(Summary of professional accomplishments, Attachment no. 4)

Substance P peptide and trastuzumab monoclonal antibody radiobioconjugates labelled with corpuscular emitters for targeted radionuclide therapy

Agnieszka Majkowska-Pilip, PhD Eng.

Institute of Nuclear Chemistry and Technology, Centre of Radiochemistry and Nuclear Chemistry

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1. Name and surname: Agnieszka Majkowska-Pilip

2. Academic diplomas and degrees:

- PhD in chemistry (20 of April 2010)

Doctoral dissertation „^{44}/^{47}Sc complexes with multidentate ligands as radiopharmaceutical precursors" – thesis defended with honours

Supervisor: prof. dr hab. Aleksander Bilewicz

Centre of Radiochemistry and Nuclear Chemistry, Institute of Nuclear Chemistry and Technology, Warsaw

- MSc in chemistry (22 of June 2004)

Master thesis „Synthesis and study of polythiophene derivatives containing oligoaniline groups in the side chain" – thesis defended with honours

Supervisor: prof. dr hab. Małgorzata Zagórska

Warsaw University of Technology, Faculty of Chemistry, Department of Polymer Chemistry and Technology

3. Information on employment in research institutes:

- 01.06.2010 – present adjunct, Institute of Nuclear Chemistry and Technology, Warsaw
- 16.09. 2010 – 15.09.2013 post-doc, European Commission, Joint Research Centre, Karlsruhe, Germany
- 01.11.2006 – 31.05.2010 assistant, Institute of Nuclear Chemistry and Technology, Warsaw
- 02.01.2005 – 31.10.2006 chemist, Institute of Nuclear Chemistry and Technology, Warsaw

4. Description of the achievements, set out in art. 219 para 1 point 2 of the Act:

a) title of scientific achievement

Substance P peptide and trastuzumab monoclonal antibody radiobioconjugates labelled with corpuscular emitters for targeted radionuclide therapy

b) list of scientific publications constituting the basis for scientific achievement

The source material for the description of my scientific achievements, which are the basis for applying for the habilitation degree, is the publication cycle below, consisting of 8 papers with a total IF of 28.912 (MNiSW = 500 points) according to the year of publication and IF of 29.594 (MNiSW = 680 points) for 2019.
For articles published in 2020 and 2021, the IF is included from 2019.

[H1] **A. Majkowska-Pilip***, M. Rius, F. Bruchertseifer, C. Apostolidis, M. Weis, M. Bonelli, M. Laurenza, L. Królicki, A. Morgenstern. *In vitro* evaluation of ^{225}Ac-DOTA-Substance P for targeted alpha therapy of glioblastoma multiforme. *Chem. Biol. Drug Design*, **2018**, 92, 1344.

$IF_{2018/2019}$ = 2.256/2.548; $MNiSW_{2018/2019}$ = 25/70 points

My participation consisted in proposing the concept of obtaining the [^{225}Ac]Ac-DOTA-SP radiobioconjugate, developing the method of its synthesis, performing stability studies, conducting most of the biological experiments, analyzing and interpreting the obtained results as well as preparing and sending the manuscript. I also prepared responses for reviewers.

[H2] **A. Majkowska-Pilip***, P. Koźmiński, A. Wawrzynowska, T. Budlewski, B. Kostkiewicz, E. Gniazdowska. Application of Neurokinin-1 receptor in targeted strategies for gliomas treatment. Part I: synthesis and evaluation of Substance P fragments labeled with ^{99m}Tc and ^{177}Lu as potential receptor radiopharmaceuticals. *Molecules*, **2018**, 23(10), 2542.

$IF_{2018/2019}$ = 3.060/3.267; $MNiSW_{2018/2019}$ = 30/100 points

My participation consisted in proposing a research concept for the preparation of Substance P bioconjugates labelled with the β^- emitter - ^{177}Lu, developing methods for their syntheses, performing stability studies, conducting biological experiments, taking care of student A. Wawrzynowska, who performed research as part of her master's thesis, analysis and interpretation of obtained results, preparation and sending the manuscript. I also prepared responses for reviewers.

[H3] S. Ostrowski, **A. Majkowska-Pilip**, A. Bilewicz, J. Dobrowolski. On Au_nAt clusters as potential astatine carriers. *RSC Adv.*, **2017**, 7(57), 35854.

$IF_{2017/2019}$ = 2.936/3.119; $MNiSW_{2017/2019}$ = 35/100 points

My participation consisted in the analysis and interpretation of the calculation results and participation in the preparation of the publication.

[H4] Ł. Dziawer, **A. Majkowska-Pilip***, D. Gaweł, M. Godlewska, M. Pruszyński, J. Jastrzębski, B. Wąs, A. Bilewicz. Trastuzumab-modified gold nanoparticles labeled with ^{211}At for local treatment of HER2-positive breast cancer. *Nanomaterials*, **2019**, 9(4), 632.

IF_{2019} = 4.324; $MNiSW_{2019}$ = 70 points

My participation consisted in proposing the concept of obtaining [^{211}At]At-AuNPs-PEG-trastuzumab radiobioconjugate, developing a method of its synthesis, conducting some biological experiments, analyzing and interpreting the obtained results, and preparing and sending the manuscript. I also prepared responses for reviewers.

[H5] **A. Majkowska-Pilip***, P. Halik, E. Gniazdowska. The significance of NK1 receptor ligands and their application in targeted radionuclide tumour therapy. *Pharmaceutics*, **2019**, 11(9), 443.

IF_{2019} = 4.421; $MNiSW_{2019}$ = 100 points

My participation consisted in developing the concept of a review of the NK1 receptor, describing chapters (3.1-3.2) on labelled SP agonists used in both diagnosis and therapy, preparation and sending the manuscript. I also prepared responses for reviewers.

[H6] **A. Majkowska-Pilip***, W. Gawęda, K. Żelechowska-Matysiak, K. Wawrowicz, A. Bilewicz. Nanoparticles in Targeted Alpha Therapy. *Nanomaterials*, **2020**, 10, 1366.

$IF_{2020/2019}$ = no data/4.324; $MNiSW_{2020/2019}$ = no data/70 points

My participation consisted in the development of the review concept using nanoparticles in targeted α-therapy, description of the chapter of nanoparticles labelled with ^{225}Ac and ^{227}Th radionuclides, preparation and sending the manuscript. I also prepared responses for reviewers.

[H7] A. Cytryniak, E. Nazaruk, R. Bilewicz, E. Górzyńska, K. Żelechowska-Matysiak, R. Walczak, A. Mames, A. Bilewicz, **A. Majkowska-Pilip***. Lipidic cubic-phase nanoparticles

(cubosomes) loaded with doxorubicin and labeled with ^{177}Lu as a potential tool for combined chemo and internal radiotherapy for cancers. *Nanomaterials*, **2020**, 10(11), 2272.

$IF_{2020/2019}$ = no data/4.324; $MNiSW_{2020/2019}$ = no data/70 points

My participation consisted in proposing a research concept for the ^{177}Lu radionuclide-labelled cubosomes, planning experiments of the chemical (synthesis of DOTAGA-OA conjugate), radiochemical (labellings, stability and lipophilicity studies) and radiobiological parts (cells studies), analyzing and interpreting the obtained results, preparing and sending the manuscript. I also prepared responses for reviewers.

[H8] K. Wawrowicz, **A. Majkowska-Pilip***, D. Gaweł, E. Chajduk, T. Pieńkowski, A. Bilewicz. Au@Pt core-shell nanoparticle bioconjugates for the therapy of HER2+ breast cancer and hepatocellular carcinoma. Model studies on the applicability of ^{193m}Pt and ^{195m}Pt radionuclides in Auger electron therapy. *Molecules*, **2021**, 26, 2051.

$IF_{2020/2019}$ = no data/3.267; $MNiSW_{2020/2019}$ = no data/100 points

My participation consisted in proposing a research concept for the preparation of a bioconjugate of gold nanoparticles covered with a platinum monolayer and attached to trastuzumab, planning biological experiments, analysis and interpretation of the obtained results, preparation and sending the manuscript. I also prepared responses for reviewers.

c) description of scientific achievement

Research objectives

My research has contributed to the design and testing of new, effective radiobioconjugates for targeted radiotherapy of two of the most aggressive and drug-resistant neoplasms: glioblastomas multiforme (GBM) and breast and ovarian tumours overexpressing HER2 receptors. In the case of GBM, I was involved in the synthesis and study of the physicochemical and biological properties of radiobioconjugates based on substane P (SP), which are showing affinity for NK1 receptors. In the case of tumours overexpressing HER2 receptors, I was involved in the synthesis and study of the radiobioconjugates based on the monoclonal antibody: trastuzumab. In the synthesised radiopharmaceuticals, both SP and

trastuzumab were carriers of radionuclides emitting β^- radiation (^{177}Lu), α-radiation (^{225}Ac, ^{211}At) and that of Auger electrons (^{193m}Pt, ^{195m}Pt).

An important element of this study was also to find a radiopharmaceutical showing radiotoxicity to the stem cells of these tumours. According to recent hypotheses, these cells show exceptional drug resistance and are responsible for the metastasis of these neoplasms.

The stages leading to obtaining potential radiopharmaceuticals included the following research issues:

- designing new radiopharmaceuticals, also with the use of nanotechnology;
- development of conjugate synthesis schemes and their labelling with radionuclides;
- determination of the physicochemical properties and stability of the synthesised preparations in biological fluids;
- characteristics of the obtained bioconjugates in terms of their biological properties on human tumour cell lines overexpressing NK1 and HER2 receptors (*in vitro* studies of receptor affinity, internalisation, and cytotoxicity).

Introduction

The intensive development of medical diagnostic methods in the last two decades mainly computed tomography (CT), functional magnetic resonance (fNMR), single photon emission tomography (SPECT) and positron tomography (PET), allows to detect neoplastic changes at a very early stage of their development, and therefore, start treatment much earlier than with classic diagnostic methods. Unfortunately, the effectiveness of therapy does not follow the enormous progress in cancer diagnostics. The drug and radiation resistance of cancer cells becomes the biggest problem. Currently, the most frequently used methods in anti-cancer therapy, apart from surgery, are radiotherapy and chemotherapy. Unfortunately, in the case of many patients, the use of chemotherapy is practically impossible due to the stage of cancer and the coexistence of other diseases. In such cases, chemotherapy can lead to the occurrence of serious side effects. In addition, treatment with cytostatics targets rapidly proliferating cells, while cancer stem cells (a small population of resistant cancer cells) divide much more slowly and are not destroyed. They are responsible for maintaining the tumour mass and are the cause of metastases and relapses of the neoplastic disease [1]. Recently, increasingly better results in the treatment of these tumours have been obtained with the use of immunotherapy, targeted radionuclide therapy (*TAT*), as well as targeted delivery of chemotherapeutic agents to tumours. The last two methods are based on the use of radionuclides or chemotherapeutic agents combined with appropriate carrier molecules, such as monoclonal antibodies and their

fragments, peptides, and other active small biological molecules, that are located in the sites of neoplastic lesions, delivering doses of the drug or ionizing radiation to these therapeutic areas (Fig. 1).

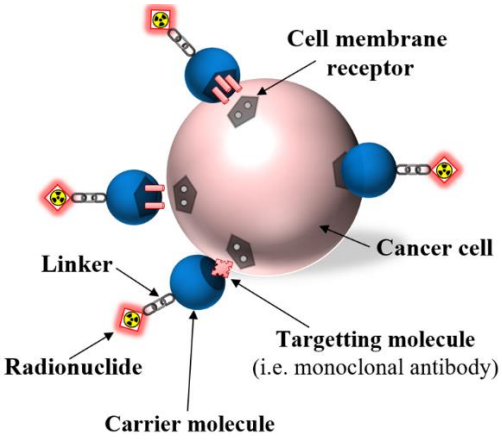


Fig. 1. The scheme of receptor radiopharmaceutical action.

The limitation of the use of 'targeted' radiotherapy is the need to find appropriate receptors on cancer cells and biologically active molecules showing high affinity for these receptors. However, according to the literature [2], it can be expected to find suitable biologically active molecules that can be used in 'targeted' radiotherapy for about half of the types of cancer. Depending on the size of the neoplastic lesion and its stage, the selection of an appropriate radionuclide is a key factor in the effectiveness of the therapy. Large solid tumours are usually treated with high- and medium-energy β^- radiation radionuclides (^{90}Y, ^{188}Re, ^{166}Ho) for which the β-particle range is 0.5–12 mm, representing ~10–100 cell layers. In the case of small neoplastic lesions, low-energy β^- emitters with a range of about 1 mm (for example, ^{177}Lu). However, α and Auger emitters show much greater cytotoxicity than β-particles in the treatment of small tumours, metastases, and early-stage neoplasms (Fig. 2).

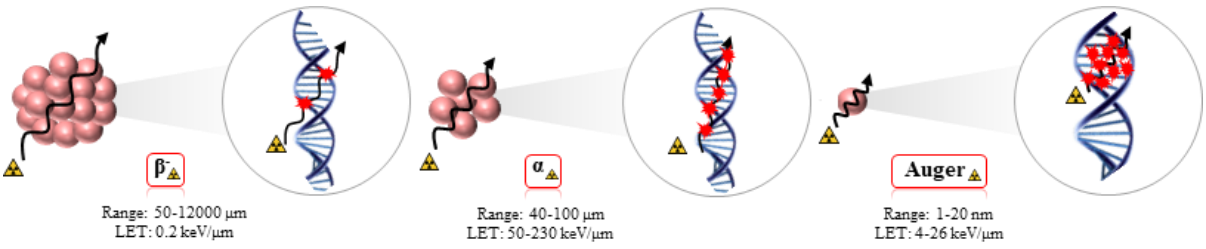


Fig. 2. Range of β, α and Auger electrons radiation.

Due to their short range, α-particles have very high values of linear energy transfer (LETα ≈ 100 keV/μm), which makes them capable of inducing double-strand breaks in DNA, resulting in complete death of the cancer cell, while β-radiation mainly damages just one strand of DNA, and then the cancer cell is able to rebuild itself. Moreover, the short range of α-radiation in the tissue (40–90 μm), which corresponds to 2–10 layers of cells, does not destroy the healthy tissues surrounding the tumour. α-radiation is very effective in terms of radiotoxicity because it works regardless of the degree of oxygenation of the cell (cell damage under hypoxia) and the phase of its cycle. Moreover, the *in vitro* studies demonstrated high radiotoxicity of α-radiation in relation to cancer stem cells (*CSC*) [**H1**].

Auger electrons are characterised by a short tissue range just like α-particles (1–10 nm) but a large LET (~20 times greater LET compared to β^- particles), also causing double-stranded DNA breaks. The emission of low-energy electrons occurs as a result of two nuclear decays, electron capture (EC) and internal conversion (IC). Both processes lead to the creation of free places in the internal electron shells, which is energetically disadvantageous. Striving to achieve the ground state, atoms undergo a series of electron transitions from higher to lower shells, which is associated with the emission of excess energy in the form of characteristic X-rays, Auger electrons, or Coster-Kronig electrons. In the EC and IC processes, several to several dozen electrons are emitted with energies ranging from a few eV to about 1 keV [3]. However, due to the very short range of Auger electrons, it is important to provide their emitter near the DNA in the cell nucleus, so it is necessary to internalise it inside the cell.

Unfortunately, some radionuclides that are very attractive from the medical point of view, such as ^{198}Au, ^{223}Ra, and ^{211}At, do not form stable chelates, hence it is difficult to bind them with a biologically active molecule. Other radionuclides, for example, bioconjugates labelled with an astatine radionuclide, do not show high stability *in vivo*. These difficulties are met by nanotechnology, which allows for the preparation of, for example, radioactive gold nanoparticles, adsorption of the astatine radionuclide on the surface of gold nanoparticles, and the immobilisation of ^{223}Ra α-emitter into the structure of the nanozeolite through ion exchange between sodium ions in the nanozeolite spaces and radium ions in the solution. These issues are dealt with by the team at the Institute of Nuclear Chemistry and Technology in which I work.

In recent years, radioactive nanoparticles have been increasingly used in both diagnostics and medical therapy [**H6**]. Their main advantage is the fact that each nanoparticle contains at least hundreds of radionuclide atoms, unlike conventional radiopharmaceuticals,

where the targeting biomolecule is labelled with one or more radioisotope atoms [4, 5]. This allows for much greater activities to be used in radiotherapy and is of particular importance in Auger electron therapy.

The future of nanoparticles in medicine is also related to their multi-functionality, consisting in combining diagnostics and therapy in one radiopharmaceutical. It results in simultaneous detection of neoplastic changes, monitoring the effectiveness of therapy and treatment. Nanomedicine also allows for the simultaneous use of a number of therapeutic methods, for example, chemo- and radiotherapy in one nanoparticle, which is extremely important due to the high drug resistance of cancer cells caused by their production of multiple defense mechanisms. It is also worth notice that the nanoparticles themselves have the ability to accumulate within a cancerous tumour through the so-called passive transport defined as 'increased permeation and retention' (*EPR*). It is related to the leakage of the tumour blood vessels into which nanoparticles easily penetrate from the circulatory system [5]. Nanoparticles are attached to a targeting biomolecule, for example, a peptide, monoclonal antibody, fragment thereof, or other small biologically active molecules to increase the accumulation of nanoparticles within a cancerous tumour without destroying normal tissues. Nanoparticles with the attached vector bind to the receptor or enter the interior of cancer cells by endocytosis, that is active transport, thus increasing the effectiveness of the therapy and at the same time reducing the toxicity to healthy cells.

In my research leading to the production of new targeted radiopharmaceuticals, I used two vectors: **substance P** and the monoclonal antibody: **trastuzumab**. I selected these biomolecules because of their affinity for two of the most aggressive cancers: glioblastoma (SP) and breast and ovarian cancers expressing HER2 receptors (trastuzumab).

SP is an endogenous neuropeptide widely distributed in the peripheral and central nervous system [6]. This biomolecule consists of 11 amino acids (Arg^{1}-Pro^{2}-Lys^{3}-Pro^{4}-Gln^{5}-Gln^{6}-Phe^{7}-Phe^{8}-Gly^{9}-Leu^{10}-Met^{11}-NH_2, Fig. 3) and belongs to the group of neuropeptide tachykinins with affinity for NK1 receptors. NK1 receptor agonists and antagonists, including SP, used in classical and nuclear medicine, have been described in detail in my review [**H5**].

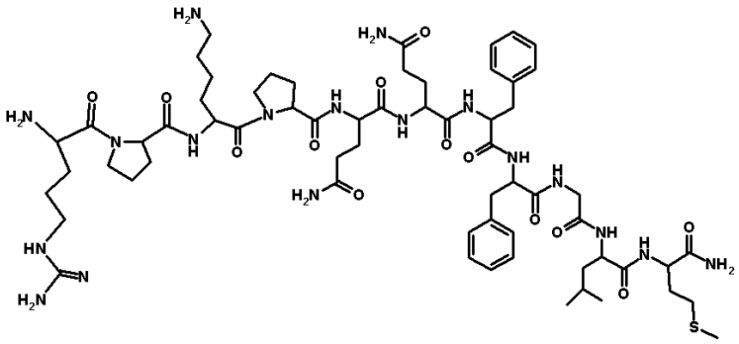


Fig. 3. The structure of SP(1-11).

The SP fragment responsible for binding to the NK1 receptor is the sequence of five amino acids located at the C-terminus of the peptide (Phe^{7}-Phe^{8}-Gly^{9}-Leu^{10}-Met^{11}-NH_2) [7-9]. Tachikinins are degraded by the neural endopeptidase enzymes (NEPs) and the angiotensin-converting enzyme (ACE) [10], therefore the biological life of SP in blood and tissues is 1.5 hour, while in plasma it is several hours [11].

SP plays an important role in the aetiology of many diseases, such as: depression, asthma, psoriasis, inflammatory bowel disease, schizophrenia, neurosis, migraine, and other diseases of the nervous system in which pain is the dominant symptom [12-17]. Moreover, the results of former research also confirm the influence of SP on the functioning of the immune [18-21], respiratory [22, 23], digestive [24, 25], bone [26, 27] and cardiovascular systems [28, 29]. Identification of NK1 receptors on many tumour cells (for example, astrocytoma, neuroblastoma, melanoma, pancreatic cancer), including GBM [30-32], has enabled the use of SP in their treatment.

In *in vivo* studies on patients, SP-based bioconjugates were labelled with β^- (^{90}Y, ^{177}Lu) and α- (^{213}Bi, ^{225}Ac) emitters. The first treatment of GBM was performed on twenty patients with the use of ^{90}Y, ^{177}Lu, and ^{213}Bi radionuclides. Radiopharmaceuticals were administered to the postoperative cavity or directly to the tumour using an appropriately selected catheter [33]. The therapy was safe and well-tolerated without side effects and led to necrosis of the cancer cells. The large infiltration of distant brain regions by glioblastoma cells imposed neoadjuvant therapy in subsequent studies, where the radiopharmaceutical [^{90}Y]Y-DOTAGA-SP was applied to the tumour mass before its surgical removal [34]. This treatment resulted in better encapsulation of the tumour (separation of the tumour from neuronal tissue) and a reduction in intraoperative bleeding, thus enabling a more accurate tumour resection. However, as mentioned earlier, ^{90}Y radionuclide is a high-energy β^-emitter of a medium range in tissues of about 2.5 mm. Hence, it can damage healthy cells in the case of glioblastomas located in eloquent parts of the brain, where the speech, vision, hearing, and movement centres are

located. Therefore, in subsequent clinical trials, ^{213}Bi (α-emitter) with a much shorter range in the tissues (~80 μm) was used. The first pilot studies were conducted on five patients with the use of the SP analogue - [Thi^{8}, Met $(O_2)^{11}$]SP(1-11) of higher stability in the organism [35]. The treatment had no side effects and was continued in a larger group of patients [36, 37]. Due to its short half-life ($T_{1/2}$ = 45.6 min), the effectiveness of ^{213}Bi treatment was not satisfactory in the case of larger tumours.

As part of my postdoctoral intership, I conducted the first *in vitro* study using the ^{225}Ac α-emitter [**H1**]. ^{225}Ac is a radionuclide with a longer half-life ($T_{1/2}$ = 9.92 d) and emits much more energy (E_α =27.6 MeV).

Labelling and *in vitro* biological studies of [^{225}Ac]Ac-DOTA[Thi^{8},Met$(O_2)^{11}$]SP radiobioconjugate (H1 publication)

The aim of this study was to obtain a potential radiopharmaceutical useful in targeted therapy of malignant brain tumours.

Obtaining high appropriate activity of preparation is a crucial parameter in the synthesis of radiopharmaceuticals and brings better treatment effects. The appropriate activity of the compound is expressed in radionuclide activity units per mole or gram of conjugate (for example, GBq/nmol). Low appropriate activity makes the unlabelled bioconjugate block receptors on cancer cells. As a result, the radioactive molecule has a limited possibility to combine with the receptor and destruct the tumour. So on the first stage of my study, I focused on developing the conditions for labelling DOTA-[Thi^{8}, Met$(O_2)^{11}$]SP with the ^{225}Ac radioisotope, using various amounts of both ^{225}Ac and the bioconjugate, which led to the highest level of appropriate activity possible and high efficiency of the labelling process. I bound actinium as a 3+ cation with DOTA macrocyclic ligand (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) conjugated directly with the SP biomolecule. DOTA chelator is widely known complexing agent mainly for lanthanides, forming with them kinetic and thermodynamic stable conjugates. As a targeting vector I used an SP analogue with a much longer half-life ($T_{1/2}$ = 7.8 ± 0.5 h) [38] than that of the SP naturally occurring in the organism. This peptide was modified by replacing phenylalanine (Phe) and methionine (Met) amino acids with thionine (Thi) and oxidised methionine (Met(O_2)) without changing the biological properties of the molecule [39].

As a result of optimisation of labelling conditions, I obtained a radiobioconjugate of high radiochemical purity (> 99%) without the need to further purify it, which was confirmed

with *instant thin layer chromatography (ITLC*), and of high appropriate activity (3.3 MBq/nmol) (Fig. 4).

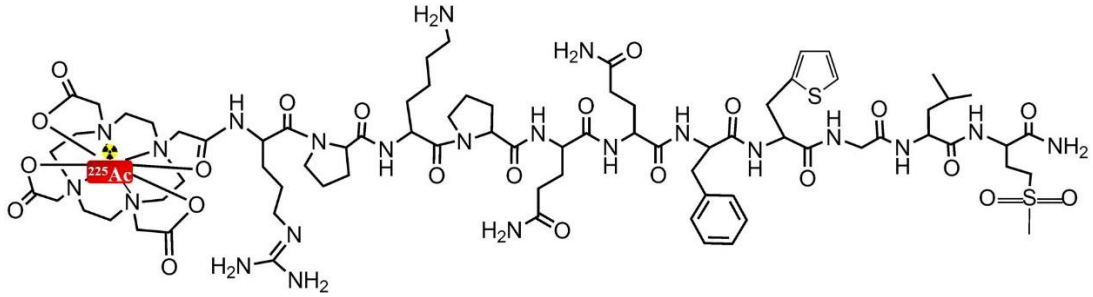


Fig. 4. Structure of [^{225}Ac]Ac-DOTA[Thi^{8},Met(O_2)^{11}]SP radiobioconjugate.

The preparation obtained was highly stable in the cerebrospinal fluid (CSF) and in 0.9% saline solution with added 1000 excess acyclic DTPA ligand (penthetic acid) (Fig. 2a; **H1**).

I studied the biological properties of the radiobioconjugate obtained on three human brain tumour cell lines (T98G, U87MG, U138MG) and glioblastoma stem cells (*GSC*), isolated directly from the tumour with the $CD133^{+}$ phenotype.

Molecular RT-PCR (Fig. 2b; **H1)** and Western blot (Fig. 2c; **H1**) tests confirmed overexpression of NK1 receptors on the T98G and GSC lines. The radiobioconjugate showed high affinity for NK1 receptors (Fig. 2d; **H1**). The value of constant dissociation K_d (concentration of ligand that binds at equilibrium to 50% of the receptors) was small (19.2 nM), and the number of receptors on the cell was $4.6*10^{4}$.

When I obtained positive results regarding SP selectivity in relation to NK1 receptor, I conducted cytotoxicity studies with MTS and clonogenic tests. MTS test constitutes measuring protein mitochondrial activity, that is, succinate dehydrogenase, which transforms orange, water-soluble tetrazole salt (bromide3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenylotetrazole) to formazan, which is a coloured substance. The intensity of its coloration (absorbance) was measured with a plate reader. It is worth notice that formazan is produced only in the case of living cells. Clonogenic test allows for defining the ability of cells to proliferate and thus to form colonies.

The experiments performed indicated toxicity of the preparation depending on the dose used (10, 50, 100 kBq/mL) and incubation time (3, 4, 5, 6 days) (Fig. 3a, 3b, 3c, 3d; **H1**). I also conducted tests with temozolomide (Temodal, TMZ) – a chemotherapeutic agent widely used in the therapy of patients with malignant glioblastoma. As a derivative of decarbazin, temozolomid undergoes a rapid transformation in physiological pH into an active metabolite

showing an alkylating behaviour in relation to the nucleic acid of DNA (forming O^6-methylguanine in the strand of DNA). This leads to its structural changes and fragmentation, and, as a result, to interfering with DNA, RNA, and protein production, as well as preventing cell divisions by blockage in the G2/M cycle phase, ultimately leading to the death of cells. A longer incubation time and a higher dose of the radiobioconjugate caused a significant reduction in viability as opposed to the chemotherapeutic drug, which showed a lesser toxicity with the increase in incubation time. It complies with various functioning mechanisms of both preparations.

The results of the clonogenic test revealed that cancer cells hardly formed colonies after adding the radiobioconjugate (Fig. 4a; **H1**), whereas the use of a cytostatic in a high concentration (500 μM) did not significantly influence the reduction in proliferation, especially in the case of the T98G line (Fig. 4b; **H1**).

The next stage of my *in vitro* tests was defining whether glioblastoma cells would undergo apoptosis or quick destruction by necrosis after being treated with various doses of radioactivity. In my experiments, I implemented flow cytometry with V-FITC annexin and propidium iodide. Apoptosis is a natural, programmed death of the cell, while necrosis is caused mainly by mechanical damage accompanied by an inflammatory reaction. It is worth notice that cancer cells show a characteristic, specific resistance to apoptosis and, what follows, a limited sensitivity to the applied therapies. The results of my experiments showed that the radiobioconjugate induced late apoptosis after 72 and 96 hours of incubation. A shorter period of incubation (24 hours) had a small effect on the programmed cell death (Fig. 5; **H1**). The following experiment analysed the effect of the [^{225}Ac]Ac-DOTA[Thi^8,Met(O_2)^{11}]SP preparation on the cycle of glioblastoma cells with the type of cytometry, which uses the phenomenon of change of DNA amount level in the cell during the cycle. Disturbances in the cellular cycle are used in cancer therapy, where the mechanisms responsible for this process act as a target for the drug (for example, a cytostatic). The results showed that the radiobioconjugate caused the stopping of the cycle in the G2/M phase, thus preventing cells from reaching the phase of M mitotic division (Fig. 6; **H1**). A similar phenomenon was observed in the case of DOTATOC somatostatin analogue, which was also labelled with the ^{225}Ac radionuclide [40].

My pioneer achievement was the analysis of the effect of α-radiation on the viability of glioblastoma stem cells ($CD133^+$). Cancer stem cells are scarce among other cancer cells found in the tumour mass (1–30% in the case of glioblastoma, depending on the degree of malignancy) and are responsible for its development, progression, metastases, and relapses of the neoplastic disease [41-44]. Moreover, the widely used therapeutic methods, such as chemotherapy,

radiotherapy, and immunotherapy, fail to destruct these cells. Therefore, an effective cancer therapy should eliminate not only differentiated cells but also the population of cancer stem cells. My experiments on the viability of the cancer cells with [^{225}Ac]Ac-DOTA[Thi^{8}, Met(O_2)^{11}] conjugate indicated its toxicity with regard to GSC. However, despite using higher doses of the preparation (100, 250, 500 kBq/mL), the percentage of apoptotic cells was lower than that of glioblastoma cells, which is probably due to their higher resistance. I also observed a blockage of the cellular cycle in the G2/M phase (Fig. 7; **H1**).

In summary, my synthesised [^{225}Ac]Ac-DOTA[Thi^{8},Met(O_2)^{11}]SP radiobioconjugate led to lower metabolic activity of glioblastomia cells depending on the dose and incubation time, initiated apoptosis and stopped the cellular cycle in the G2/M phase. Additionally, the preparation showed toxicity at higher doses of radioactivity for glioblastoma stem cells, resistant to conventional treatment methods. The effectiveness of TMZ, even at a high concentration (500 μM), was low compared with the effectiveness of [^{225}Ac]Ac-DOTA[Thi^{8}, Met(O_2)^{11}]SP. According to the literature data, the TMZ concentration found in the cerebrospinal fluid in patients undergoing TMZ therapy was about 5 μM [45]. When used *in vivo*, the high concentrations I used *in vitro* on glioblastoma cells would cause serious side effects, including patient's death.

My *in vitro* experiments led to initiation of a scientific collaboration between the Joint Research Centre of the European Commission (Directorate for Nuclear Safety and Security, Karlsruhe, Germany), where I worked for three years on my postdoctoral intership, and the Department of Nuclear Medicine of the Medical University of Warsaw and implementation a radiobioconjugate in the treatment of patients with GBM as part of medical experiment. This study is being continued, with a safe therapy and very promising results [46].

Synthesis, physicochemical and *in vitro* biological studies of SP analogs labelled with ^{99m}Tc and ^{177}Lu emitters (H2 publication)

To consider the suggestions by some clinicians, I conducted this study to find a more lipophilic radiopharmaceutical that could easily penetrate deeper areas of the tumour or cavity walls after surgery than the labelled DOTA[Thi^{8},Met(O_2)^{11}]SP bioconjugate and showing affinity for NK1 receptors on glioblastoma cells. I selected a natural SP(1-11) and its four analogues for the physicochemical tests (Fig. 5). A modified SP(1-11) was the referential biomolecule due to its common use in medical experiments. Labelling with the ^{99m}Tc and ^{177}Lu radionuclides had to be possible. To achieve that, biologically active molecules were first

attached to DOTA-NHS (ester of the -1,4,7,10-tetraacetic-1,4,7,10-tetraazacyclododecane-acid) and CN-BFCA (ester succynyloimidylate 4-izocyanobutyric acid) bifunctional ligands – with which these radioisotopes form stable and inert complexes, respectively.

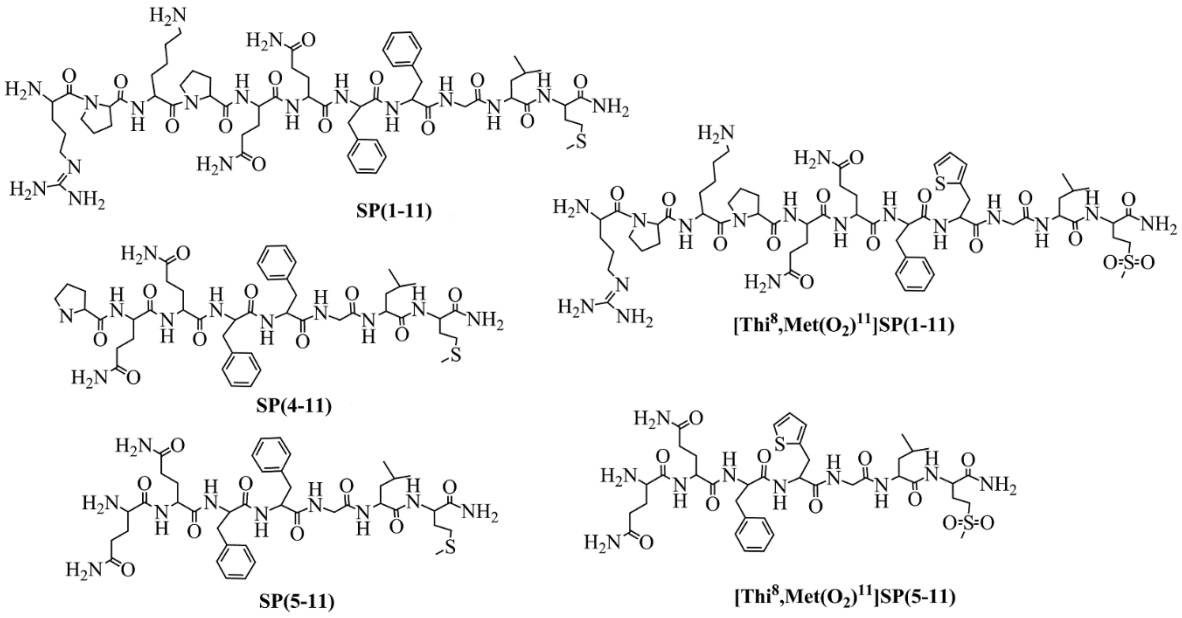
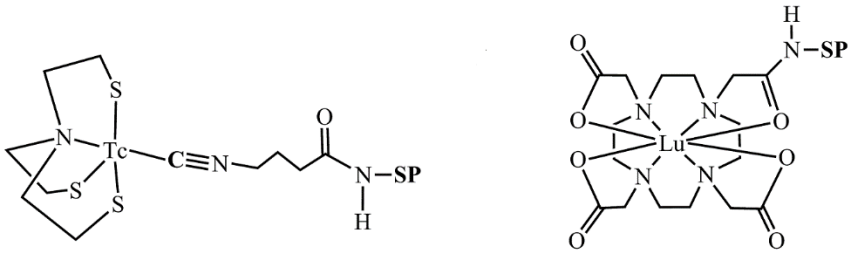


Fig. 5. Structures of SP(1-11) and its analogues.

The bioconjugates obtained were purified on a semi-preparative column with high-performance liquid chromatography (*HPLC*) (Fig. 8, 9; **H2**). The efficiency of the coupling reaction (achieved by forming an amide bond between the chemically active ester of the ligand and the amino group of the peptide) was ~85–95%. Additionally, the characteristics of these compounds were defined with mass spectrometry. When binding a biomolecule with a bifunctional linker, it must be remembered that the amino group of the biologically active molecule was outside its pharmacophore fragment so that it does not affect the molecule-receptor interaction.

The next stage of my study was labelling the bioconjugates with commercially available radionuclides: ^{99m}Tc (analogue of the ^{188}Re therapeutic radioisotope, γ emitter, $T_{1/2}$ = 6.01 h, $E_{(\gamma)max}$ = 0.141 keV) and ^{177}Lu (β^- emitter, $T_{1/2}$ = 6.71 d, $E_{(\beta)}$max = 496 keV) (Fig. 6) and determining their characteristics with the HPLC (Fig. 3; **H2**). The efficiency of the labelling process was measured with the ITLC as 96–98%. The radiobioconjugates exhibit high stability in the PBS buffered saline solution (10^{-2} M) and in the cysteine and histidine solutions (10^{-3}M) with the chemically active SH, NH_2, and COOH groups.



[^{99m}Tc]Tc-NS$_3$/CN-SP **[^{177}Lu]Lu-DOTA-SP**

Fig. 6. The structures of obtained radiobioconjugates.

Considering the potential use of SP and its analogues in GBM therapy, I determined the stability of labelled ^{177}Lu bioconjugates in the cerebrospinal fluid and blood serum isolated directly from the patients. I examined the stability of the preparations with the ITLC and HPLC. In the case of HPLC, I precipitated protein compounds with ethanol and then eliminated them with centrifugation. The results indicated high stability of the radiobioconjugates in the cerebrospinal fluid (96%) even after fifteen days of incubation, while their stability in the serum was very low (4–17% after 24 hours) (Fig. 4, 5, 6; **H2**). It is worth notice that protein concentration in the serum is about twenty times higher than that in the cerebrospinal fluid [46]. The results showed that shorter fragments of SP underwent a faster and stronger enzymatic degradation than radiobioconjugates with a full sequence of amino acids, which may result from a better adjustment of the shortest peptide to the active places of the enzyme. The unmodified SP(5-11) fragment was the least stable.

The next parameter important in the evaluation of a radiopharmaceutical is its lipophilicity, which affects its possibility to pass through cell membranes, its distribution and transport, both in the whole organism and in the tumour area, and the way of its excretion. I determined lipophilicity (logP, where P is the partition coefficient) of the radiobioconjugates in the *n*-octanol/PBS (pH = 7.4) phase. Technetium preparations had a several times higher lipophilicity than lutetium preparations, which is directly related to the hydrophobic properties of NS_3 tetradental ligand (*tris*(2-merkaptoethylo)amine) as opposed to the hydrophilic properties of DOTA chelator (presence of COOH groups). Moreover, shorter SP labelled fragments were more lipophilic, whereas the replacement of the amino acids in the 8 and 11 positions of the peptide (biological time elongation) reduced its lipophilicity (Table 1, 2; **H2**).

The last stage of my study consisted in experiments on receptor affinity of three radiobioconjugates: [^{177}Lu]Lu-DOTA[Thi^8, Met(O_2)^{11}]SP(1–11), [^{177}Lu]Lu-DOTA-SP(4–11),

and [^{177}Lu]Lu-DOTA[Thi^{8}, Met$(O_2)^{11}$]SP(5–11) on U373MG human glioblastoma cell line, characterised with overexpression of NK1 receptors (Rys. 7; **H2**). The results suggest that all the preparations bound specifically and showed high affinity for the receptor (K_d values in nM) (Table 3; **H2**). Just as I expected, the values of K_d constant dissociation for [^{177}Lu]Lu-DOTA [Thi^{8},Met$(O_2)^{11}$]SP(1–11) (K_d = 11.1 nM) were similar to those of the same bioconjugate labelled with ^{225}Ac radionuclide (K_d = 19.2 nM; **H1**), despite using various cell lines.

In summary, I have obtained new radiobioconjugates based on shorter SP fragments, which can be used in targeted radionuclide therapy of glioblastomas due to their affinity for NK1 receptors and higher lipophilicity compared with SP(1-11) and its modified analogue. I did not observe the enzymatic degradation of these preparations in the cerebrospinal fluid. Unfortunately, their unsatisfactory instability in the serum limits their use. However, we can hope that they would not be degraded, taking to account that they would be administered shortly after tumour resection right to its cavity and assuming that there would be no blood in the postoperative cavity when subsequent doses would be administered. However, it must be remembered that even a small amount of blood will cause SP cut. For this reason, these therapies should be personalised and adjusted to the individual course of the disease. Moreover, precise diagnostics should be an indispensable step before beginning any therapy.

My following papers on new potential radiopharmaceuticals useful in therapies of aggressive and drug-resistant neoplasms considered **trastuzumab** as a targeting biomolecule against tumours expressing HER2 receptors.

Trastuzumab (Herceptin®) is a recombined, humanised IgG1 monoclonal antibody with a molecular mass of 145 kDa used in the treatment of highly aggressive and drug-resistant breast and ovary cancers with metastases [48] and of HER2+ metastatic stomach cancer or that of gastroesophageal junction. Trastuzumab combines selectively with the extracellular IV domain (*ECD*) of *human epidermal growth factor receptor 2 (HER2*). Overexpression of HER2 receptors occurs in about 20–30% of all breast cancers [49]. Moreover, HER2 receptor is overproduced in about 22% of patients suffering from stomach cancer. Overexpression of this antibody blocks the ability of cells to proliferate by stopping the cycle in the G1 phase. It also weakens the process of angiogenesis and stimulates *antibody-dependent cellular cytotoxicity*, which depends on antibodies with overexpressed HER2 receptors. According to literature data, trastuzumab combined with other cytostatics (for example, cisplatin, thiotepa, etoposide/doxorubicin, paclitaxel, methotrexate, and vinblastine) shows synergy or additivity,

increasing the degree of response to treatment. Its therapeutic activity consists in blocking repair mechanisms of cancer cells damaged by chemotherapy [50, 51]. The combination of an antibody with radionuclides (*radioimmunotherapy – RIT*) also leads to a stronger therapeutic effect. This effect is related to the destruction of cancer cells by ionising radiation and toxic activity of the antibody, which also serves as the targeting vector.

Au_nAt clusters as potential ^{211}At carriers (H3 publication)

^{211}At is one of the α-emitters with suitable nuclear properties, which can be used in radionuclide cancer therapy. It is obtained cyclotronically by irradiation of metallic bismuth target with α-particles ($^{209}Bi(\alpha,2n)^{211}At$). Astatine should be in the form of a 1- anion At^- characteristic for the halogen group as an element belonging to the 17 groups of the periodic table. However, astatine has a more metallic character and behaves differently than other elements in the halogen group, taking to account changes in chemical properties of elements belonging to this group. Its chemical properties also significantly differ from those of the neighbouring iodine. The strength of its bond with carbon is much lower than that of iodine, so the methods of direct labelling biomolecules with radioiodine failed to give the expected results. They were unsatisfactory due to a lack of stability and release of ^{211}At under *in vitro* and *in vivo* conditions [52]. Research on alternative methods of binding ^{211}At with biomolecules is conducted in various centres for many years. So far, no stable astatine radiopharmaceuticals were obtained.

It is known that the strength of bonds with halogens decreases lengthwise along with the group, which makes the bonds with iodine and astatine the weakest. A similar phenomenon, yet to a lower degree, was observed in boron, offered as an alternative to ^{211}At. It was, however, noticed that iodine shows high affinity for precious metals, such as Ag, Au, and Pt, and the stability of X-M bonds increases downwards in the group: F<Cl<Br<I. The tests on Cl^-, Br^-, I^- ion adsorption on the surface of AuNPs indicated the formation of a covalent Au-X bond [53]. Therefore, we formulated the thesis that At-Au bond strength should be higher than Au-I bond strength. As a result, we could obtain stable ^{211}At radiobioconjugates with nanotechnology.

The initial results of the test on ^{211}At adsorption on gold nanoparticles confirmed these assumptions. A conjugation of astatine to gold surface was more stable than all the other astatine bonds tested so far. To find the explanation for the character of this bond and the reasons for stable astatine-gold bonds, prof. Dobrowolski from our institute performed quantum-mechanical calculations determining Au-At bond strength on gold nanoparticles (nanoclusters)

[**H3**]. Unfortunately, high complexity of the system made it impossible to make calculations for the nanoparticles of 5 nm selected for further research. Calculations were made for combinations of At, a single Au atom, and a gold cluster of 12 atoms (Au_{12}) of about 1.2 nm. Moreover, calculations were made for the gas phase. The calculations helped in formulating the mechanism of astatine adsorption on the surface of gold nanoparticles.

The determined value of Au-X bond energy was unexpectedly the least negative, which would suggest that gold would form the strongest bonds with fluorine and the weakest bonds with astatine. However, the calculations were made for the gas phase, and halides usually occur in the anion form in aqueous solution. Thus, as we have noticed, three subsequent reactions are needed to form Au-X bond:

I) oxidation of the halogen anion to the zero degree;

$$X^- \rightarrow X^0 + e^-$$

II) water reduction;

$$H_2O + e^- \rightarrow ½H_2 + OH^-$$

III) conjugation of the halogen in the first degree of oxidation to the gold atom;

$$X^0 + Au \rightarrow AuX$$

The total reaction is as follows:

$$X^-_{aq} + Au^0 + H_2O \rightarrow AuX + ½H_2 + OH^-$$

Energies of anion hydratation and their reduction potentials were considered to define the strength of binding halogen anions on the gold surface. At^- anion shows the lowest solvation energy and the least negative standard reduction potential in the halogen group, which gives the free energy of ΔG reaction the most negative value. This explains why the adsorption on gold is strong in the case of astatides and the weakest in the case of fluorides. These calculations revealed the essence of strong At chemisorption and demonstrated that strong astatine adsorption on gold is related to its high electrode potential.

Theoretically obtained results led to the initiation of the experimental studies on the possibility to synthesise a potential therapeutic radiopharmaceutical – gold nanoparticles labelled with ^{211}At radionuclide and conjugated with trastuzumab - targeting biomolecule [**H4**].

Gold nanoparticle bioconjugates labelled with an α emiter - ^{211}At (H4 publication)

The complexes of ^{211}At and the biomolecule show low *in vivo* stability (low the stability of C-At bond), and the former quantum-mechanical calculations [**H3**] and experimental studies [54] showed that astatine is strongly chemisorbed on the gold surface. Hence, for the purpose of this study, I decided to make a stable bond of ^{211}At ($T_{1/2}$ = 7.21 h; $E_{\alpha(mean)}$ = 6.7 MeV) with the biologically active trastuzumab molecule, using gold nanoparticles (AuNPs) as carriers.

The first stage was the synthesis of gold nanoparticles of 5 nm [55]. The size of nanoparticles significantly affects the time of their blood circulation, the speed of achieving the tumour, as well as its effective penetration and the possibility of its excretion through kidneys. The size, shape, and morphology of the nanostructures obtained were defined with the DLS (*Dynamic Light Scattering*) and TEM (*Transmission Electron Microscopy*). TEM micrographs showed that the synthesis led to the creation of small spherical nanoparticles of coloidal gold of not more than 5 nm (Fig. 2; **H4**).

Nanostructures should have hydrophilic surface to avoid detection by macrophages [56]. One method of increasing their hydrophilicity consists in covering the surface of nanoparticles with a polymer, such as polyethylene glycol (PEG) [57, 58]. Additionally, modification of their surface significantly affects the pharmacokinetics of the nanoparticles and their biodistribution in the organism. Hence, the next stage was developing a simple and effective method of functionalising the surface. To this end, HOOC-PEG-SS-PEG-COOH bifunctional linker was used as the one with a disulphide bridge (to bind with AuNPs on the basis of sulphur-gold affinity) and carboxyl groups (to form a bond with the biologically active molecule). The AuNPs-PEG-COOH molecules modified by activating the –COOH group (NHS active ester obtained) were conjugate with trastuzumab, which led to the formation of an amide bond with lysine amino group (Fig. 1; **H4**).

To define the average number of antibody molecules attached to one AuNP nanoparticle, trastuzumab was labelled with ^{131}I radionuclide and added during bioconjugate synthesis. Protein iodisation consists in electrophilic binding iodine to tyrosine in the protein with an oxidising factor, for example, iodogen. The results of radiometric method showed that 4 trastuzumab molecules were found on the surface of a single AuNP nanoparticle of 5 nm. Additionally, the DLS measurements of the size and zeta potential confirmed a conjugation of trastuzumab with AuNPs (Table 1; **H4**). I obtained ^{211}At isotope in the U200-P cyclotrone in the Heavy Ion Laboratory of the University of Warsaw in the ^{209}Bi(α,2n)^{211}At reaction, which

was then separated from the target material using dry distillation. I also used ^{131}I radionuclide as the ^{211}At surogate because of its chemical similarity and wide availability.

The next stage was labelling the bioconjugate obtained with ^{131}I and ^{211}At isotopes, characterised with high efficiency (>99%) (Table 2; **H4**) (Fig. 7). The labelling mechanism consisted in the adsorption of the radionuclides on the surface of gold nanoparticles.

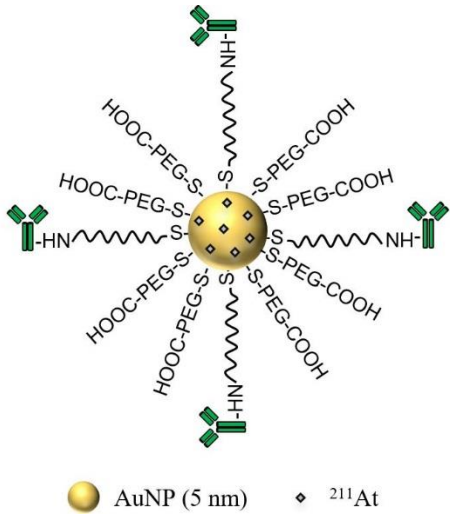


Fig. 7. [^{211}At]At-AuNPs-PEG-trastuzumab radiobioconjugate.

The tests on the stability of [^{211}At]At-AuNPs-PEG-trastuzumab and [^{131}I]I-AuNPs-PEG-trastuzumab radiobioconjugates in the blood serum indicated a small leakage of radionuclides, which was much bigger in the case of the bioconjugate labelled with ^{131}I radioisotope (~9%) (Table 3; **H4**). Comparison of the stability of astatine bioconjugates based on gold nanoparticles with that of radiobioconjugates whose ^{211}At was conjugated with a phenyl ring [59], Rh(S4) complex [60], or boronic clusters [61, 62] demonstrates a much better stability of bioconjugates labelled with ^{211}At using AuNPs.

I conducted biological *in vitro* tests to define the practical use of the radiopharmaceuticals. Experiments on receptor affinity demonstrated that nanoparticles conjugated with trastuzumab undergo a specific reaction with HER2 receptors of the cancer cells of the SKOV-3 line. K_d parameter defining the concentration of antibody needed to saturate half of the present receptors had a value of 16.6 ± 4.1 nM and was close to the K_d value determined comparatively for iodised trastuzumab (K_d=10±3.2 nM) under the same conditions (Fig. 3; **H4**).

Tests using confocal microscopy were performed to illustrate and confirm the specificity of bioconjugate-HER2 bond and its penetration of SKOV-3 cancer cells. In cells incubated with the bioconjugate, a signal from AuNPs and trastuzumab was indicated, while AuNPs without

antibody were not visible. The results demonstrate the effective penetration (internalisation) of AuNPs-PEG-trastuzumab compound of the perinuclear surface of SKOV-3 cells. Additionally, some combined signals were also observed in the nucleus, which may indicate that the tested bioconjugate penetrate the nuclear membrane (Fig. 4; **H4**).

Tests on cytotoxicity using the MTS made it possible to determine the effect of the synthesised bioconjugates on the metabolic activity of cancer cells. The toxicity of the radiobioconjugate to SKOV-3 cells depended on its dose and incubation time. The median value of the lethal dose (LD_{50}) for [^{111}At]At-AuNPs-PEG-trastuzumab was 0.55 MBq / mL. LD_{50} parameter was much higher for the nanoparticles labelled with the radionuclide without the vector (LD_{50}=1.3 MBq/mL) (Fig. 5; **H4**). These results proved the very high specificity of the interaction of trastuzumab - HER2 receptor. Conjugation with 5 nm Au nanoparticle does not significantly reduce the receptor interaction of trastuzumab. Moreover, the cytotoxicity obtained with ^{211}At α-emitter was about 7 times stronger than when AuNPs-PEG-trastuzumab molecules were labelled with ^{177}Lu β^- emitter, although the half-life of ^{211}At is 22 times shorter than that of ^{177}Lu [63].

In summary, my tests led to finding an innovative radiobioconjugate ([^{211}At]At-AuNPs-PEG-trastuzumab), which binds specifically to HER2 receptors, internalizes inside the cell, and shows cytotoxicity to the SKOV-3 cells overexpressing these receptors. The potential use of this pharmaceutical in targeted cancer therapy requires *in vivo* research. Additionally, the systemic application is non-recommended due to the unspecific accumulation of preparations based on nanoparticles in various phagocytal organs, such as the liver and spleen. These compounds can be only injected directly into the tumour or cavity after its resection. This concept resembles the classic brachytherapy with the exception that nanoparticles bound with an additional biologically targeting molecule are used instead of microspheres labelled with radionuclides. The advantage of such therapy in comparison with the ‘classic’ local application of the radiopharmaceutical is its much more effective accumulation of the nanobioconjugate within the tumour. It is worth notice that research with the use of gold nanostructures labelled with ^{211}At continue in the foremost radiopharmaceutical centre at the Duke University, USA, by Prof. M. Zalutsky.

Application of lipid nanoparticles - cubosomes, as doxorubicin and β$^-$ emiter - ^{177}Lu carriers for simultaneous chemo- and radiotherapy of cancers (H7 publication)

Drug-resistance of cancer cells is a huge problem in effective treatment. Chemotherapeutics and radiotherapy reduce tumour capacity only by destroying a part of cancer cells. Therefore, modern drugs with a higher therapeutic potential are sought and combined therapies based on several treatment methods are used (for example, chemotherapy combined with radiotherapy). **I wanted to obtain a stronger therapeutic effect, therefore I attempted to synthesise a potential multimodal radiopharmaceutical containing both doxorubicin (DOX) chemotherapeutic and ^{177}Lu β$^-$ emitter. The carrier were cubosomes, which are lipid liquid-crystalline nanoparticles (LCNPs).** It was the first study to make cubosomes the platform for simultaneous chemotherapy and radiotherapy.

Cubosomes have much larger appropriate surface (400 m^2/g) than the widely used liposomes, which allows for introducing higher amount of drugs into their structure. They also show higher stability and resistance to mechanical damage. Drug release from cubosomes to the cancer cell is induced by a change in environment, in this case, a change in pH. The tumour microenvironment has a lower pH value (~5.8) than healthy tissues. The role of cubosomes is to transport the cytostatic to cancer cells and release the drug in its destination in an effective concentration. Additionally, the use of LCNP limits toxicity and adverse side effects of the chemotherapeutic agent.

As part of my collaboration with the Faculty of Chemistry of the University of Warsaw, cubosomes of size about 100 nm (hydrodynamic diameter: 181 ± 10 nm) with the 'top-down' method were synthesized [64]. The size of the synthesises nanoparticles was confirmed with the DLS, and their structure was confirmed with the SAXS (*small-angle X-ray scattering*).

The first stage of obtaining radioactive doped cubosomes was a synthesis of p-NSC-benzyl-DOTA-GA-OA-oleylamina (DOTAGA-OA) conjugate by forming NHCSNH bond between the NCS group of p-NSC-benzyl-DOTA-GA bifunctional ligand and NH_2 group of oleylamina (Fig. 1 c; **H7**). Metallic complexes with DOTAGA have a hydrophilic character. Therefore, in order to be able to incorporate the chelator into the lipid layer of the cubic structure, it was attached to a hydrophobic amine - oleylamine. The product obtained was determined with ^{1}H NMR and ^{13}C NMR spectroscopic methods (Fig. S1, S2; **H7**) and the MS (mass spectrometry) (Fig. S3; **H7**).

The test on lipophilicity of the synthesised [^{177}Lu]Lu-DOTAGA-OA radioconjugate performed in the *n*-octanol/PBS pH = 7.4 phase system confirmed the possibility of

incorporation the compound into the cubic structure (logP=-0.84±0.14), where the possibly radioactive [^{177}Lu]Lu-DOTAGA complex is placed in a water channel, while oleylamina coupled with it is placed in a double lipid layer.

The next stage involved optimisation of doping conditions for DOX and DOTAGA-OA conjugate using their various quantities of weights (Table 1; **H7**). The effect of doping on the structure of cubosomes was determined with the following physicochemical methods: DLS, SAXS, and cryo-EM (*electron cryomicroscopy*).

The process of labelling the doped nanoparticles with regard to the use of DOTAGA macrocyclic ligand was performed at an increased temperature of 95°C, but it did not cause significant changes in the structural parameters of cubic phases (Table 2; **H7**). The degree of labelling the radiobioconjugates obtained was defined with the ITLC, and the efficiency of the reaction was >99%. The tests on their stability in human serum and PBS buffer after 24 hours of incubation showed their stability at the levels of 78% and 86%. Unfortunately, a longer incubation time weakened their stability.

Biological tests with the MTS on the HeLa cell line (cervical cancer) showed that empty cubosomes with the monoolein concentration of 54 μg/mL were non-toxic (Fig. 5A; **H7**). Immobilisation of DOX in liquid nanoparticles caused the death of cells, depending on the concentration of the chemotherapeutic drug. The determined IC_{50} parameter was 0.5 and 0.2 μg/mL after 48 and 72 hours of incubation, respectively (Table S2; **H7**). The cubosomes labelled with β^- emitter showed toxicity depending on the dose and incubation time. The highest reduction of cancer cell viability was observed for the highest dose (15 kBq/mL) and the longest incubation time (72 h, ~53%) (Fig. 5A; **H7**).

In the case of the nanostructures doped both with the cytostatic and ^{177}Lu radionuclide, no synergic effect was obtained in comparison with the effectiveness of those compounds when used separately. The cell viability after 72 hours of incubation was 36.5±2.33% for the radiobioconjugate – not much less than for the nanoparticles with DOX (0.21 μg / mL, 43.6 ± 4.26%) and cubosomes with the radionuclide (15 kBq/mL, 47.4 ± 3.54%) (Fig. 5; **H7**). The stronger effect of the radioconjugate was obtained after 24 hours of incubation, which may be caused by the fact that lipid structures do not disintegrate after such a short time.

In summary, I obtained a radioactive conjugate doped with a chemotherapeutic drug and ^{177}Lu low-energy β^- emitter. The preparation showed limited stability in biological fluids (PBS and human serum), which affected the tests on cytotoxicity. Unfortunately, no synergic or additive effect was observed during the incubation of cancer cells and the radioconjugate. Toxicity was only slightly higher than in the case of the compounds used separately. The half-

life of ^{177}Lu ($T_{1/2}$ = 6.71 d) and a relatively low stability of cubosomes made us plan further research with ^{213}Bi, α– radionuclide of a much shorter half-life ($T_{1/2}$= 45.6 min). Moreover, ^{213}Bi is an α-emitter, and thus its therapeutic effect is much stronger than that of ^{177}Lu, which was used in these experiments. Additionally, combination of trastuzumab or the prostate-specific membrane antigen (PSMA) and cubosomes should ensure precise delivery of the nanostructure to the specified cancer cell types overexpressing receptors without damage to the healthy tissues.

Gold nanoparticle bioconjugate covered with platinum (Au@Pt-PEG-trastuzumab) for targeted electron Auger therapy (H8 publication)

In recent years, good effects of treatment with α-emitters are being observed. Many clinical studies led to spectacular results of using the small PSMA-617 molecule labelled with ^{225}Ac α-emitter in the treatment of tumour metastases caused by prostate cancer [65-67]. Application of [^{225}Ac]Ac-PSMA-617 radiopharmaceutical led to a complete recovery despite the earlier resistance of the neoplasm to other treatment methods [68]. Moreover, other studies demonstrated a huge α-potential of radiotherapy. It was confirmed in my **H1** study in the case of the destruction of cancer stem cells. Unfortunately, the availability of α-emitters is limited, which only allows for several clinical studies per year. However, Auger electrons have similar properties to the emitted α-particle. Auger electrons have a short range in the tissue and a high linear energy transfer (LET), so they could be used in the therapy of small neoplastic changes, micrometastases, and single cancer cells. In future, they could replace α-particle emitters due to their wider availability.

Large quantities of Auger electrons emitted by ^{193m}Pt ($T_{1/2}$ = 4.33 d) and ^{195m}Pt ($T_{1/2}$ = 4.03 d) Auger emitters (26 and 36 electrons, respectively) justify further research on their use. There were attempts to label platinum complexes (cisplatinum, carboplatinum) with ^{193m}Pt / ^{195m}Pt radionuclides due to the cytotoxic properties of the former. The radioconjugates obtained were destroying both healthy and diseased tissues just as cisplatinum, which demonstrated their low selectivity. The next inspiration for research with platinum were the results of the study [69], which revealed that certain cancer cells with high oxidative potential, for example, hepatocellular carcinoma cells (*HepG2*) cause oxidation of platinum nanoparticles to Pt^{2+} iones (dilution of nanoparticles), and then the liberated Pt^{2+} cations cross the membrane, reach the cell nucleus and bind with the DNA causing cytotoxicity. According to literature data, a higher concentration of hydrogen peroxide (H_2O_2) is also present in the cells of ovary cancer (for

example, SKOV-3), which show overexpression of HER2 receptors [70]. **The aim of my next study was the synthesis of gold nanoparticles targeted at HER2 receptors, covered with a thin layer of platinum and the determination of their biological properties. This was an initial study with non-radioactive platinum, whose next stage will be obtaining a radioactive bioconjugate – nanoparticles of the 'core-shell' type useful in targeted radionuclide therapy with Auger electrons.**

The first stage was the synthesis of gold nanoparticles of 30 nm, which were then covered with platinum by reducing the salt of platinum hexachloride ($Na_2PtCl_6 \cdot 6H_2O$) in ascorbic acid solution. The HR-TEM (*high-resolution transmission electron microscopy*) (Fig. 1; **H8**) and DLS (Table 1, 2; **H8**) confirmed the size of the nanoparticles obtained. The amount of platinum attached to the surface of the nanostructures was determined with the ICP-MS (*inductively coupled plasma mass spectometry*) by measuring its concentration in the supernatant after centrifuging Au@Pt (Fig. 3; **H8**). Obtaining a monoatomic layer of platinum on the surface of AuNPs will be an important aspect in further research on the use of radioactive platinum because of the short range of Auger radiation. Optimalisation of the conditions of combining various amounts of platinum led to a thin covering of even one layer of atoms **(**Table 1, Fig. 3; **H8**).

The bioconjugate synthesis involved using adequate OPSS-PEG-NHS linker with a PEG of the 5000 Da molecular mass and an active ester, which was used to attach NH_2 group of lysine to trastuzumab. Additionally, the molecule was stabilised with a PEG (HS-PEG-COOH, 5 kDa) of the carboxylic group (Fig. 8). A radiometic method was used with trastuzumab labelled with ^{131}I radionuclide and added to the bioconjugate synthesis to determine the amount of molecules of the antibody conjugated with the gold nanoparticle covered with platinum. The results showed that one nanostructure contains about 22 antibody molecules.

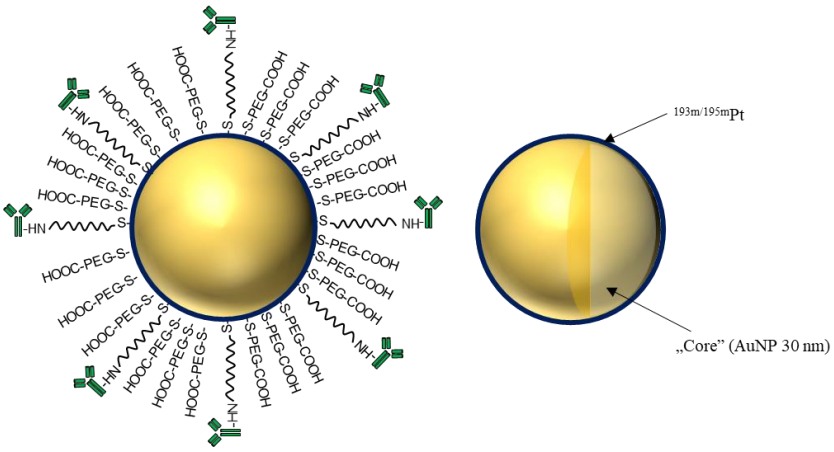


Fig. 8. Au@Pt-PEG-trastuzumab bioconjugate.

Every stage of the surface functionalisation was confirmed with a measurement of the nanoconjugate size and zeta potential with the DLS (Table 2; **H8**). The tests performed with this method also indicated that the synthesised bioconjugate was stable in PBS buffer and saline solution. Agglomeration of the nanoparticles after 7 days at the temperature of 37°C was not observed (Fig. 6; **H8**).

The results of receptor affinity unequivocally indicated a specific bond of the antibody attached to the surface of the nanoparticles and the receptors on the surface of SKOV-3 cells (Fig. 7A; **H8**). The binding effect was not observed in the case of the HER2 negative MDA-MB-231 cell line **(**Fig. 7B; **H8)**, which was confirmed by the selectivity of the compound obtained.

An important aspect of therapy with Auger electrons is the necessity of delivering the radiopharmaceutical in the vicinity of the neoplastic DNA. Thus, its internalisation is required. The experiments with the SKOV-3 cell line indicated that the bioconjugate undergoes about 95% of internalisation already after an hour of incubation (Fig. 9; **H8**). Tests with confocal microscopy were performed to illustrate accumulation of the compound in the cell. The results suggest that after entering the cell membrane, the bioconjugate accumulates in the perinuclear space between the external and internal membrane (Fig. 10; **H8**) and partially in the nucleus (Fig. 12; **H8**). This effect was not observed for the HER2 negative MDA-MB-231 line (Fig. 11; **H8**).

The results of the MTS test on cytotoxicity show that the effect of the bioconjugate on the metabolic activity of SKOV-3 and MDA-MB-231 cells is minimal (Fig. 14; **H8**). Taking to account literature data, where gold nanoparticles were covered with a mono-/multilayer of platinum [71], tests on Au@Pt cytotoxicity were also made for higher concentrations of

platinum attached to the surface of than those in the synthesised bioconjugate that is 10 μg/mL and 145 μg/mL. The results showed that the platinum concentrations used were not lethal to SKOV-3 cancer cells, whereas a reduction in HepG2 viability was observed for 145 μg/mL of platinum (Fig. 15; **H8**). Liver cancer cells have a huge oxidative potential and cause Pt^{2+} release and cytotoxicity by oxidising platinum. These results are an inspiration for next chemical-biological experiments (for example, a test on the concentration of reactive oxygen species *(ROS*)), which could define the therapeutic mechanism of the bioconjugate resultant from platinum attachment.

In summary, I obtained Au@Pt-PEG-trastuzumab bioconjugate, which is stable, exhibits HER2 receptor affinity and internalises into the vicinity of the nucleus. Additionally, Au@Pt nanoparticles with higher amounts of platinum on their surface cause cytotoxicity of liver cancer cells with a higher concentration of hydrogen peroxide. The next stage of this study will be the replacement of the 'non-radioactive' platinum with ^{193m}Pt and ^{195m}Pt radionuclides emitting Auger radiation and biological tests on the SKOV-3 and HepG2 cell lines. It is worth notice that the effectiveness of Auger radiation is related to the direct reaction leading to damage of the DNA or other cellular structures or depends on the indirect generation of reactive oxygen forms. If the dissolution effect of platinum nanoparticles in cancer cells with high oxidizing potential (e.g. HepG2) is confirmed, then in the case of radioactive nanoparticles containing the Auger ^{193m}/^{195m}Pt emitter, we will be able to achieve selective destruction of tumor cells by Auger electrons. Such an effect was not reached by other radiopharmaceuticals used in therapy with Auger electrons. An additional aspect increasing the cytotoxicity and limiting side effects of the therapy is attachment of a biologically active molecule targeted at specific receptors, in this case, HER2 receptors. Unfortunately, the tests on receptor affinity with SP94 peptide ([^{177}Lu]Lu-DOTA-SP94), which overexpresses GRP78 receptor on liver cancer cells according to the literature data [72], failed to show a specific binding of this conjugate with the receptors on the HepG2 line cells (Fig. 8; **H8**), which prevents its further use in the targeted therapy. The obtained therapeutic effect derived directly from Auger radiation and dilution of platinum nanoparticles may be smaller due to the lack of a selective vector with affinity for liver cancer cells.

Summary of the most important achievements constituting the basis for my habilitation process:

My studies presented in a cycle of publications on the radiobioconjugates targeted at the therapy of the most aggressive and drug-resistant brain, breast, and ovary cancers contributed to the proposal of new, more effective methods of their treatment. It is worth notice that design of new receptor radiopharmaceuticals is an interdisciplinary field, which requires skills and knowledge of, among others, organic and coordinate chemistry, radiochemistry, and radiobiology. Hence, numerous multiple biological tests were needed to authenticate my achievements and innovative solutions in radiopharmaceutical chemistry.

Of these, the most important are:

- design, synthesis, and chemical-biological characterization of the innovative [^{225}Ac]Ac-DOTA-SP radiopharmaceutical of use in brain cancer therapy, which showed cytotoxicity not only against cancer cells but also against glioblastoma stem cells resistant to conventional treatment methods, which is highly significant. This is the first study demonstrating the effectiveness of α-radiation in destroying highly resistant GSC. The preparation is currently used as part of the medical experiment and administered directly to the tumour cavity after its resection;
- demonstration that shorter SP fragments – SP(4-11) and SP(5-11) are more lipophilic than [Thi^{8}, Met(O_2)^{11}]SP(5-11) conjugate used so far, which allows them to easily penetrate cell membranes and preserve their affinity to NK1 receptors. This would allow for a systematic application of radiopharmaceuticals based on these biomolecules. Unfortunately, radiobioconjugates based on short SP fragments have low stability in serum, which may limit their use in systemic glioblastoma therapy and local therapy because there are cases of serous fluid exudation after tumour resection owing to damage of the blood-brain barrier. However, the test results suggest the use of the preparation based on SP(4-11) fragment. [^{177}Lu]Lu-DOTA-SP(4-11) radiobioconjugate shows almost twice higher lipophilicity and no effect on stability compared with the currently used preparation based on DOTA- [Thi^{8}, Met(O_2)^{11}]SP(1-11) bioconjugate;
- determination of the mechanism of binding astatine on the surface of gold nanoparticles with the use of quantum-mechanical calculations. This mechanism consists in forming a

strong Au-At covalent bond, whose source is the least positive potential of oxidising At^- to At^0;

- design and synthesis of a new astatine radiopharmaceutical on the basis of quantum-mechanical results of astatine-gold interaction. The [^{211}At]At-AuNP-trastuzumab radiobioconjugate obtained was stable under *in vitro* conditions and showed high affinity for HER2 receptors and high toxicity;
- design and obtainment of a multimodal radiopharmaceutical with both a chemotherapeutic agent and ^{177}Lu β^- emitter, whose lipid nanoparticles (cubosomes) are the carriers. Unfortunately, no synergic or additive effect was observed due to the limited stability of cubosomes. However, there was higher toxicity than in the case of using these compounds separately. The study will continue with the short-lived ^{213}Bi α-emitter;
- development of the method of covering gold nanoparticles with a thin layer of platinum and attachment of trastuzumab to obtain a receptor radiobioconjugate useful in therapy with Auger electrons. Additionally, the toxicity of the compound against liver cancer cells with a high oxidative potential was observed. The study continues with ^{193m}Pt and ^{195m}Pt Auger emitters instead of non-radioactive platinum. Such radiobioconjugate is possibly the first radiopharmaceutical based on Auger electron emitters which binds selectively with cancer cells. It seems possible in the light of the initial results of diluting platinum nanoparticles in biological fluids with a high ROS concentration recorded in recent literature.

Recently, research on the treatment of advanced tumours involves mainly studies directed at finding modern targeted drugs due to a large number of serious side effects of chemotherapy. The radiobioconjugates I designed are preparations targeted at specific NK1 and HER2 receptors. Taking to account the results of my study, I can certify that α an Auger radionuclides, are some of the most promising corpuscular radiation emitters due to their short range and high linear energy transfer (LET). The results of my experiments show that α-particles and supposingly Auger electrons also destroy cancer stem cells, which appeals for their use in my further research. So far, no preparation causing the death of resistant stem cells was found. These studies can give hope to the patients suffering from the most severe neoplasms. That is why I am planning to extend my research on the cytotoxic effect of radiopharmaceuticals based on α and Auger electron emitters on cancer stem cells.

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5. Presentation of significant scientific activity carried out at more than one university, scientific institution, especially at foreign institutions:

In the years 2010–2013, I underwent postdoctoral intership in the team of Prof. A. Morgenstern at the Joint Research Centre (JRC) of the European Commission in Karlsruhe, Germany. The group is among a few teams in the world that use α-emitters (mainly ^{213}Bi and ^{225}Ac) in nuclear medicine.

The aim of one project with my participation was to devise a new radiopharmacetucial based on SP molecule, which shows overexpression of NK1 receptors for its potential use in targeted glioblastoma therapy. The radiobioconjugate I synthesised ([^{225}Ac]Ac-DOTA-SP) showed affinity and cytotoxicity to brain cancer cells [**H1**]. Moreover, this was the first study in which DOTA-SP compound labelled with an α-emitter was effective against cancer stem cells - cells resistant to standard treatment methods, such as radio-, chemo-, and

immunotherapy. This study helped me initiate clinical research with [^{225}Ac]Ac-DOTA-SP radiopharmaceutical in brain cancer therapy, which is conducted at the Department of Nuclear Medicine of the Medical University of Warsaw. The preparation is administered directly to the tumour cavity after its resection.

My collaboration with the JRC still continues, which is testified by our common publications. Moreover, I conduct the study of [^{225}Ac]Ac-DOTATATE radiopharmaceutical on patients suffering from neuroendocrine tumours (neoplasms showing overexpression of SST2 somatostatin receptors) as part of the consortium grant of the Agency for Medical Research on research and development in terms of non-commercial clinical research. The study is entitled: "Evaluation of the effectiveness of treating patients with neuroendocrine neoplasms and non-resectional metastases to the liver with the use of somatostatin analogue labelled with [^{225}Ac]Ac-DOTATATE α-emitter." This has been the first therapy given in Europe since November 2019 and its results are promising.

Scientific cooperation before obtaining the doctoral degree:

- Institute of Nuclear Chemistry, Johannes Gutenberg University, Mainz, Germany - prof. Frank Roesch
- Institute of Nuclear Technologies, Lisbon, Portugal - dr Maria Neves
- National Laboratory of Energy and Geology, Lisbon, Portugal - dr Fatima Teixeira

Scientific cooperation after obtaining the doctoral degree:

- Joint Research Centre, European Commission, Karlsruhe, Germany - prof. Alfred Morgenstern, dr Frank Bruchertseifer
- NCSR "Demokritos", Radiochemical Studies Laboratory INRASTES, Athens, Greece - dr Penelope Bouziotis
- Karlsruhe Institute of Technology (KIT), Germany - dr Damien Hudry
- Institute of Nuclear Sciences, Ege University, Izmir, Turkey - prof. dr Perihan Ünak
- Centre of Postgraduate Medical Education, Warsaw, Poland - dr hab. Damian Gaweł
- Faculty of Chemistry, University of Warsaw, Poland - dr hab. Ewa Nazaruk
- Warsaw University of Life Sciences, Warsaw, Poland - dr hab. Marta Grodzik
- Institute of Physical Chemistry, Polish Academy of Sciences, Warsaw, Poland - dr Tomasz Ratajczyk

- Central Clinical Hospital of the MSWiA in Warsaw, Poland - dr n. med. Tadeusz Budlewski

6. Presentation of teaching and organizational achievements as well as achievements in popularization of science:

before obtaining the doctoral degree:

- participation in the Science Picnic, Science Festival and Museum Night as a representative of the Institute of Nuclear Chemistry and Technology in Warsaw (2005-2009)
- conducting laboratories with postgraduate students of "Nuclear Power Engineering" (2009-2010)
- tutor of 2 students carrying out internships at INCT

after obtaining the doctoral degree:

- auxiliary supervisor of 6 doctoral theses, main supervisor of 4 master's theses and 1 bachelor's thesis

  - auxiliary supervisor of the doctoral dissertation of Emilia Górzyńska, "Radiobioconjugates of gold nanoparticles labelled with Auger electron emitters ^{197}Hg/ ^{197m}Hg for anticancer therapy" carried out at the Institute of Nuclear Chemistry and Technology in Warsaw under the supervision of prof. dr hab. Aleksander Bilewicz
  - auxiliary supervisor of the doctoral dissertation of Kinga Żelechowska-Matysiak „Radioactive gold cluster bioconjugates with chemotherapeutic agent for targeted radionuclide therapy" carried out at the Institute of Nuclear Chemistry and Technology in Warsaw under the supervision of prof. dr hab. Aleksander Bilewicz
  - auxiliary supervisor of the doctoral dissertation of Kamil Wawrowicz „Radiobioconjugates of gold nanoparticles labelled with ^{193m}/^{195m}Pt and trastuzumab for targeted therapy with Auger electrons" carried out at the Institute of Nuclear Chemistry and Technology in Warsaw under the supervision of prof. dr hab. Aleksander Bilewicz
  - auxiliary supervisor of the doctoral dissertation of Adrianna Cytryniak „Nanostructured liquid crystalline lipid carriers of chemotherapeutic agents and emitters of corpuscular radiation for targeted cancer therapy" carried out at the Institute of

Nuclear Chemistry and Technology in Warsaw and University of Warsaw under the supervision of prof. dr hab. Renata Bilewicz

- auxiliary supervisor of the doctoral dissertation of Michał Żuk „Gold-198 nanoparticle radiobioconjugates for simultaneous targeted radiotherapy and radiofrequency hyperthermia" carried out at the Institute of Nuclear Chemistry and Technology in Warsaw and University of Warsaw under the supervision of prof. dr hab. Paweł Krysiński

- supervisor of master thesis of Antonina Matuszyńska „DOTATATE biomolecule labelled with ^{135}La radionuclide for Auger electron therapy of neuroendocrine tumors" (defense planned for June 2021)

- supervisor of master thesis of Emilia Górzyńska, „Synthesis and *in vitro* studies of ^{198}AuNPs-PEG-DOX-Octreotide radiobioconjugate for targeted cancer therapy" (defense – 30 of June 2020)

- supervisor of bechelor thesis of Emilia Górzyńska „^{198}AuNP-Octreotide radioconjugate for targeted therapy of neuroendocrine tumors" (defense 2018). <u>The thesis was awarded 1st prize of the Polish Nuclear Society in 2019 for the best bechelor thesis in the field of nucleonics</u>

- auxiliary supervisor of the doctoral dissertation of Łucja Dziawer, „Gold nanoparticle bioconjugates as ^{211}At carriers in targeted alpha therapy" carried out at the Institute of Nuclear Chemistry and Technology in Warsaw under the supervision of prof. dr hab. Aleksander Bilewicz (defense 2018)

- supervisor of master thesis of Anna Wawrzynowska „Synthesis and *in vitro* studies of Substance P bioconjugates labelled with ^{177}Lu radionuclide"(defense 2016)

- supervisor of master thesis of Marcelina Bednarczyk „Radiotoxicity of bioconjugate labelled with ^{223}Ra against glioblastoma cells and glioblastoma stem cells" (defense 2016). <u>The thesis was awarded in the competition for theses with the greatest commercialization potential organized by the University Center for Technology Transfer (UCTT).</u>

7. Description of other scientific and research achievements:

a) received awards and distinctions

<u>before obtaining the doctoral degree:</u>

- receiving the award of the INCT Director for the progress achieved in the implementation of the doctoral thesis and professional activity (2006 i 2007)
- 1st prize of the Marshal of the Mazowieckie Voivodeship, doctoral dissertation received the highest evaluation of experts in terms of science and usefulness for the further development of scientific and research activity in Mazovia (2009)

after obtaining the doctoral degree:

- 1st prize of the Polish Society of Nuclear Medicine for conducting innovative scientific research in nuclear medicine („*Radiolabelling and biological evaluation of* ^{225}*Ac-DOTA-SubstanceP for targeted alpha therapy of gliomas*"), 13th Congress of the Polish Society of Nuclear Medicine, 19-22 of September 2012, Kielce, Poland
- award of the INCT Director for a series of publications on the use of scandium radionuclides in diagnostics and therapy (2014)
- team award achievement „*The therapeutic radiopharmaceutical based on astatine-211-labelled gold nanoparticles and method of its preparation*" Ł. Janiszewska, M. Pruszyński, P. Koźmiński, **A. Majkowska**, A. Bilewicz – Diploma and Special Award of the Romanian Inventors Forum – Show IWIS 2016, Warsaw, Poland, 10-12 of October 2016
- team award achievement „*The therapeutic radiopharmaceutical based on astatine-211-labelled gold nanoparticles and method of its preparation*" Ł. Janiszewska, M. Pruszyński, P. Koźmiński, **A. Majkowska**, A. Bilewicz – Platinum Medal – Show IWIS 2016, Warsaw, Poland, 10-12 of October 2016
- team award achievement „*Method for obtaining diagnostic amounts of* ^{43}*Sc radionuclide*" - A. Bilewicz, R. Walczak, **A. Majkowska-Pilip**, Gold medal at the International Warsaw Invention Show IWIS 2017, Warsaw, Poland, 9-11 of October 2017
- awards of the INCT Director for a series of publications on the use of nanoparticles in targeted radionuclide therapy and new potential radiopharmaceuticals and chemotherapeutic agents in the treatment of cancer (2018, 2019).

A. Majkowska-Pilip