitis, bladder cancer, etc.). These works are conducted within the collaboration with teams of doctors from: Medical University of Gdansk (professor Zbigniew Zdrojewski, Marcin Matuszewski, PhD), Department of Oncological and General Urology, Regional Hospital in Bialystok (Tomasz Guszcz, PhD) and the Institute of Lung Diseases and Tuberculosis in Warsaw (professor Joanna Chorostowska-Wynimko).

Magdalena Wysocka