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(PHD)

Radiolabeling of small molecules and peptide derivatives for PET imaging

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13:00, 3 November 2021.

1. Introduction and aims of study

Melanoma malignum is one of the most aggressive skin tumors with an extremely poor prognosis in the advanced state. This negative trait is due to its increased metastatic and angiogenic propensity as well as its resistance to various treatments. Induction of angiogenesis is a critical point in the development of most human tumors, including malignant melanoma. It also occurs in many inflammatory diseases and malignancies (more than 90% of solid tumors). Despite the early detection and the subsequent surgical removal of the primary tumor, and the ongoing screening programs, the number of patients and those at advanced stages of the disease is increasing continuously. The aggressiveness of melanomas results from their ability to grow rapidly toward local capillaries and then form distant metastases in multiple organs (e.g., lung, brain, kidney, liver, and intestine), even when the size of the primary tumor is not spectacular. The presence of metastases greatly reduces life expectancy, so early detection and localization of these metastases is critical to patients' chances of survival. Medical imaging techniques can play a key role in prolonging patients' lives. Among the non-invasive imaging methods, positron emission tomography (PET) is a useful method for the detection and staging of malignancies due to its

high sensitivity and resolution. The most commonly used radiopharmaceutical for imaging primary tumors and metastases is ^{18}F-fluoro-2-deoxy-D-glucose (^{18}FDG). ^{18}FDG is a glucose analogue that accumulates in cells with high metabolic activity, such as tumors. Despite its widespread use, ^{18}FDG has certain limitations: it is not specific for malignant melanoma, in addition it is also accumulated by inflammatory lesions and some organs (for example the brain), making it difficult to distinguish between healthy and diseased tissues on PET images. These limitations of ^{18}FDG require the synthesis and characterization of specific tracers to improve the diagnosis of melanoma, which increase the efficiency of the use of PET images.

Generator-based radioisotopes are convenient alternatives to cyclotron-produce isotopes. One such PET isotope, ^{68}Ga, which has nearly ideal physical properties (89% β^+; $t_{1/2}$ = 68 min; E_{av} (β^+) = 740 keV), provides easy on-site availability due to the commercially available ^{68}Ge/^{68}Ga generators. The physical and chemical properties of ^{68}Ga allow for efficient radiolabeling when using small molecules, mainly peptides, but for antibodies and proteins - due to its short half-life - the implementation of indirect labeling can be more useful. Reliable radiolabeling methods are already available from the

literature, which have already resulted in compounds used in clinical practice and research. However, the efficient, fast, and high accurate mapping of various tumors as well as physiological processes requires the development of even more specific radiopharmaceuticals, which is a serious challenge for contemporary PET radiochemistry. This is a really delicate task, requires multidisciplinary collaboration from the fields of radiochemistry, radio- and molecular biology.

The main goal of our research is to develop radioligands with specific interactions toward the targeted tissues, which are more efficient in terms of diagnostics and include the possibility of developing theragnostic analogues:

- Production of small molecules for the detection of melanin in melanoma and radiolabeling with ^{68}Ga.
- Visualization of angiogenesis, a key physiological process in metastasis formation, through highly expressed receptors.

The set goals were achieved in two major projects:

Project 1: The melanin pigment-specific benzamide analogue, procainamide, was conjugated to macrocyclic and open-chain chelators, and the resulting compounds were radiolabeled with ^{68}Ga. The radioligands were tested *in vitro, in vivo,* and *ex vivo*

methods. To perform the biological evaluation, healthy, control mice; melanin-producing B16F10 cell lines and B16F10 tumor-bearing mice; and melanin-free Melur and A375 cell lines, as well as animal models of tumors induced using these cells were applied.

Project 2: We have developed PET radioligands for the detection of various molecular targets that are suitable for imaging angiogenesis.

a) Development and testing of ^{68}Ga-NODAGA-YEVGHRC for targeting the Aminopeptidase N (CD13) receptor.
b) Production and assays of ^{68}Ga-NODAGA-APRPG-COOH and ^{68}Ga-NODAGA-APRPG-NH_2 capable of detecting the VEGFR-1 receptor
c) A heterodimeric derivative was prepared with a subunit specific for Aminopeptidase N and the other to the VEGFR-1 receptor. The multimerization concept can be useful for improving the quality of PET imaging. This radioligand is ^{68}Ga-NOTA-NGR-APRPG.

The peptide derivatives prepared in the second project were conjugated with chelator, radiolabeled with ^{68}Ga, a quality control protocol was developed, and then tested for their imaging properties in *in vivo ex vivo* PET measurements in B16F10 melanoma tumors and healthy animals.

2. Materials and methods

2.1 Tracing of small molecules for PET imaging

2.1.1. Conjugation reaction of 4-Amino-N- (2-diethylaminoethyl) benzamide hydrochloride with NODAGA-NHS ester chelator

The first melanoma selective small molecule was prepared from 4-amino-*N*-(2-diethylaminoethyl)benzamide-hydrochloride. The substance was conjugated to a macrocyclic NODAGA-NHS-ester chelator in a 3:1 mixture of acetonitrile/sodium carbonate buffer (pH 9). The pH of the mixture was maintained during the reaction between 8.5 to 9 with 2% NaOH. The resulting NODAGA conjugated benzamide analog (NODAGA-PCA) was purified by semi-preparative RP-HPLC, and the fractions were collected and then were lyophilized.

2.1.2. Synthesis of HBED-CC-tris(tBu)tetrafluorophenyl ester followed by conjugation with 4-amino-N-(2-diethylaminoethyl)benzamide-hydrochloride

The first step for the synthesis of HBED-CC-PCA was the preparation of the active ester analogue of the chelator. For this reaction *N,N'*-bis[2-hydroxy-5-(carboxyethyl)-

benzyl]ethylenediamine-*N*,*N'*-diacetic acid tris tert-butyl ester and 2,3,5,6-tetrafluorophenol were dissolved in acetonitrile. Then, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDAC) and *N*,*N*-diisopropylethylamine (DIPEA) were added to the mixture. The solution was stirred at room temperature for 2 hours and then evaporated. The mixture was dissolved, and a normal phase column chromatography was applied to purify the product. The pure material was recovered from the stationary phase with ethyl acetate/methanol 4:1. The organic phases containing HBED-CC-tris (tBu) tetrafluorophenyl ester were combined and evaporated. The resulting product was then conjugated to 4-amino-N- (2-diethylaminoethyl) benzamide hydrochloride, and the protecting groups (-tBu) were cleaved with TFA. The NODAGA-PCA was purified by means of a semi-preparative RP-HPLC. The chemical structure of the molecule was assessed by ESI-MS and ^{1}H-NMR. For the radiolabeling reactions a stock solution of 3 mmol/dm3 was prepared using ultrapure water.

2.1.3. Radiolabeling of NODAGA-PCA and HBED-CC-PCA with ^{68}Ga

For radiolabeling of NODAGA-PCA and HBED-CC-PCA, an Obninsk-type ^{68}Ge/^{68}Ga generator containing TiO_2

stationary phase was used, which was eluted with 5 mL of 0.1 M HCl. To increase the activity concentration of ^{68}Ga and decrease the amount of the potentially existing other metal ions, a fractionated elution was used. The highest activity containing fraction (≈350 MBq) was collected (in 1 mL volume) and then buffered with 0.16 mL of ultrapure NaOAc (1 mol/dm^3), the pH of the reaction mixture was adjusted to 4.5 with 0.06 mL of 2% NaOH. Then, 5 μL of 3 mmol/dm^3 precursor (NODAGA-PCA or HBED-CC-PCA) was added to the reaction mixture, which was incubated for 5 minutes at 95°C. The reaction solution was allowed to cool to room temperature and then transferred to a preactivated OASIS HLB 1cc 30 mg packed column (the column was preconditioned with 400 μL of 96% EtOH and then it was rinsed with 2 mL of water). After immobilization of the radioactivity, the column was flashed with 2 mL of water to remove any non-radioactive metal contaminants and the free ^{68}Ga. The ^{68}Ga radiolabeled procainamide derivatives were recovered from the column with 0.5 mL of isotonic sodium chloride solution/96% ethanol 2:1. The radiolabeled product was further diluted with 2 mL of saline to reduce the alcohol content to less than 10% and then filtered through a sterile filter. In each case, the radiochemical purity (%) of the final product was checked by analytical RP-HPLC using a combination of UV and radiodetector.

2.1.4. Semi-preparative and analytical HPLC

Purification of procainamide derivatives conjugated to different chelators was performed using a KNAUR HPLC system on a semi-preparative Supelco Discovery® Bio Wide Pore C18 column (150 mm * 10 μm; particle size: 10 μm) at a flow rate of 4.4 mL/min. Peaks were detected at 254 nm using a UV detector.

KNAUER HPLC as described above was used to control the quality of radiolabeled compounds, but using a dual detection (radiodetector and UV detector parallel), and the column was replaced to an analytical one, Supelco Discovery® Bio Wide Pore C18 column (250 mm * 4.6 mm; particle size: 10 μm), and the flow rate was reduced to 1.2 mL/min.

2.1.5. Determination of the octanol/PBS partition coefficient (LogP) of ^{68}Ga-labeled small molecules

The partition coefficient (LogP) values of ^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-PCA are indicative parameters for the water solubility of the compounds at a given pH. They were determined in octanol/PBS environment for both procainamide derivatives. The radioactivity was measured with a Perkin Elmer Packard Cobra gamma counter, and the result

was obtained in cpm, from the ratio of the different layers the LogP values were calculated.

2.1.6. Determination of the in vitro stability of ^{68}Ga-labeled small molecules

The stability of ^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-PCA was tested at 37°C in rat and mouse serum at different time points, and the radiochemical purity of the compounds was measured by HPLC. The method was identical we have written during the quality control.

2.1.7. In vitro saturation binding assays

Melanin-producing cell line B16-F10 was used for *in vitro* binding assays. Cells were grown in 24-well plates for 24-hour intervals. Different concentrations (20-1600 nmol/dm3) of ^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-PCA were added to each well. After an incubation time (60 min in a CO_2 incubator at 37°C), the medium was removed, the cells were washed twice with both PBS and glycine solution, and then lysed with NaOH for 10 minutes at 37°C. The radioactivity of the samples was measured with a gamma counter.

2.1.8. Cell uptake and outflow assays

B16-F10, A375 and MELUR cells were grown as monolayer cell culture in tissue culture flasks (2 x 10^5

cells/flask) for 24 hours. Cell uptake and efflux assays were then performed.

2.1.9. The tumor models

C57BL/6J and CB17 SCID mice were used for the measurements. Adult female CB17 SCID (n = 30) and C57BL/6J (n = 20) mice were available for induction of *in vivo* tumor models. Upon induction of amelanotic tumor models, 1 * 10^5 amelanotic MELUR or A375 tumor cells were injected subcutaneously into the left shoulder region of mice in 0.9% NaCl solution (100 μL). To generate melanin-producing melanoma, C57BL/6J mice were injected with B16-F10 tumor cells (1 * 10^5 cells: in 100 μL NaCl) subcutaneously in the left shoulder area. *In vivo* and *ex vivo* experiments were performed 21 ± 2 days after subcutaneous injection of tumor cells.

2.1.10. PET/CT and PET/MRI imaging

Control and tumor-bearing animals were injected with 7.0 ± 0.2 MBq of ^{68}Ga-NODAGA-PCA or ^{18}FDG through the tail vein 20 ± 2 days after implantation of B16-F10 or Melur cells, followed by whole-body PET measurement (10 min recording time) was performed using the MiniPET-II scanner.

The PET/MRI examination of the animals was performed in the Preclinical Laboratory of the Translation

Research Center of ScanoMed Kft. in Debrecen. Control and tumor-bearing animals were injected with 10.3 ± 0.3 MBq of ^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-PCA through the lateral tail vein. Whole body PET scans (20 min static PET scan) were performed on the preclinical nanoScan PET/MRI device.

2.1.11. Ex vivo biodistribution studies

The day after *in vivo* PET/MRI imaging, control and tumor-bearing mice were injected intravenously with 10.3 ± 0.3 MBq of ^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-PCA. After the administration of ^{68}Ga-labeled procainamide derivatives, mice were anesthetized with 5% forane. Samples were taken from each organ and tissue, and they were weighed with an analytical balance. The radioactivity of the samples were measured by a gamma counter, and the DAR (Differential Absorption Rate) was calculated.

2.2. Radiolabeling of peptide derivatives for PET imaging

2.2.1. Preparation of APRPG-NH_2 and APRPG-COOH peptides by peptide synthesis

The synthesis of the APRPG-NH_2 and APRPG-COOH peptides was performed for the research Prof. Dr. Gábor Mező,

the head of the MTA-ELTE Peptide Chemistry Research Group, based on the structure we designed.

The APRPG-NH_2 peptide was prepared on Rink-Amid MBHA resin, while the APRPG-COOH peptide was synthesized on 2-ClTrt resin by SPPS method using standard Fmoc chemistry. The crude peptides were purified by RP-HPLC, and after the purification step, the identity of the products (APRPG-NH_2 and APRPG-COOH) were assessed by analytical RP-HPLC-MS (electron spray ionization-mass spectrometry).

The NGR-APRPG and YEVGHRC peptides were ordered from the Bankpeptide Biological Tech Co., LTD (Hefei, China) based on request.

2.2.2 Preparation of NODAGA-APRPG-COOH, NODAGA-APRPG-NH_2, NOTA-NGR-APRG and NODAGA-YEVGHRC peptide derivatives

To prepare the NODAGA-APRPG-COOH and NODAGA-APRPG-NH_2 peptide precursors, an amine-selective chelator p-SCN-Bn-NODAGA was applied.

Different macrocyclic chelator, the Maleimide-NODAGA moiety, was used to prepare the NODAGA-YEVGHRC peptide derivative. The covalent bond was formed

between the maleimide subunit and the cysteine side chain. During the precursor-formation from the heterodimeric compound (NOTA-NGR-APRPG), an amine-selective NOTA chelator was applied. For each compound prepared, the crude product was purified by semi-preparative RP-HPLC on a semi-preparative Supelco Discovery Bio Wide Pore C18 column. After purification, the fractions containing the products were collected in a flask and lyophilized. The compounds were characterized by ESI-MS and analytical RP-HPLC. Subsequently, a stock solution of 3 mmol/dm^3 was prepared from each compound.

2.2.3 Radiolabeling of the prepared peptide derivatives with ^{68}Ga

A GalliaPharm® ^{68}Ge/^{68}Ga generator (Eckert-Ziegler, Berlin, Germany) was used for radiolabeling. After fractional elution, 1 mL from the eluate was buffered with 0.16 mL (1 mol/dm3) of ultrapure sodium acetate to adjust the pH of the reaction mixture to 4–4.1 for each compound. Subsequently 5 μL volume from the stock solutions were introduced to the reaction mixture. The radiolabeling reaction for the NODAGA-YEVGHRC and NOTA-NGR-APRPG peptide derivatives was carried out at 95 °C for 5 minutes. During our measurements, it was found that the NODAGA-APRPG-COOH and NODAGA-

APRPG-NH_2 peptides can presumably more sensitive to radiolysis, and this can affect detrimentally the radiochemical purity. To eliminate the formation of by-products, radiolabeling was performed at room temperature for 5 minutes, and 0.1 mL of absolute EtOH was added to the reaction mixture due to its radical scavenging properties. The purification-formulation step was performed on the OASIS HLB 1 cc 30 mg column. Peptide derivatives labeled with ^{68}Ga were eluted from the column with 0.2 mL of 2:1 isotonic saline/96% ethanol. In each case, the radiochemical purity (%) of the final product was checked and determined by analytical RP-HPLC using a combination of UV and radiodetector. The octanol/PBS partition coefficient (logP) values of the radioligands as well as their *in vitro* stability were determined as described in sections *2.1.5* and *2.1.6*.

2.2.4 Biological systems and conditions

The receptor binding properties of our ligands capable of detecting angiogenesis were tested in mice bearing B16-F10 tumors. During the cell culture process, B16F10 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) medium with 10% FBS (fetal calf protein) serum supplemented with 1% (v/v) MEM (non-essential amino acid), 1% MEM vitamin, 1% antibiotic and antimycotic solutions were added.

For the *in vivo* series of experiments to test the imaging properties of our ^{68}Ga-labeled peptide derivatives, 12-week-old male C57BL/6 mice (n = 60) were purchased from Animalab Ltd. (reseller of The Jackson Laboratory). To establish the tumor, $5 * 10^6$ B16-F10 mouse-derived melanin-producing cells were injected in 150 µL saline into the left shoulder region of the mice.

2.2.5 In vivo animal testing and quantitative PET analysis

Imaging was performed on day 8 or 9 after B16-F10 tumor induction for each peptide derivative using a miniPET camera. After anesthesia of tumor-bearing (n = 40) and healthy, control (n = 20) mice, 5.5 ± 0.7 MBq ^{68}Ga-NODAGA-APRPG-COOH, ^{68}Ga-NODAGA-APRPG-NH2, ^{68}Ga-NODAGA-YEVGHRC and ^{68}Ga-NOTA-NGR-APRPG were injected through the lateral tail vein. After 90 minutes of incubation, the animals were anesthetized with 3% isoflurane (Forane) using a special inhalation device. While maintaining anesthesia, a static PET scan (t = 20 min) was performed on the MiniPET-II. using a camera. The recordings were reconstructed using BrainCad software.

2.2.6 Ex vivo biodistribution studies

Ex vivo biodistribution studies were performed as mentioned above (Chapter 2.1.11 according to *Ex vivo* biodistribution studies), but with different radioligands here ^{68}Ga-NODAGA-APRPG-COOH, ^{68}Ga-NODAGA-APRPG-NH2, ^{68}Ga-NODAGA-YEVGHRC and ^{68}Ga-NOTA-NGR-APRPG compounds were injected, and the results were expressed in ID%.

3. Results and Discussion

3.1. Interpretation of imaging properties of compounds used to detect melanoma malignum

To demonstrate the diagnostic efficacy of procainamide-type compounds, two chelator-conjugated derivatives with different structures were prepared. In our work, the 4-amino-*N*-(2-diethylaminoethyl) benzamide hydrochloride backbone was coupled to a NODAGA-NHS ester macrocyclic chelator and an acyclic HBED-CC-tris (tBu) tetrafluorophenyl ester.

Radiolabeling of NODAGA-4-amino-*N*-(2-diethylaminoethyl) benzamide was performed with ^{68}Ga (^{68}Ga-NODAGA-PCA). The complete synthesis took 15 minutes, including the final formulation step, which provided a convenient synthesis time frame for working with the short half-life radioisotope. The radiochemical purity of the radiolabeled compound exceeded 98%, with a molar activity ranging from 15 to 17 GBq/μmol. Therefore, this radiosynthesis method allowed us to robustly produce melanin-specific ^{68}Ga-NODAGA-PCA for *in vitro* and *in vivo* applications, and the parameters of the radioligands were suitable for successful preclinical studies.

It has previously been reported in the literature that a radioactive compound with a similar chemical structure, was enriched in B16-F10 cells as a function of time. Our *in vitro* measurements confirmed this observation, as we found monotonically increasing accumulation in B16-F10 cells with ^{68}Ga-NODAGA-PCA during the examined period, but this difference was not significant.

The melanin specificity of ^{68}Ga-NODAGA-PCA was demonstrated *in vitro* using melanin-positive B16-F10 and melanin-negative Melur cell lines. Accumulation of radioligand in cells was significantly higher in melanin-containing B16-F10 cells than in amelanotic Melur cells at the time points studied (30, 90 min). Therefore, our cellular experiments confirmed the melanin specificity of ^{68}Ga-NODAGA-PCA. We were interested in also the fate of the bound activity within the cell. Therefore, we have performed "efflux studies" with ^{68}Ga-NODAGA-PCA for both cell lines (B16-F10, Melur). During the efflux assay of B16-F10 and Melur cells, after 30 or 90 incubation times, the cells were incubated for another 10 min, but in a clear medium, without radiolabeled compound. We have found that the amount of bound ^{68}Ga-NODAGA-PCA decreased after the extra 10 min period. It was hypothesized that after saturating the melanin content of the cells, the excess

radiolabeled compound would be cleared from the cells. However, the uptake rates of melanin-positive B16-F10 and melanin-negative Melur cell lines did not differ significantly from the initial rates. Because that the cellular uptake of benzamide derivatives can be dependent from the water solubility logP value was also determined. During our measurement, this parameter of ^{68}Ga-NODAGA-PCA was -2.79 ± 0.10, suggesting that the radioligand is highly hydrophilic.

In vivo and *ex vivo* biodistribution studies in healthy animals have shown that ^{68}Ga-NODAGA-PCA is excreted via the urinary tract. This behavior was expected based on the logP value. The accumulation of the radiolabeled compound was low in healthy tissues and organs, and the liver produced also negligible accumulation after an incubation period of 90 min. These results correlated well with the outcome of other studies investigating radiolabeled benzamides, structurally like ^{68}Ga-NODAGA-PCA.

The melanin specificity of ^{68}Ga-NODAGA-PCA was confirmed by *in vivo* and *ex vivo* biodistribution measurements using subcutaneously growing melanotic B16-F10 (C57BL/6 mice) and amelanotic Melur tumors (SCID mice) 20 ± 2 days after tumor cell inoculation. 10 min static PET images were

taken 90 min after intravenous administration of the radioactive injection. Subcutaneously growing B16-F10 tumors became clearly visible on the miniPET images with low background activity, and after quantitative analysis of PET images, we found that the accumulation of ^{68}Ga-NODAGA-PCA in the melanin-containing B16-F10 tumor was significantly ($p \leq 0.01$) higher than in amelanotic Melur tumor. These *in vivo* results correlate with *ex vivo* biodistribution studies that confirmed the melanin specificity of ^{68}Ga-NODAGA-PCA also. Our results are consistent with measurements in the literature; benzamide derivatives labeled with PET isotopes (^{18}F, ^{68}Ga) showed relatively high accumulation in B16 tumors with excellent tumor/background values.

In our work, the efficacy of ^{68}Ga-NODAGA-PCA in lung metastases induced by B16-F10 cells was also investigated in a mouse model. The syngeneic animal model C57BL/6, which was used, is widely accepted in preclinical melanoma research. In our study, lung metastases were induced after intravenous injection of $1x10^5$ B16-F10 melanoma cells. Analysis of miniPET/CT images revealed metastatic tumor lesions in the lung, 90 min after the ^{68}Ga-NODAGA-PCA injection. Low background accumulation was experienced. After quantitative evaluation of PET images, approximately 6-

8-fold higher ^{68}Ga-NODAGA-PCA activity was observed in B16-F10 metastases than in background (lung or muscle).

Although in subcutaneously growing B16-F10 tumors (SUV scale: 0.35 ± 0.09, SUVmax: 2.03 ± 0.87) or lung metastases (SUV scale: 0.15 ± 0.04, SUVmax: 0.42 ± 0,06) found only moderate accumulation with ^{68}Ga-NODAGA-PCA, however, the low activity of other organs allowed the preparation of high-quality images with low background activity and high tumor/muscle or tumor/lung ratios.

In some previous studies, the accumulation of benzamide- and nicotinamide-based radioligands in experimental melanoma tumors was compared to ^{18}F-FDG using PET imaging. ^{18}F-FDG has often been used to diagnose, stage, and following the therapeutic effort of these types of malignancies. However, its non-specific interaction with the tumor makes the evaluation difficult. Moreover ^{18}F-FDG-PET images due to the relatively high background uptake in metabolically active healthy tissues make the diagnosis complicate. After intravenous injection of ^{18}F-FDG for the assessment of the existence of the experimental tumors, we also found high uptake values in both subcutaneously growing amelanotic Melur and melanin-positive B16-F10 tumors and in B16-F10 lung metastases. After quantitative analysis of PET

images, B16-F10 melanoma tumors showed significantly ($p \leq 0.01$) higher ^{18}F-FDG uptake than ^{68}Ga-NODAGA-PCA. Despite this outcome, when the ^{68}Ga-NODAGA-PCA uptake values of the tumors were compared with the background (muscle or lung) activity, accumulation was approximately twice or three times higher than that of ^{18}F-FDG. Due to the specific binding to melanin and lower background accumulation, ^{68}Ga-NODAGA-PCA provided better contrast than ^{18}F-FDG.

HBED-CC-4-amino-*N*-(2-diethylaminoethyl)-benzamide was also radiolabeled with ^{68}Ga. As we experienced during the previous compound the time requirement of this radiolabeling was also 15–20 min, including purification and reformulation steps. Subsequent quality control measurements confirmed that the radiochemical purity of the compound was also reliable for this ligand, better than 98%. To our surprise, the three stereoisomers of the product also separated on the non-chiral stationary phase, which is clearly visible on the radio chromatogram. The molar activity of the radiolabeled derivative ranged from 13 to 16 GBq/μmol. Similar decay-corrected yields (about 65-68%) were obtained for both melanin-specific compounds. The results obtained from the radiolabeling of ^{68}Ga-HBED-CC-PCA and the quality control

measurements provided us with a rapid and simple preparation-protocol of the radiopharmaceutical candidate for preclinical applications with excellent radiochemical purity and high molar activity. The enzymatic stability of the new radioligand was tested. After 2 hours of incubation in rat serum, the 75% of parent compound remained intact from the ^{68}Ga-HBED-CC-PCA. Interestingly, this finding does not support the observation of Eder et al. that the use of HBED-CC instead of the NODAGA chelator may increase the metabolic stability of the radioactive substance. Despite the slow metabolization ^{68}Ga-HBED-CC-PCA is still a very stable molecule, suitable for biological measurements.

To examine the melanin specificity of the ^{68}Ga-labeled various chelator-conjugated procainamide derivatives, melanin-positive (B16-F10) and melanin-negative melanoma cell lines (MELUR, A375) were used in monolayer and suspension cell cultures. Previous studies have shown that transport processes (uptake/efflux) are different when cells are used in a single layer or in suspension for *in vitro* studies. In our measurements, we also found differences in the uptake of melanin-specific radiolabeled compounds when we compared the suspension technique with the monolayer technique. Higher uptake of ^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-PCA was

found at each time point when melanoma cells were applied in a single layer. Based on these *in vitro* data, we concluded that the physiological property of monolayer cell culture for adherent melanoma cells is closer to that of a living system. When ^{68}Ga-HBED-CC-PCA uptake was examined by both *in vitro* methods, significantly ($p \leq 0.05$ and $p \leq 0.01$) higher accumulation was found in melanin-producing B16-F10 cells than in for amelanotic MELUR or A375 cells, and the uptake increased over time and persisted after 10 min of efflux phase. Despite the relatively low uptake values, these results confirmed the melanin specificity of the new compounds. Our results are consistent with the literature where it is concluded that the ^{68}Ga-labeled benzamide derivatives (^{68}Ga-SCN-NOTA-BZA and ^{68}Ga-SCN-DOTA-PCA) specifically bind to melanin in B16-F10 melanoma cells and the time dependence of the binding is also detectable. It is generally accepted that the uptake of ^{68}Ga-labeled procainamide happens by passive diffusion, so the logP values of the labeled compounds can be informative in the estimation of the rate of the process. A ^{68}Ga-NODAGA-PCA had a logP of -2.79 ± 0.10 and ^{68}Ga-HBED-CC-PCA had a logP of -2.19 ± 0.12. Compared to ^{68}Ga-NODAGA-PCA, this means that ^{68}Ga-HBED-CC-PCA has a slightly less hydrophilic character, but they are highly polar. As it was expected, ^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-

PCA are eliminated similarly, mainly via the urinary system. Furthermore, we have experienced rapid clearance from the bloodstream, as low accumulation was observed in other organs and tissues after an incubation period of 90 min. These *in vivo* and *ex vivo* results correlate well with several studies investigating the behavior and distribution of other radiolabeled (^{18}F, ^{68}Ga, ^{125}I) benzamide derivatives in the body.

The specificity of ^{68}Ga-HBED-CC-PCA was tested in a mouse model carrying subcutaneous melanoma tumor and compared with the results obtained with ^{68}Ga-NODAGA-PCA. Melanotic B16-F10 (in C57BL/6 mice) and amelanotic A375 and Melur tumors (in SCID mice) were examined 21 ± 2 days after tumor cell inoculation. 20-minute static PET/MRI images were taken 90 minutes after injection of the radiopharmaceutical. Melanin-containing B16-F10 tumors became clearly visible by PET/MRI imaging with low background activity for both compounds. In contrast, when amelanotic MELUR and A375 tumors were used for PET imaging, very low ^{68}Ga-HBED-CC-PCA and ^{68}Ga-NODAGA-PCA uptake were found, which confirmed the melanin specificity of the radiolabeled compounds. After quantitative SUV analysis of *in vivo* PET/MRI images and processing *of ex vivo* DAR data, we found that ^{68}Ga-HBED-CC-PCA and ^{68}Ga-

NODAGA-PCA uptake were significantly ($p \leq 0.01$) higher in B16-F10 tumors than in amelanotic MELUR or A375 tumors. A moderate accumulation - ^{68}Ga-HBED-CC-PCA (SUV mean: 0.13 ± 0.01, SUVmax: 0.56 ± 0.11) and ^{68}Ga-NODAGA-PCA (SUV value: 0.46 ± 0.06, SUVmax: 1.93 ± 0.25) was observed in subcutaneously growing B16-F10 tumors, however, due to the low activity of non-targeted tissues, we found that their use resulted in high contrast, high quality images. We have found that our newly prepared compounds showed a higher tumor/muscle ratio (^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-PCA had a T/M SUV of 40.7 ± 4.23 and 11.43 ± 1.24, respectively) than ^{68}Ga-SCN-DOTA-PCA (9.47 ± 2.36) a relevant example from the competitor radioligands.

In our work, we have found evidence that the uptake of radioligands bearing the procainamide moiety is melanin-dependent, however, the properties of the bifunctional chelators affect the amount of the labeled compound in melanin-positive melanoma cells and tumors. The choice of the appropriate chelating agent depends largely on the isotope used, but considerations should be taken to optimize the pharmacokinetic properties of the ligands also. However, our results demonstrated that HBED-CC is not necessarily a better chelator universally than the various elements of the NOTA family,

since our ligand had less *in vitro* stability and presumably due to a lower transport rate lower uptake than its NODAGA counterpart. Another possible issue with the application of HBED-CC that it forms three diastereoisomers upon complexation with gallium. The proportion of isomers formed depends on temperature, pH and the concentration of chelator during complex formation. It was recently reported that using the standard labeling protocol (pH 4.4, temperature 95 ° C) for ^{68}Ga-PSMA-HBED-CC, mainly the more thermodynamically favorable diastereoisomeric form was formed; however, measurable amounts of the other isomers were found in the final product. This property may be the main limiting factor prior to the widespread use of HBED-CC in clinical practice, as the potentially different biological behavior of diastereoisomers has a great importance.

3.2 Interpretation of imaging properties of compounds used to detect angiogenesis

One of the basic processes in the spatial growth of tumors is angiogenesis (new angiogenesis), which plays an important role in the metastasis formation of tumors. Several molecular targets regulate new angiogenesis, such as vascular endothelial growth factors (VEGF), integrins (e.g., αvβ3), matrix metalloproteinases (MMPs), or aminopeptidase N

(CD13). These molecular targets can be visualized by PET imaging if, for example, they are targeted with a specific peptide derivative labeled with ^{68}Ga. Thus, this method may be suitable for both the detection of tumors and the monitoring of their therapeutic response.

In our research group, we have previously prepared several compounds, carrying Asn-Gly-Arg peptide motifs, which showed specific binding to the APN/CD13 receptor. But the NGR sequence is prone to non-enzymatic deamidation. In this project we intend to find new sequences with improved chemical stability, which can serve as new lead compounds for the research of the detection of neo-angiogenesis.

In our work, we have developed four new ^{68}Ga-labeled radiopharmaceuticals and tested their efficacy to detect the expression of APN and VEGFR-1 in an experimental tumor model. We have found it important to broaden the scope of our research and consider not only the APN, as a molecular target, but also another receptor to visualize angiogenesis. Furthermore, following the multimerization concept that has been successful in ligand development, we also generated a heterodimeric peptide derivative that simultaneously targets APN and VEGFR-1 receptors - within a compound. One subunit of this analog carries the NGR peptide motif, while the

other subunit is APRPG. The YEVGHRC peptide derivative is a linear ligand targeting the APN receptor. The third and fourth compounds are APRPG-COOH and APRPG-NH_2, which are selective agents for the VEGFR-1 receptor.

Heterodimeric NGR-APRG and YEVGHRC peptides were ordered from the Bankpeptide Biological Tech Co., LTD (Hefei, China) based on the structural formulas provided by us. The NGR-APRPG heterodimeric peptide derivative was reacted with p-SCN-Bn-NOTA chelator to give NOTA-NGR-APRPG. The YEVGHRC peptide derivative underwent a conjugation step with the chelator on the thiol group of cysteine, resulting in the NODAGA-YEVGHRC ligand.

The two APRPG peptide derivatives were synthetized by Prof. Dr. Gábor Mező, from the MTA-ELTE Peptide Chemistry Research Group. The APRG-NH_2 and APRPG-COOH peptides were constructed by SPPS, the former on Rink-Amide-MBHA resin and the latter on 2-ClTrt resin. The only side chain protected amino acid was on the Fmoc-Arg-(Pbf)-OH, the Fmoc protecting groups were removed with a mixture of 2% DBU and 2% piperidine in DMF. A DIC/HOBt coupling agent was used to couple the amino acids. The unprotected crude peptides were cleaved from the resins with a mixture of 95% TFA, 2.5% water and 2.5% TIS. The linear APRPG-

COOH and APRPG-NH_2 peptides were conjugated with the *p*-SCN-Bn-NODAGA chelator via the free amino group on the alanine subunit at our institute to give NODAGA-APRPG-COOH and NODAGA-APRPG-NH_2. The prepared compounds (NOTA-NGR-APRPG, NODAGA-YEVGHRC, NODAGA-APRPG-COOH, NODAGA-APRPG-NH_2) were successfully labeled with ^{68}Ga, in most cases with a radiochemical purity of more than 95%, except for ^{68}Ga-NODAGA-APRPG-NH_2, where the radiochemical purity reached 90%. The specific activity of the compounds ranged from 14.68 to 19.47 GBq/μmol.

The partition coefficients of the four new peptide derivatives were determined, and the results show that, although to a different extent, our compounds have strong hydrophilic properties. The following logP values were obtained from the assays: ^{68}Ga-NOTA-NGR-APRPG -3.981; ^{68}Ga-NODAGA-APRPG-NH_2 -3.024; ^{68}Ga-NODAGA-APRPG-COOH became -2,370 and ^{68}Ga-NODAGA-YEVGHRC became -4.421. The stability of radioligands in rat and mouse serum was tested after incubation for 2 hours at 37°C. After the end of the measurement, more than 80% of the starting compounds remained intact. Based on these, ^{68}Ga-NOTA-NGR-APRPG, ^{68}Ga-NODAGA-APRPG-NH_2, ^{68}Ga-NODAGA-APRPG-

COOH and ^{68}Ga-NODAGA-YEVGHRC proved to be suitable compounds for the biological assays.

Biological testing of the compounds in *in vivo* and *ex vivo* biodistribution studies in control animals revealed a high accumulation in the kidneys of ^{68}Ga-NOTA-NGR-APRPG, ^{68}Ga-NODAGA-APRPG-NH_2 ^{68}Ga-NODAGA-APRPG-COOH and ^{68}Ga-NODAGA-YEVGHRC radioligands. As there was a significant difference in the logP values of the analogs, we also expected different distribution-patterns of the compounds inside the body. The partition coefficient (logP: -2.37) of ^{68}Ga-NODAGA-APRPG-COOH suggests a less hydrophilic character, which was also supported by *ex vivo* biodistribution studies, as significantly ($p \leq 0.05$ and $p \leq 0.01$) higher ID%/g in blood, spleen, stomach, lungs, heart, and fat than ^{68}Ga-NOTA-NGR-APRPG, ^{68}Ga-NODAGA-APRPG-NH_2 and ^{68}Ga-NODAGA-YEVGHRC compounds. For the radioligand ^{68}Ga-NODAGA-APRPG-COOH, the increased activity of the non-targeted organs and tissues allowed for lower quality imaging due to the higher background (present in the circulation for a longer time. The ID%/g values of ^{68}Ga-NOTA-NGR-APRPG and ^{68}Ga-NODAGA-YEVGHRC were significantly higher in the kidney (ID%/g of ^{68}Ga-NOTA-NGR-

APRPG value of 3.048, while that of ^{68}Ga-NODAGA-YEVGHRC was 5.785) compared to other organs and tissues.

Further biological studies were performed with the compounds in tumor-bearing mice. A subcutaneously induced mouse tumor model from B16-F10 cells was tested for APN/CD13 and VEGFR-1 binding. Previous studies have shown that B16-F10 tumor cells show APN/CD13 and VEGFR-1 positivity, and this evidence was demonstrated by molecular biological methods and PET imaging. *In vivo* PET imaging affirmed the positions of the subcutaneous B16-F10 tumors in C57BL/6 mice in each case with the ^{68}Ga-labeled compounds, so these radioligands were able to detect new, increased angiogenesis near to the tumor-environment, but there were significant differences. The ^{68}Ga-NOTA-NGR-APRPG heterodimeric compound had the best imaging properties based on the *in vivo* and *ex vivo* measurements we performed. The heterodimeric compound showed a significantly higher SUV mean (0.193 ± 0.05) under the conditions used in the study, compared to the other three compounds. This outstanding result is presumably due to the ability of the compound to bind to two different molecular targets simultaneously with its two subunits, making the receptor-ligand complex more stable for detecting angiogenesis. Further optimization of the multimeric

ligand is necessary, but we successfully induced an improvement in the quality of PET imaging using the multimerization process. For the other compounds, the following uptake values were obtained in B16-F10 tumors, which were significantly lower ($p \leq 0.01$) compared to the heterodimeric compound: ^{68}Ga-NODAGA-YEVGHRC (SUVmean: 0.03 ± 0.01); ^{68}Ga-NODAGA-APRPG-NH2 (SUVmean: 0.04 ± 0.003); ^{68}Ga-NODAGA-APRPG-COOH had a SUVmean of 0.097 ± 0.01. Previous studies have shown that YEVGHRC peptide-functionalized liposomes effectively target APN/CD13 positive tumors. In our study, the compound containing radiolabeled YEVGHRC did not show adequate targeting properties. The *in vivo* and *ex vivo* results correlated well with each other, showing that ^{68}Ga-NOTA-NGR-APRPG and ^{68}Ga-NODAGA-APRPG-COOH were found to have significant uptake values compared to ID%/g: 1.33 ± 0.31 the latter ID%/g: 1.35 ± 0.12. Significantly lower uptake was observed for the other two radioligands: ^{68}Ga-NODAGA-YEVGHRC (ID%/g: 0.13 ± 0.06), ^{68}Ga-NODAGA-APRPG-NH_2 (SUVmean: (ID%/g: 0, 23 ± 0.05).

In the present work, we investigated the efficacy of four, new PET radioligands not yet used by other research groups to detect angiogenesis. Although NGR-APRPG,

APRPG-COOH and APRPG-NH_2 and YEVGHRC are not yet optimized vectors, a greater or lesser accumulation of radioligands was found in the animal model B16-F10 tumor, and even the heterodimeric compound (^{68}Ga-NOTA-NGR-APRPG) specific binding was observed. It is believed that these compounds may be promising candidates for further development to optimize the pharmacokinetic properties of the derivatives.

4. Summary

^{68}Ga is a generator isotope that provides simple, reliable, fast, and easy access to the development of PET radiopharmaceuticals. In our work, we focused on the preparation, characterization and preclinical testing of small molecules and peptide derivatives which can accumulate based on specific interactions of certain tumors. We hope that our results will contribute to the most effective detection of malignant melanoma and angiogenesis.

In our first project, we have prepared two new compounds (^{68}Ga-NODAGA-PCA and ^{68}Ga-HBED-CC-PCA) for the detection of melanin-positive melanoma, and after their successful radiolabeling, we have used these radioligands on a wide range of *in vitro*, *in vivo* and *ex vivo* preclinical biological studies. Based on these experiments, it has become clear that the newly produced radiolabeled compounds bind specifically to melanin in melanoma malignum in both cell and animal models, their excess is excreted through the urinary system, and they do not bind to non-melanin-producing melanoma cell lines and

tumors. We have also collected evidence, that the properties of the applied chelating agents can dramatically affect the radioligand uptake in melanin-containing cell lines and tumors. This observation is presumably the result of the different transport rates.

In another project, we have targeted receptors that are overexpressed during the angiogenesis with various ^{68}Ga-labeled compounds. ^{68}Ga-NODAGA-YEVGHRC was intended to detect Aminopeptidase N; ^{68}Ga-NODAGA-APRPG-COOH and ^{68}Ga-NODAGA-APRPG-NH_2 were used for visualization of VEGFR-1, while the ^{68}Ga-NOTA-NGR-APRPG heterodimer was used to further improve imaging parameters cell lines expressing both proteins. We successfully prepared, radiolabeled, and characterized the compounds listed above in a control and B16-F10 tumor-bearing animal model, and examined their PET imaging properties. Each derivative was suitable for tumor imaging, however, the imaging properties of the heterodimeric compound were outstanding.

Our novel radioligands were promising in both phases of the research and proven to be effective in preclinical PET imaging. We have successfully presented proof of concept studies with the new substances and the further application of these compounds are possible, but the realization of the diagnostic value of these radioligand requires further optimization.

5. New scientific results of the dissertation

• We successfully prepared two procainamide-based derivatives, and they were efficiently radiolabeled with ^{68}Ga. The radioligands were characterized in biological systems (^{68}Ga-NODAGA-PCA and ^{68}Ga-HBEDD-CC PCA).

• *In vitro*, we have found that the rate of entry into the cells and the clearance from them differs in the case of the two derivatives, which may be due to the use of two different chelators.

• Melanin specificity of radioligands was demonstrated *in vivo* and *ex vivo* in control and tumor animal models.

• We have demonstrated that radioligands may be suitable for melanin-positive melanoma PET imaging.

• We have prepared four peptide derivatives containing macrocyclic chelators with different sequences for the detection of angiogenesis, which were efficiently radiolabeled with ^{68}Ga.

• Receptor specificity of radiolabeled peptide derivatives *in vivo* and *ex vivo* was assessed in control and tumor-bearing animal models. The molecular targets of the compounds were different.

• We have found that the applied radiolabeled peptide sequences may be suitable for the detection of angiogenesis, and especially after optimization they can be good candidates to improve efficacy of angiogenesis-detection with PET.

6. List of publications

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Registry number: DEENK/418/2021.PL
Subject: PhD Publication List

Candidate: Noémi Dénes
Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology
MTMT ID: 10057296

List of publications related to the dissertation

1. **Dénes, N.**, Kis, A., Péli-Szabó, J., Jószai, I., Hajdu, I., Arató, V. Z., Enyedi, K. N., Mező, G., Hunyadi, J., Trencsényi, G., Kertész, I.: In vivo preclinical assessment of novel 68Ga-labelled peptides for imaging of tumor associated angiogenesis using positron emission tomography imaging.
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2. Trencsényi, G., **Dénes, N.**, Nagy, G., Kis, A., Vida, A., Farkas, F., Péli-Szabó, J., Kovács, T., Berényi, E., Garai, I., Bai, P., Hunyadi, J., Kertész, I.: Comparative preclinical evaluation of 68Ga-NODAGA and 68Ga-HBED-CC conjugated procainamide in melanoma imaging.
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DOI: http://dx.doi.org/10.1016/j.ejps.2017.06.026
IF: 3.466

Total IF of journals (all publications): 23,756
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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