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The role of radiolabeled cyclodextrins in the *in vivo* PET imaging of metastases in experimental tumors

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The PhD Defense takes place at the Lecture Hall of the Institute of Internal Medicine “A” building, Faculty of Medicine, University of Debrecen, 21st November 2025, 13:00

1. Introduction and aims of study

According to a report of the World Health Organization (WHO), nearly 10 million deaths were caused worldwide by various cancer types in 2020. Despite significant research efforts over the past decades to find treatments for more than the 120 types of cancer, available therapeutic options are often limited. Therefore, early diagnosis of these conditions using imaging techniques such as positron emission tomography (PET) is crucial for long-term survival. PET imaging requires radionuclides that must meet several criteria, such as: (i) the radiation energy must be in a range high enough to escape the patient's body but low enough to be detected by the scanners (511 keV in the case of PET); (ii) the half-life ($t_{1/2}$) of the radionuclide must be long enough to allow examination of the targeted lesion or biological process after the radiopharmaceutical is produced and administered, but short enough to avoid significant damage to the examined tissues and organs; (iii) the radionuclides should be inexpensive and readily available.

Malignant melanoma (MM) is a major public health problem, with increasing incidence and mortality among the Caucasian population. Therefore, its early detection is essential, thus the research in this field has dynamically progressed in recent decades. In addition to the increasing incidence, melanoma poses a serious risk due to its high metastatic potential, which can begin in very early stages of the disease. Often, even when the primary tumor is still small, metastases already appear in multiple organs (e.g., brain, liver, lungs, etc.). This makes early detection crucial, as the formation of metastases significantly reduces the probability of survival.

The most commonly used PET imaging agent to date is 2-[^{18}F]fluoro-2-deoxy-D-glucose ([18F]FDG), a glucose derivative with excellent potential for detecting lesions with increased metabolic activity. However, [18F]FDG has its drawbacks, it also accumulates significantly during inflammatory processes and is strongly taken up by central nervous system cells, making image analysis and differentiation between healthy and diseased tissue more difficult. Additionally, [^{18}F]FDG is considered a general tumor diagnostic agent, as it is not specific to any of cancer type and therefore distributes evenly throughout the body.

Achieving organ- or tissue-specific imaging requires the use of tracers (target molecules) that have a high affinity for specific lesion types and can bind the radiopharmaceutical stably to target cells in order to increase the effectiveness of PET imaging.

In my doctoral work, we produced and studied radiopharmaceuticals labeled with isotopes ^{52}Mn, ^{61}Cu, and ^{213}Bi, examining their tumor-targeting abilities and diagnostic potential for PET applications. The produced compounds included different chelators for binding the radioactive metal ions: cyclodextrin (for ^{52}Mn), DOTA (for ^{213}Bi), and 15aneN_5-based chelator (for ^{61}Cu). To achieve tumor specificity, we used NAPamide for ^{52}Mn and ^{61}Cu as well as NAPamide, FOLDamide, HOLDamide, and MARSamide target molecules for ^{213}Bi.

Objectives of my doctoral research:

- The primary aim was to investigate the *in vivo* tumor-targeting ability and biological behavior of a newly synthesized manganese-52-labeled, randomly methylated beta-cyclodextrin ([^{52}Mn]Mn-

DOTAGA-RAMEB). The results were compared to those of previously described radiopharmaceuticals, namely the melanocortin-1 receptor (MC1-R) specific [^{68}Ga]Ga-DOTA-NAPamide and the PGE2 receptor-selective [^{68}Ga]Ga-DOTAGA-RAMEB.

- We sought to evaluate the tumor-targeting efficiency of the MC1-R-specific [^{61}Cu]Cu-KFTG-NAPamide, developed for melanoma detection, using a B16-F10 melanoma model and its thoracic metastases.
- We aimed to examine the biological distribution and tumor-targeting properties of newly synthesized MC1-R ligands labeled with bismuth-213 (^{213}Bi), including DOTA-NAPamide, HOLDamide, MARSamide, and FOLDamide, in experimental melanoma models.
- Additionally, we aimed to collect comparative data on the targeting capabilities of the PGE2-specific [^{68}Ga]Ga-DOTAGA-RAMEB and [^{52}Mn]Mn-DOTAGA-RAMEB, as well as the MC1-R-specific [^{68}Ga]Ga-DOTA-NAPamide and [^{61}Cu]Cu-KFTG-NAPamide, in the context of thoracic melanoma metastases.

2. Materials and methods

2.1. Cell lines

The MC1-R positive B16F10 mouse melanoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Merck Life Science Ltd., Budapest, Hungary) supplemented with 1% (v/v) MEM Non Essential Amino Acid solution (Merck Life Science Ltd.), 1% MEM Vitamins solution (Merck Life Science Ltd.), 10% Fetal Bovine Serum (FBS, GIBCO Life Technologies, Billings, MT, USA) and 1% Antibiotic and Antimicotic solution (Merck Life Science Ltd.). The cells were kept in humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Animal housing

The C57BL/6J mice (n=5, twelve-week-old female) purchased from Charles River Laboratories (Animalab Ltd., Budapest, Hungary) were kept under standard laboratory conditions. All mice were housed in Individually Ventilated Cages (5/cage, Techniplast, Akronom Ltd., Budapest, Hungary) at 24±2°C and relative humidity of 55±10% with 12 h light–dark cycle under sterile conditions. Sterile semi-synthetic feed (Akronom Ltd., Budapest, Hungary) and drinking water were provided ad libitum. The laboratory animals were kept and treated in accordance with all applicable sections of the Hungarian Laws and regulations of the European Union. The Ethics Committee for Animal Experimentation of the University of Debrecen, (Debrecen, Hungary) approved this study (study number: 28/2023/DEMÁB).

2.3. *In vivo* melanoma tumor model

For the biodistribution studies, *in vivo* melanoma tumormodel C57BL/6 J mice (ten-week-old female, n = 65) were used. For the melanoma tumor induction, the mice were subcutaneously (in the left shoulder area) or in the metastasis model, intravenously (into the lateral tail vein) injected with 1×10^6 MC1- R positive B16-F10 tumor cells in 100 μL saline. *In vivo* and *ex vivo* experiments were performed 14±1 days post-administration of the tumor cells at tumor volume of 115±10 mm^3.

2.4. Radiotracers used in the experiments

2.4.1. Synthesis of [^{18}F]FDG

The production of [^{18}F]FDG is performed routinely at the Department of Nuclear Medicine, primarily for the diagnostic imaging of oncology patients. The required ^{18}F is generated in a cyclotron by proton bombardment of ^{18}O-enriched water. The resulting ^{18}F-ion is transferred to an automated FDG synthesis module, where the precursor (trifluoromethanesulfonyl-β-D-mannose (TATM) dissolved in anhydrous acetonitrile) undergoes radiolabeling via nucleophilic substitution. The reaction between [^{18}F]fluoride and TATM occurs at 85 °C. The product is 1,3,4,6-(TA-[^{18}F]FDG), which contains protective acetyl groups that are subsequently removed by acid hydrolysis using hydrochloric acid. The hydrolysis is performed at elevated temperature (120 °C) and under high pressure in a sealed reaction vessel. The final product, 2-[18F]fluoro-*β*-D-deoxyglucose, is diluted with saline solution to achieve the desired concentration.

2.4.2. Synthesis of ^{52}Mn-DOTAGA-RAMEB

[^{52}Mn]Mn-DOTAGA-RAMEB radiolabeling. A sample of ^{52}Mn in acidic (0.1 M HCl) solution (100 µl) was mixed with sodium-acetate (2 M) buffer (V=500 µl) to set the pH to 7.0. Thereafter, in a reaction vial, the ^{52}Mn solution was mixed with 10 µl DOTAGA-RAMEB (c=3 mM) solution. The vial was heated at 95 °C for 10 min and cooled to ambient temperature (5 min).

For the formulation, the solution was loaded onto a Light C18 Sep-Pak Cartridge (Waters Ltd., Budapest, Hungary) and washed with water (1 mL). The [52Mn]Mn-DOTAGA-RAMEB product was then eluted with 96% ethanol (300 µL). The entire eluate was evaporated to near dryness at 95 °C under nitrogen flow and subsequently redissolved in isotonic NaCl solution.

Instant thin layer chromatography (iTLC-SG, Varian) was carried out applying sodium citrate 0.5 M (pH 5.5) as a mobile phase to determine the RCY. 3 µl [^{52}Mn]Mn-DOTAGA-RAMEB solution was deposited on iTLC-SG strips at 2 cm from the bottom and the solvent was allowed to run to 10 cm from the bottom. The free ^{52}Mn migrated with the solvent front, while the radiotracer remained at the starting point, The strips were then analyzed using the MiniGITAa TLC scanning device (Elysia-raytest GmbH, Straubenhardt, Germany). The product was filtered to be sterile and diluted with isotonic (0.9%) saline solution.

2.4.3. Radiolabeling of DOTA-NAPamide with ^{68}Ga

The ^{68}Ge/^{68}Ga generator was eluted with 5 mL of 0.05 M ultrapure HCl. From the aliquot with the highest activity, 375 µL of the ^{68}Ga solution (80–130 MBq) was measured and mixed with 120 µL of ammonium acetate buffer (0.5 M, pH 4) and 5 µL of DOTA-NAPamide stock solution (1 mg/mL, 0.673 mM). The reaction mixture was incubated at 95 °C for 15 minutes. After incubation, the solution was loaded onto a preconditioned Strata-X cartridge (1 mL 96% ethanol, 5 mL water, and 1 mL 0.25 M ammonium acetate buffer, pH 4), and subsequently washed with 1 mL of water. Following buffer removal, the product was eluted with 300 µL of 96% ethanol. The eluate was evaporated to dryness at 95 °C under a stream of nitrogen. The dry residue was reconstituted in 200 µL of PBS.

The radiochemical purity (RCP) of the product was assessed by radio-HPLC using a Waters Acquity BEH C18 column (1.7 µm, 3 × 50 mm). The mobile phases were: (A) 0.01 M oxalic acid (pH 3) and (B) acetonitrile:water (9:1, v/v). The flow rate was set to 0.9 mL/min with the following gradient profile: 0 min: 100% A; 1 min: 100% A; 2.5 min: 100% B; 3 min: 100% B; 3.1 min: 100% A.

2.4.4. Radiolabeling of DOTAGA-RAMEB with ^{68}Ga

1 mL of ^{68}Ga eluate extracted from a ^{68}Ge/^{68}Ga generator was buffered with sodium acetate (1 M, 160 µL) to ensure the appropriate pH value (pH = 4.3–4.5). Subsequently, an aqueous solution of DOTAGA-RAMEB (5 µL, 3 mM) was added. The reaction was incubated at 95 °C for 10 minutes. After the incubation, the solution was loaded onto a Light C18 Sep-Pak column, than that was washed with 2 mL of water. The ^{68}Ga-labeled product ([^{68}Ga]Ga-DOTAGA-RAMEB) was eluted with a mixture of 96%

EtOH and isotonic NaCl solution in a 1:2 ratio. Radiochemical purity was determined using an RP-HPLC system combined with a radio detector. The product was diluted with 0.9% NaCl solution to reduce the ethanol content below 10%, followed by sterile filtration prior to animal experiments.

2.4.5. Synthesis of [^{61}Cu]Cu-KFTG-NAPamid

An HBTU solution (anhydrous DMF, 7 mg, 0.018 mmol, 1 mL), DIPEA (3 μL, 0.017 mmol) and a solution of *bis*(Boc)-KFTG (anhydrous DMF, 5 mg, 0.007 mmol, 1 mL) were added to the protected, resin-bounded NAPamide [Ac-Nle-Asp(*t*Bu)-His(Trt)-(*D*-Phe)-Arg(Pbf)-Trp(Boc)-Gly-Lys-(Rink Amide MBHA resin)] (30 mg, 0.0045 mmol). The mixture was shaken for 3 h at room temperature then washed with anhydrous DMF. The hydrolysis was performed in 95 % trifluoroacetic acid (TFA, 1 mL). After shaking for 6 h, the liquid part was evaporated, and the crude product was purified by HPLC. For the purification, semipreparative RP-HPLC equipped with a Luna C18(2) 100 Å10 μm (250 × 10 mm) column was used at a flow rate of 4 mL/min using the following solvents: A: 0.1 % HCOOH, B: 95 % acetonitrile, gradient: 0 min: 100 % A, 2 min: 100 % A, 32 min: 100 % B, 40 min 100 % B. The product was collected between 15 and 16 min. After lyophilization, KFTG-NAPamide was gained as a white powder (1.3 mg, yield: 20 %). HRMS ESI (electrospray ionization high-resolution mass spectrometry) calc. for: C72H105N21O12, 728.9204 $[M + H]^{2+}$ and 486.2829 $[M + H]^{3+}$, Found: 728.9174 $[M + H]^{2+}$, 486.2832 $[M + H]^{3+}$.

^{61}Cu was produced from natural zinc foil (Sigma Aldrich, 99.999 % purity, 10x10x0,1 mm size, 0.25 mm thickness) irradiated by 15.5 MeV proton beam (30 min., 20 μA) in a GE PETtrace cyclotron. Following the

separation from gallium isotopes the typical ^{61}Cu activity was 250–330 MBq. The irradiated target material was dissolved in 7 M HNO_3 (2 mL) and mixed with 5 mL, 2.5 M ammonium formate solution to adjust the pH to 2. Then, the sample was dropped to a small CU resin column (6 × 9 mm). After washing with 10 mL of water, the trapped activity was eluted with 0.5 mL of 7 M HCl and evaporated to dryness. The ^{61}Cu was dissolved in 0.01 M HCl or HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH = 7).

In the labeling experiments, 5 μL of KFTG-NAPamide solution (1 mg/mL) was added to circa 100 MBq ^{61}Cu in 50 μL HEPES (1 M) to set the pH to 7 and than the sample was heated at 95 °C for 15 min in an Eppendorf tube. The solution was diluted with water (1 mL), and loaded to a Waters light C18 SPE cartridge. After washing with water, the labelled peptide was eluted with 500 μL saline-ethanol 1:1 mixture. The solvent was evaporated with heating in nitrogen stream to near dryness and diluted with saline solution. RCY was determined with radio-TLC on ITLC stripes (ammonium acetate – methanol 1:1). The molar activity of the labelled peptide fell into the range 52–58 MBq/nmol.

2.4.6. Production of $^{205/206}$Bi and ^{213}Bi, followed by radiolabeling of DOTA-conjugated peptides

Production and purification of $^{205/206}$Bi was the first step. A natural lead foil (99.995% purity, 0.9 × 0.9 cm, 0.25 mm thick) was irradiated with a 16 MeV proton beam (60 minutes, 10 μA) using a GE PETtrace cyclotron. The irradiated Pb target was dissolved in 2 mL of 7 M suprapur HNO_3 and evaporated to a final volume of 1 mL, when precipitation of $Pb(NO_3)_2$ was observed. To separate the solid phase, the solution was mixed with 10 mL

of ultrapure water and filtered through a 0.22 μm Millipore membrane. The resulting solution was loaded onto a column packed with 150 mg TK 200 resin (preconditioned sequentially with 1 mL 0.7 M s.p. HNO_3, 1 mL 7 M s.p. HNO_3, and 5 mL 0.7 M s.p. HNO_3). The column was washed with 5 mL of 0.7 M s.p. HNO_3, and the $^{205/206}$Bi isotopes were eluted in 1 mL fractions using 7 M s.p. HNO_3. Fractions containing the $^{205/206}$Bi isotopes (~30 MBq) were evaporated to dryness and subsequently dissolved in 200 μL of 0.1 M ultrapure HCl.

Elution of the Actinium-225/Bismuth-213 (225Ac/213Bi) generator: The ^{225}Ac/^{213}Bi generator (180 MBq) was purchased from the Institute for Transuranium Elements (Germany, ITU). Before the eluation, the column was washed with 0.01 M hydrochloric acid. During elution, 600 μL of ^{213}Bi was collected using a mixture of 0.1 M sodium iodid /0.1 M HCl. The column was stored in 0.01 M HCl.

For the radiolabeling of DOTA-conjugated peptides, stock solutions (1 mg/mL) were prepared from the DOTA-conjugated peptides. For *in vivo* studies, 5 μL of DOTA-NAPamide (3.4 nmol) or DOTA-HOLDamide (3.2 nmol), and 10 μL of DOTA-MARSamide (5.6 nmol) or DOTA-FOLDamide (5.6 nmol) solutions were mixed with 600 μL of ^{213}Bi (96.01 ± 17.49 MBq), 135 μL of 2 M TRIS buffer (final concentration: 0.34 M), and 50 μL of 20% ascorbic acid (72 mM) (pH = 8.7).

In order to determine the Log*P* values and the stability of the tracers, 20 μL of $^{205/206}$Bi was added to sodium citrate-buffered (pH 5.5) stock solutions of the radiolabeled peptides (30–35 μmol; 10.1 nmol DOTA-NAPamide, 9.6 nmol DOTA-HOLDamide, 8.4 nmol DOTA-MARSamide and DOTA-FOLDamide). Reactions were incubated at 95 °C for 5 minutes.

After cooling to room temperature, 400 μL of 1 M ammonium acetate (NH_4OAc, pH 4) was added to the reaction mixture, which was then processed through a solid-phase extraction (SPE) column (Oasis HLB 1 cc (30 mg); Waters), preconditioned with ethanol and water. The column was washed with 1 mL of water, and the radiolabeled products were eluted dropwise with 200 μL of ethanol. The eluate was evaporated to dryness at 60 °C and reconstituted either in 200 μL of phosphate-buffered saline (PBS) for *in vivo* studies or in 30 μL of PBS for Log*P* and stability measurements.

The RCP of the samples was assessed by instant thin-layer chromatography (iTLC) using silica gel impregnated iTLC-SG strips (Varian) and radio-TLC detection. A 0.1 M sodium citrate solution (pH 5.5) was used as the mobile phase.

2.5. Determination of Log*P* values

The octanol/PBS partition coefficient (indicating the solubility of the molecules in water at physiological pH (pH = 7.4) of our radioisotope-conjugated compounds was determined in every case. The log*P* value provides preliminary information about the behavior and pharmacokinetic properties of the labeled compounds in the target organism. For each compound, 10 μL of those was mixed with a combination of 500 μL of 1-octanol and 490 μL of PBS (pH = 7.4) in a centrifuge tube. The mixture was then shaken for 20 minutes using a vortex shaker (600 rpm), followed by centrifugation at 20,000 rpm for 5 minutes at 4 °C, until complete phase separation. From both phases, three 100 μL aliquots were transferred into test tubes, and their radioactivity was recorded using a calibrated Perkin

Elmer Packard Cobra gamma counter. The log*P* value was calculated by averaging the result of the 3 measurement.

2.6. *In vivo* and *ex vi*vo studies

The ethical approval for animal experiments was given by Animal Welfare Committee of University of Debrecen (16/2020/DEM´AB). Study design and the animal experiments were in accordance with the ARRIVE guideline.

2.6.1. PET imaging of [^{52}Mn]Mn-DOTAGA-RAMEB on B16-F10 melanoma mouse model

PET imaging measurements were performed using the MiniPET-II scanner in the preclinical laboratory of the Department of Nuclear Medicine (University of Debrecen, Debrecen, Hungary).

As a first step, the mice bearing B16-F10 tumors, were intravenously administered with 6.90 ± 1.19 MBq [^{68}Ga]Ga-DOTAGA-RAMEB. The PET imaging was performed after 60 minutes post-injection. 24 hours after the first scan, 20-minute PET scans were conducted on all experimental mice, 80 minutes after intravenous administration of 10.07 ± 0.07 MBq [^{68}Ga]Ga-DOTA-NAPamide. One day later, the mice received 3.50 ± 0.16 MBq [^{52}Mn]Mn-DOTAGA-RAMEB, and 20-minute static PET scans were carried out 1 hour, 4 hours, and 3 days post-injection. Isoflurane-induced anesthesia was maintained throughout all imaging periods [3% isoflurane (Forane), AbbVie, Budapest, Hungary; OGYI-T-1414/01].

2.6.2. *Ex vivo* biodistribution study of [^{52}Mn]Mn-DOTAGA-RAMEB on B16-F10 melanoma mouse model

For the biodistribution studies, healthy control mice were sacrificed at 30, 60, 90, and 240 minutes after i.v. administration of 3.50 ± 0.16 MBq [^{52}Mn]Mn-DOTAGA-RAMEB, and the major organs were collected to record their radioactivity by means of a calibrated gamma counter (PerkinElmer). To measure the radioactivity of B16-F10 tumors *ex vivo*, the same procedure was applied in tumor-bearing mice at 60 minutes post-injection.

2.6.3. PET imaging of [^{61}Cu]Cu-KFTG-NAPamid on melanoma mouse model

For the *in vivo* imaging studies, mice bearing B16-F10 tumors were intravenously injected with 10.3 ± 0.3 MBq [^{61}Cu]Cu-KFTG-NAPamide via the lateral tail vein, and dynamic PET scans were performed under 1.5% isoflurane (Forane, AbbVie) anesthesia (Tec3 Isoflurane Vaporizer, Eickemeyer Veterinary Equipment, United Kingdom).

2.6.4. *Ex vivo* biodistribution study of [^{61}Cu]Cu-KFTG-NAPamid on B16-F10 melanoma mouse model

8±1 days after tumor cell inoculation, mice bearing B16-F10 tumors were intravenously injected with 10.3 ± 0.3 MBq [^{61}Cu]Cu-KFTG-NAPamide. After incubation periods of 30, 60, 90, and 180 minutes, the mice were euthanized using 5% isoflurane overdose. Tissue samples were collected from selected organs, and the weight and radioactivity of the samples were measured using a calibrated gamma counter (Hewlett Packard Cobra II Autogamma Gamma Counter, USA).

2.6.5. *Ex vivo* biodistribution studies of [^{213}Bi]Bi-DOTA-conjugated NAPamide, HOLDamide, FOLDamide, and MARSamide on B16-F10 Melanoma Mouse Model

Ex vivo biodistribution studies were performed 90 minutes after i.v. administration of 3.81 ± 0.32 MBq [^{213}Bi]Bi-DOTA conjugated with NAPamide, HOLDamide, FOLDamide, and MARSamide. Following the euthanization with 5% isoflurane, tissue samples were collected from both healthy (control) and B16-F10 tumor-bearing experimental animals. The radioactivity of various organs was measured using a gamma counter (Packard, Cobra II), and the values were calculated relative to tissue mass. The radioisotope accumulation was then expressed as %ID/g of tissue.

2.7. Data processing

All PET images were analyzed using the BrainCad image analysis software (version: 1.124). After image reconstruction, regions of interest (ROIs) were carefully defined on the tumors and major tissues/organs, and the radioactivity concentrations within the ROIs were expressed as SUVmean and SUVmax values (g/mL). The T/M SUVmean and T/M SUVmax values were calculated from the tumor and background muscle tissue activity.

In the biodistribution studies, the measured gamma radiation values of the organs were recorded, and the decay-corrected radioactivity concentrations were expressed as a percentage of the injected dose per gram of tissue (% ID/g). The data are presented as mean ± standard deviation (SD).

For statistical analyses, the MedCalc 18.5 software package (MedCalc Software, Mariakerke, Belgium) was used, and all statistical data are presented as mean ± SD. Mann-Whitney U-test, student's two-tailed t-test, and two-way ANOVA were applied for statistical significance assessment with a p-value of less than 0.05 considered statistically significant.

3. Results and Discussion

3.1. [^{52}Mn]Mn-DOATAGA-RAMEB

Given the high mortality associated with melanoma, early diagnosis using tumor-selective PET radiopharmaceuticals is at the forefront of current research. Thanks to its favorable physical properties and long half-life ($t_{1/2}$), ^{52}Mn has recently attracted increased attention in nuclear medicine as a potential radioisotope for the development of oncological PET imaging radiopharmaceuticals. With its 5.56 days half-life, this isotope facilitates work with systems of longer pharmacokinetics and allows extended acquisition times. In the recent years, several ^{52}Mn-labeled PET tracers, including [^{52}Mn]Mn-PyC3A, [^{52}Mn]Mn-DOTAGA-bevacizumab, or [^{52}Mn]Mn-BPPA-trastuzumab, have been successfully applied in preclinical studies. Therefore, we investigated the *in vivo* diagnostic potential of a novel ^{52}Mn-labeled RAMEB in MC1-R positive melanoma models and compared it with other tumor-specific PET radiopharmaceuticals used in melanoma PET imaging ([^{52}Ga]Ga-DOTA-NAPamide and [^{52}Ga]Ga-DOTAGA-RAMEB).

Consistent with previous literature findings, we observed significant [^{68}Ga]Ga-DOTA-NAPamide accumulation in our MC1-R positive B16-F10 melanoma tumors, confirming the receptor specificity of the examined compound and its high diagnostic potential in MC1-R positive melanoma imaging. In earlier PET/MRI studies using the same preclinical models, [^{68}Ga]Ga-DOTA-NAPamide demonstrated MC1-R selectivity and outstanding tumor-targeting ability. Furthermore, beside ^{68}Ga, other radionuclides such as copper-64/61, fluorine-18, bismuth-213, or scandium-44 can also be used to radiolabel MC1-R-affine NAPamide

molecules for melanoma detection. Similarly, alternative α-MSH analogues labeled with bismuth-213 (HOLDamide, FOLDamide, MARSamide) are considered promising vector candidates for the treatment of MM.

I*n vivo* results showed that tumors growing subcutaneously can be clearly delineated using both ^{68}Ga- and ^{52}Mn-labeled cyclodextrin molecules, demonstrating the potential applicability of the tested radiotracers for *in vivo* PET imaging of MM. Additionally, considering RAMEB's ability to form complexes with PGE2 receptors, our findings may also indicate PGE2 positivity of the tumors studied. Although the complete receptor profile of B16-F10 tumors is yet to be fully elucidated, earlier immunohistochemical data reported strong PGE2 positivity in such tumor types. Furthermore, findings by Kim et al. related to B16-F10 cells showed that treatment with timosaponin AIII was associated with decreased PGE2 and EP receptor levels, supporting the presence of PGE2 and its receptors on melanoma tumor cells. While PGE2 expression in the melanoma examined may be associated with the accumulation of radiolabeled cyclodextrins, further research is required to explore the link between radiotracer uptake and receptor expression in melanoma cells.

In agreement with the present findings, Szabó et al. also demonstrated significant tumor uptake in a proof-of-concept study using ^{68}Ga-labeled cyclodextrin derivatives ([^{68}Ga]Ga-NODAGA-RAMEB and [^{68}Ga]Ga-NODAGA-HPβCD) in an experimental melanoma model. The similar accumulation of ^{52}Mn-labeled RAMEB and [^{68}Ga]Ga-NODAGA-RAMEB in B16-F10 tumors, measured 1 hour after intravenous administration of the radiopharmaceuticals, suggests that the type of radionuclide does not influence the tumor-targeting ability of cyclodextrins ([^{52}Mn]Mn-

DOTAGA-RAMEB: 0.06±0.01 and 0.09±0.02 for SUVmean and SUVmax; [^{68}Ga]Ga-NODAGA-RAMEB: SUVmean: 0.06±0.02 and SUVmax: 0.10±0.03).

Nevertheless, significantly higher tumor uptake was recorded with [^{68}Ga]Ga-DOTAGA-RAMEB (SUVmean: 0.16±0.04; SUVmax: 0.24±0.05) compared to PET scans with the radiotracer linked via a NODAGA chelator ([^{68}Ga]Ga-NODAGA-RAMEB: SUVmean: 0.06±0.02; SUVmax: 0.10±0.03), which is likely related to structural differences or the differing stability of the chelators used (NODAGA vs. DOTAGA). Despite the differences in SUV values, we can state that, replacing the linker (NODAGA with DOTAGA) has no significant effect on the tumor-targeting ability of cyclodextrin-based tracers.

We hypothesize that the significantly lower accumulation of [^{52}Mn]Mn-DOATAGA-RAMEB observed at later time points may be associated with temporal changes in surface receptors of tumor cells during tumor growth and development (SUVmean and SUVmax of 0.02±0.01 and 0.03±0.01 at 4 hours, and 0.01±0.01 and 0.01±0.01 at 3 days, respectively). Changes in PGE2/EP2 expression, hypoxia developing in the tumor microenvironment, and chronic inflammation may explain the uptake kinetics of ^{52}Mn-labeled compounds.

Based on all these findings, we can conclude that ^{52}Mn-cyclodextrin imaging is capable of visualizing changes in EP receptor density and PGE2 concentrations in the tumor microenvironment. A more in-depth investigation of tumor-related biomarkers (receptors, soluble molecules) could lead to the identification of new targets for both diagnostic and therapeutic applications in the long term. In addition, hypoxia and necrosis, especially in central tumor regions, may be responsible for the

heterogeneous distribution of [^{52}Mn]Mn-DOTAGA-RAMEB. Even though [^{52}Mn]Mn-DOTAGA-RAMEB activity decreased significantly at all time points compared to its ^{68}Ga-labeled counterpart, ^{52}Mn labeling may still offer certain advantages over ^{68}Ga. The long-lived ^{52}Mn enables longer detection windows, potentially facilitating the investigation of slower tumor-related pathophysiological processes and improved identification of lesions not visible at early time points. Since the rapid elimination kinetics and pronounced renal uptake of [^{52}Mn]Mn-DOTAGA-RAMEB may exclude its use for such purposes, pharmacokinetic optimization – such as incorporation of an albumin-binding moiety – may help prolong its biological half-life and thereby its tumor accumulation. Additionally, [^{52}Mn]Mn-DOTAGA-RAMEB shows capability for PET-based visualization of MC1-R-negative tumors.

As mentioned earlier, the observed differences in uptake between [^{68}Ga]Ga-DOTAGA-RAMEB and [^{52}Mn]Mn-DOTAGA-RAMEB tracers may partly be attributed to changes in PGE2 concentration and the presence of PGE2 receptors in the tumor microenvironment. It is worth noting that differences in PGE2 binding affinity between the two molecules should also be addressed. Although both the MC1-R-targeted NAPamide and the PGE2-selective cyclodextrins may be suitable for melanoma imaging, based on uptake values, the NAPamide molecule appears to be superior. The stronger MC1-R presence in melanoma tumors compared to EP2 may explain the enhanced activity of [^{52}Ga]Ga-DOTA-NAPamide. Moreover, the longer tumor retention of the NAPamide-based tracer may contribute to the higher uptake of the α-MSH analog.

Consistent with *in vivo* PET findings, the rapid renal excretion of [^{52}Mn]Mn-DOTAGA-RAMEB is supported by the low activity recorded

in the blood, significant renal uptake, and high radioactivity in the bladder at 30 minutes post-injection. Similar to our findings, the biodistribution data for [$^{205/206}$Bi]Bi-DOTAGA-RAMEB, [^{68}Ga]Ga-DOTAGA-RAMEB, and [^{68}Ga]Ga-NODAGA-HPβCD also indicate elimination via urine. The relatively high hepatic uptake indicates the possible role of the gastrointestinal system in the excretion of the tracer as well; however, further studies are needed to provide a definitive explanation for this observation. In agreement with previous data on [^{68}Ga]Ga-NODAGA-HPβCD (0.99±0.13 %ID/g), we observed relatively high retention in the lungs at early time points (1.22±0.41 %ID/g). Although the exact cause has not been fully elucidated, according to the results published by Hajdu et al., prolonged accumulation of [^{52}Mn]Mn-DOTAGA-RAMEB in water-filled pulmonary areas may hinder its return to the systemic circulation, leading to higher uptake values. In line with the results from other radiolabeled RAMEB molecules ([^{68}Ga]Ga-DOTAGA-RAMEB and [$^{205/206}$Bi]Bi-DOTAGA-RAMEB), relatively low tracer accumulation was observed in other examined organs/tissues.

In accordance with *in vivo* PET data, the increased tumor uptake of [^{52}Mn]Mn-DOTAGA-RAMEB further confirmed the tracer's tumor-targeting potential and the possible presence of PGE2 and EP receptors in the tumor microenvironment. Assessing the *ex vivo* biodistribution of B16-F10 tumors, Szabó et al. also detected significant accumulation for [^{68}Ga]Ga-NODAGA-HPβCD and [^{68}Ga]Ga-NODAGA-RAMEB, which aligned with our results. However, the markedly lower RAMEB retention (0.32±0.10 %ID/g) reported in their experiment (vs. 0.82±0.09 %ID/g in ours) is likely due to the different time points of *ex vivo* uptake measurements. While we assessed data at 60 minutes, Szabó et al.

performed detection at 90 minutes post-injection of the radiotracer, and we hypothesize that significant washout between 60 and 90 minutes may explain the observed differences in tracer concentrations. The high accumulation of [^{52}Mn]Mn-DOTAGA-RAMEB in the tumor, together with negligible background activity (except in kidneys and liver), delivered an optimal T/M ratio, which is crucial for image evaluation. Still, the presence of renal or hepatic malignancies can not be identified due to the physiologically high uptake in these organs. Overall, the *ex vivo* data also confirmed the tumor specificity of ^{52}Mn-labeled RAMEB in melanoma imaging. Although our pioneering results may be promising, further studies focused on pharmacokinetic optimization are necessary.

In summary, we demonstrated the feasibility of diagnostic application of a newly synthesized cyclodextrin PET imaging molecule labeled with ^{52}Mn in experimental melanoma tumors. Since both ^{68}Ga- and ^{52}Mn-labeled cyclodextrin PET tracers are promising radiotracers for melanoma detection, additional cyclodextrin-based radiolabeled compounds with other radionuclides should be developed and evaluated.

3.2. Evaluation of the biological behaviour of [^{61}Cu]Cu-KFTG-NAPamide

Based on our experiments with [^{61}Cu]Cu-KFTG-NAPamide (the first ^{61}Cu-based radiopharmaceutical in this application field), it can be concluded that this radiopharmaceutical possesses excellent capabilities for non-invasive PET diagnostics of malignant melanoma tumors. The rapid formation of the pristine complex ([Cu(KFTG)]$^{+}$), its high inertness, and moderate SOD activity already indicated its potential for *in vivo* applications, which is further enhanced by NAPamide's well-known affinity for melanoma tumors.

Before conducting biological studies, the plasma stability of [^{61}Cu]Cu-KFTG-NAPamide was studied by examining the decomplexation of the compound in human plasma. In a 6-hour time window, less than 4% activity loss was detected, which, considering the obtained diagnostic results, can be considered excellent.

From the *in vivo* and *ex vivo* results, one can conclude that the [^{61}Cu]Cu-KFTG-NAPamide radiotracer binds with high affinity to MC1-R positive experimental melanoma tumors, as supported by the SUV and %ID/g values.

Furthermore, the rapid clearance from the background and renal excretion enable [^{61}Cu]Cu-KFTG-NAPamide to provide outstanding image contrast, which notably improves the evaluation of PET images and the identification of MC1-R positive melanoma tumors.

3.3. Evaluation of the results of [^{213}Bi]Bi-DOTA-Conjugated Amide Derivatives

Previous research findings have clearly supported the higher *in vivo* uptake of radioactively labeled α-MSH analogs in B16-F10 melanoma compared to A375 tumors, which are characterized by low MC1-R expression. Tafreshi and colleagues developed MM-selective optical diagnostics expressing the MC1-R receptor by producing a fluorescent MC1-R-affine peptidomimetic ligand, MC1RL-800. In order to assess *in vivo* tumor specificity, they performed fluorescent imaging following intravenous administration in mice bearing A375 and A375/MC1R tumors. Tafreshi and his coworkers confirmed the diagnostic value of MC1-R as a target, since they detected significantly increased uptake values in receptor-positive tumors compared to tumors with low receptor numbers ($p < 0.05$).

In a study performed by Nagy and colleagues, the binding potential of MC1-R specific, DOTA-conjugated NAPamide labeled with ^{44}Sc and ^{68}Ga was verified both *in vitro* and *in vivo* in MC1-R positive A375 melanoma cell lines, as well as in C57BL/6 J mice bearing B16-F10 tumors and CB17 SCID mice bearing A375 tumors. The results of the *in vitro* studies showed that, both the ^{68}Ga- and ^{44}Sc-labeled NAPamide possess higher uptake in B16-F10 cells compared to A375 cells ($p < 0.01$), confirming MC1-R overexpression in the former one.

As part of their investigations, the increased uptake of [^{44}Sc]Sc-DOTA-NAPamide and the excellent PET image contrast of subcutaneously developing MC1-R-positive B16-F10 tumors were demonstrated. Translational data, however, showed increased tumor standardized uptake values and tumor-to-muscle ratios when the [^{44}Sc]Sc-DOTA-labeled

derivative was applied, reinforcing its applicability for melanoma detection. Consistent with our *in vivo* and *ex vivo* findings, increased renal accumulation was observed with all tracers studied. Similarly, previous experiments using ^{44}Sc- and ^{68}Ga-DOTA-NAPamide reported minimal radioactivity in the chest and most abdominal organs. However, we registered elevated accumulation of ^{213}Bi-HOLDamide in the intestines, stomach, and liver, which aligned with previous studies involving ^{18}F-labeled NAPamide derivatives.

Although the present experimental series did not assess the effects of time or peptide quantity on biodistribution, based on current literature evidences, several points are worth mentioning. The properties (structure and length) of peptides may influence the *ex vivo* tracer uptake profiles of the studied peptides. For instance, a peptide with a shorter sequence might be eliminated more rapidly from target organs and tissues, leading to lower background activity and thus a higher tumor-to-background ratio, significantly facilitating the visual evaluation of PET images. Furthermore, shorter molecules containing only amino acid motif responsible for receptor binding, likely reduces non-specific binding. Furthermore, *ex vivo* measurements carried out at different time points would deliver further information on the pharmacokinetics of the tracers investigated. Therefore, to gain a better insight into the uptake kinetics of the studied radiocomplexes, more extensive studies focusing on chemical structure should be conducted.

Based on our results, we observed that B16-F10 melanomas were clearly identifiable with all investigated radiotracers. The specific tumor accumulation observed for the ^{213}Bi-labeled amide derivatives suggests

that these compounds exhibit high affinity for MC1-R-expressing melanoma. Therefore, these newly synthesized tumor-specific radiotracers are excellent candidates for *in vivo* diagnostics of MC1-R-positive malignant tumors. Furthermore, ^{213}Bi-labeled alpha-emitting molecules could be suitable for targeted radionuclide therapy of melanoma. Integrating alpha-emission, receptor-targeted amide derivatives into standard human protocols could contribute to personalized melanoma treatment.

3.4. Study of chest B16-F10 melanoma metastases

We studied the targeting ability of PGE2-specific [^{68}Ga]Ga-DOTAGA-RAMEB and [^{52}Mn]Mn-DOTAGA-RAMEB, as well as melanocortin-1 receptor (MC1-R) specific [^{68}Ga]Ga-DOTA-NAPamid and [^{61}Cu]Cu-KFTG-NAPamid in mice with chest melanoma metastases.

Our results showed that (consistent with expectations), similar to the primary tumors, significant accumulation of the radio-pharmaceuticals was observed in the metastases. It was clearly established that, in line with the high MC1-R expression in B16-F10 melanoma cells, [^{68}Ga]Ga-DOTA-NAPamid and [^{61}Cu]Cu-KFTG-NAPamid showed significantly higher activity than the radio-labeled RAMEB cyclodextrins. This high receptor expression was confirmed by *in vitro* cellular and *in vivo* studies published by Cheng and colleagues in 2007, in which radionuclide-labeled α-MSH analogs demonstrated high accumulation in B16-F10 tumors.

We hypothesize that the PGE2 molecules and their receptors targeted by RAMEB are present in the metastases, just as in the primary tumors,

produced and acting in an autocrine manner, however, they are found in smaller quantities in the tumor compared to the MC1-R receptor expression. This explains the lower accumulation of [^{68}Ga]Ga-DOTAGA-RAMEB and [^{52}Mn]Mn-DOTAGA-RAMEB. Furthermore, we believe that since the amount of enzymes involved in PGE2 synthesis changes with tumor growth, and the expression of PGE2 receptors is influenced by intratumoral inflammation, hypoxia, and processes related to angiogenesis, it is possible that significant differences in RAMEB-based radiopharmaceutical accumulation could be observed in the same experimental animal with the same tumor on two consecutive days.

In conclusion, it is evident that the PGE2-specific [^{68}Ga]Ga-DOTAGA-RAMEB and [^{52}Mn]Mn-DOTAGA-RAMEB, as well as the MC1-R-specific [^{68}Ga]Ga-DOTA-NAPamid and [^{61}Cu]Cu-KFTG-NAPamid radiopharmaceuticals, are suitable for detecting B16-F10 chest metastases.

4. New scientific results of the dissertation

- We successfully developed two radiopharmaceuticals suitable for the diagnosis of malignant melanoma, namely [^{52}Mn]Mn-DOTAGA-RAMEB and [^{61}Cu]Cu-KFTG-NAPamide, along with four ^{213}Bi-labeled derivatives (DOTA-NAPamide, HOLDamide, FOLDamide, and MARSamide) intended for therapeutic applications.
- All of the synthesized radiopharmaceuticals demonstrated high binding affinity to melanoma tumors.
- We confirmed that [^{52}Mn]Mn-DOTAGA-RAMEB is effective for the diagnosis of experimental melanoma tumors.
- Our results showed that [^{61}Cu]Cu-KFTG-NAPamide provides excellent imaging contrast, significantly enhancing the evaluation of PET images and the identification of MC1-R-positive tumors, due to its rapid background clearance and renal excretion profile.
- The investigation of the ^{213}Bi-labeled amide derivatives revealed that all compounds were capable of detecting malignant melanoma tumors. Among the four derivatives, [^{213}Bi]Bi-DOTA-NAPamide exhibited the most favorable tumor-targeting properties.
- Based on these findings, we state that ^{213}Bi-labeled alpha-emitting compounds can be promising candidates for targeted radionuclide therapy of malignant melanoma, potentially contributing to the development of personalized therapeutic approaches in clinical oncology.
- The comparative analysis of PGE2-specific ([^{68}Ga]Ga-DOTAGA-RAMEB and [$_{52}$Mn]Mn-DOTAGA-RAMEB) and MC1-R-specific ([^{68}Ga]Ga-DOTA-NAPamide and [^{61}Cu]Cu-KFTG-NAPamide)

radiopharmaceuticals, conducted in a thoracic melanoma metastasis mouse model, demonstrated significant accumulation of all compounds within metastatic lesions. Moreover, [^{68}Ga]Ga-DOTA-NAPamide and [^{61}Cu]Cu-KFTG-NAPamide exhibited substantially higher tumor uptake compared to the radiolabeled RAMEB cyclodextrin derivatives.

5. Summary

During my doctoral work, our objective was to develop radiotracers with practical applicability for the PET imaging of malignant melanoma. To this end, we synthesized and characterized several radiopharmaceuticals labeled with the isotopes ^{52}Mn, ^{61}Cu, and ^{213}Bi, based on DOTAGA (^{52}Mn), DOTA (^{213}Bi), and 15aneN$_5$ (^{61}Cu) chelators. In order to achieve tumor specificity, we conjugated the radiocomplexes with RAMEB (^{52}Mn), NAPamide (^{61}Cu), and NAP-, FOLD-, HOLD- and MARSamide (^{213}Bi), respectively. The diagnostic potential of the agents was evaluated *in vivo* using a B16-F10 melanoma-bearing mouse model, and the results were compared to corresponding data of radiopharmaceuticals reported in the literature. For our studies, we used ten-week-old female C57BL/6J mice. MC1-R and PGE2-R positive tumors were established subcutaneously at the left shoulder region, while in the metastasis model, tumor cells were administered intravenously via the lateral tail vein. *In vivo* imaging was performed using a MiniPET-II scanner, while *ex vivo* studies were conducted by means of a calibrated PerkinElmer gamma counter at our department.

Based on the results of our experiments, one can concluded that, the [^{52}Mn]Mn-DOTAGA-RAMEB compound is a potential radiopharmaceutical suitable for the PET diagnosis of malignant melanoma tumors, however, its rapid elimination kinetics and high renal uptake may limit its capability in studies requiring prolonged imaging.

The results obtained with the [^{61}Cu]Cu-KFTG-NAPamide revealed that this agent shows high affinity to MC1-R-positive experimental

melanoma tumors and provides excellent image contrast and significantly facilitates PET image evaluation.

From the results of the study on ^{213}Bi-labeled, DOTA-conjugated, MC1-R-affine α-MSH analogues, we can conclude that all four radiotracers show specific binding to MC1-R in B16-F10 tumors. The highest uptake values were gained for NAP- and FOLDamide derivatives, suggesting that these molecules could play a significant role in the PET imaging of MM tumors.

Finally, our studies conducted with the PGE2-specific [^{68}Ga]Ga- and [^{52}Mn]Mn-DOTAGA-RAMEB, as well as the MC1-R-specific [^{68}Ga]Ga-DOTA-NAPamide and [^{61}Cu]Cu-KFTG-NAPamide radiotracers, aimed at identifying thoracic B16-F10 melanoma metastases, demonstrated that all four compounds exhibited significant accumulation, which was not observed in control animals. Based on previous results, it was not surprising that higher accumulation was detected for the NAPamide derivatives, which correlates with the results of sc tumor studies.

6. List of publications

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Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology

List of publications related to the dissertation

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