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In vivo study of the angiogenesis of metastases with peptide-based PET radiotracers

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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, 9 April 2024.

1 Introduction

Malignancies and metastases from tumours are a growing health problem worldwide. The US National Cancer Institute (IARC) estimates that there were 17 million new cancer cases and 9.5 million cancer deaths worldwide in 2018. As the risk of the disease increases with age, oncology care is facing an increasing challenge, given the demographic trends of recent years in Western societies (population growth, ageing). This, together with the increased demand for personalised therapies, is also raising expectations for diagnostics.

An important requirement for the progression of tumours is that the primary tumour must have access to an adequate supply of nutrients from existing blood vessels or through the formation of new blood vessels. This process - angiogenesis - creates the possibility for some of the cells of the malignant lesion to enter the blood vessel, thereby increasing the chance of so-called haematogenous metastasis. Both preclinical and clinical studies in recent years have increased our knowledge of the multistep process of metastasis, but it remains an open question whether metastases metastasise or whether they all arise from the primary lesion. A precise knowledge of the molecular expression pattern of metastases would not only provide an accurate detection of the lesion, but could also lead to a rethinking of existing therapeutic schemes.

As angiogenesis is considered the cornerstone of tumour progression and metastatic ability, non-invasive molecular imaging techniques targeting neoangiogenic mediators/molecules expressed on the cell surface of metastases and primary tumour lesions may provide valuable diagnostic tools for the timely assessment of tumour lesions and the introduction of treatments to inhibit or slow tumour progression.

During angiogenesis, the interaction between VEGF and VEGFR initiates endothelial cell proliferation and migration and capillary remodelling. Therefore, the overexpression of VEGF and its receptors (VEGFR) is associated with tumour progression, metastatic potential, microvascular density and worsening of patient prognosis. As αvβ3 integrin also promotes tumour and endothelial cell migration, its overexpression may play a critical role in the regulation of tumour growth, invasiveness and metastasis. In addition, APN/CD13 also has a close association with tumour-associated neoangiogenesis through the generation and modulation of angiogenic signals and as a marker of angiogenic blood vessels. Prostaglandin E2, on the other hand, plays a crucial role in tumorigenesis through the cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) pathway, by stimulating angiogenesis and lymphangiogenesis not only in inflammation but also during tumour progression.

With advances in molecular biology, more and more potential tumour markers are being discovered, and the number of new tumour-specific diagnostic molecules is growing accordingly. PET imaging, in particular in combination with CT or MR tools, appears to be a valuable tool for the detection of specific molecular biomarkers of neoangiogenesis, opening a new field towards a deeper understanding of the cell surface molecular pattern of primary tumours and their metastases.

In this respect, integrin $\alpha_v\beta_3$ is a growing area of investigation not only in preclinical studies but also in human clinical research. Due to the elevated expression of $\alpha_v\beta_3$ integrins typically in tumour tissues, integrin-specific RGD (Arg-Gly-Asp) sequence-containing radiolabeled peptides play a crucial role in molecular oncological imaging. In turn, NGR (Asn-Gly-Arg) peptide derivatives bind with high affinity to APN/CD13, and therefore delivery of these molecules into cancer lesions may not only enable imaging but also inhibit angiogenesis, as restriction of APN function may impede tumour vascularisation. Cyclodextrins have recently come to the forefront of nuclear medicine as potential tumour targeting molecules. These glucose-based cyclic oligosaccharides with a hydrophilic outer surface and lipophilic inner cavity have been identified as promising tools for the preparation of endocytosis-based drug delivery systems and the development of water-soluble drugs. In view of the favourable chemical properties mentioned above, radiolabelled cyclodextrins may open up a new field of *in vivo* molecular imaging of malignant tumours. Among the cyclodextrin derivatives, randomly methylated β-cyclodextrin (RAMEB) has been shown to have a high affinity for complex formation with PGE2, and hydroxypropyl beta-cyclodextrin (HPβCD) has already received considerable clinical attention in therapeutic avenues due to its efficacy, satisfactory safety profile and measurable tolerability. An additional important aspect is that the outstanding *in vivo* biodistribution and pharmacokinetics of [^{68}Ga]Ga-NODAGA-HPβCD, as well as its high radiochemical purity, may provide the potential for further pharmacokinetic measurements and may also be relevant for tumour-specific molecular imaging.

Our aims are:

- Changes in the expression of molecules involved in neoangiogenesis in primary tumours and their metastases can significantly affect the efficacy of therapies. The aim of our study was to evaluate the changes in aminopeptidase N (APN/CD13) and $\alpha_v\beta_3$ integrin receptor expression in serially transplanted mesoblastic nephroma tumour (Ne/De) metastases using ^{68}Ga-labeled NOTA-cNGR and NODAGA-RGD radiotracers with preclinical positron emission tomography (PET) imaging.

- The detailed previously published data seen above have prompted us to investigate the diagnostic value of cyclodextrin derivatives in molecular imaging of various tumours, which may bring us closer to the ultimate goal of molecular target-based antitumor therapy. Therefore, we aim to evaluate the tumour-targeting potential of [^{68}Ga]Ga-NODAGA-RAMEB and [^{68}Ga]Ga-NODAGA-HPβCD using non-invasive *in vivo* PET imaging.

2 Material and methods

2.1 Cell culturing

2.1.1 Ne/De, He/De cells:

The mesoblastic nephroma (Ne/De) tumour and the hepatocellular carcinoma (He/De) tumour used in the studies were generated in previous research at the University of Debrecen as follows. A few days after birth, Fischer 344 (F344) rats were injected intraperitoneally with 125 μg of N-nitrosodimethylamine in saline solution. After about 6 months, the tumours that had formed were removed and a cell line was established.

Dulbecco's Modified Eagle Medium (DMEM) containing 10 (v/v) % fetal bovine serum was used to culture Ne/De cells, while Iscove's Modified Dulbecco's Medium (IMDM) containing 10 (v/v) % fetal bovine serum (FBS) supplemented with 1 % antimicrobial-antibiotic solution was used to culture He/De cells. The monolayer cell cultures were maintained in a T-75 cell culture flask containing 12 ml of culture medium in an incubator (ESCO CCl-170B-8 incubator) with 5% CO_2 and 95% humidity at 37 °C. Cultures were seeded 3 times a week.

2.1.2 HT1080, B16F10, BxPC3, A20, PancTu cells

HT1080 (human fibrosarcoma) cell line was purchased from ATCC (Virginia, USA). For cell culture, Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS) supplemented with 1% antimicrobial-antibiotic solution was used.

The B16F10 (mouse melanotic melanoma) cell line was purchased from ATCC (Virginia, USA). For cell culture, Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) was used, supplemented with 1% antimicrobial antibiotic solution, MEM non-essential amino acid solution (1 (v/v) %; Gibco™) and MEM vitamin solution (1 (v/v) %; Gibco™).

The BxPC3 (human pancreatic adenocarcinoma), A20 (mouse B-cell lymphoma) and PancTu (human pancreatic adenocarcinoma) cell lines were purchased from ATCC (Virginia, USA).

Roswell Park Memorial Institute (RPMI) 1640 Medium medium containing 10% fetal bovine serum (FBS) supplemented with 1% antimicrobial-antibiotic solution was used for cell culture.

Single layer cell cultures and suspension cell culture (A20) were maintained in a T-75 cell culture flask containing 12 ml of culture medium in an incubator (ESCO CCl-170B-8 incubator) with 5% CO_2 and 95% humidity at 37°C. Cultures were seeded 3 times a week. Tumor cell engraftment was performed after five to eight seeded in each case. In all cases, cell viability was checked by trypan blue exclusion assay prior to tumour induction.

2.2 Experimental animals

Female F344 rats (n=30), weighing 250±20 g, 16 weeks old, were used to study Ne/De tumours and their metastases and He/De tumours.

C57BL6 mice were purchased to study melanoma of B16F10 mouse origin.

Animals were kept in a conventional animal house under controlled temperature (24°C±2°C) and humidity (51±10%). Artificial lighting was provided in automatically controlled 12-h circadian cycles. The mice were fed ad libitum with semi-synthetic feed (Animalab Kft., Budapest, Hungary) and given tap water.

For the human tumour study, CB17 SCID immunodeficient mice (12-week-old male mice purchased from Innovo Ltd, Hungary; n=35) were used. Animals were maintained under sterile conditions in an IVC cage system (Techniplast, Italy) at 26±3 °C, 52±10% humidity, under artificial lighting in 12-hour circadian cycles. Sterile drinking water and semi-synthetic feed (Akronom Ltd., Budapest, Hungary) were provided ad libitum to all animals.

Laboratory animals were kept and treated in compliance with all applicable sections of the Hungarian Laws and regulations of the European Union (ethical permission numbers: III/6-KÁT/2015; 16/2020/DEMÁB).

2.3 Subrenal Capsule Assay (SRCA) surgery.

The rats were anaesthetized for the intervention using an inhalation pet anaesthesia apparatus (Eickemeyer Research, Tec3, Ghislandi Ltd., Hungary). For anaesthesia, 3% Aerrane inhalation vapour and 0.4 liter/min of oxygen and 1.2 liter/min of nitrous oxide were used as carrier gas, and the amount of Aerrane vapour was reduced to 1.5% to maintain anaesthesia. The lumbar region under the ribs on the left side of the animals was shaved, disinfected and the skin was incised with surgical scissors using forceps. The subcutaneous muscle layer was then cut away to reach the left kidney region. An isolation drape was applied to the affected area and the left kidney of the animal was carefully moved outside the body. The kidney thus accessed was kept

moist using physiological saline. A small incision was made in the capsular renalis using Iris scissors and the prepared Gelaspon disc was implanted through this incision under the kidney of the experimental animals. During preparation, $1x10^6$ Ne/De cells were placed on the Gelaspon disc suspended in 10 µl of physiological saline. After implantation, the kidney was reinserted into the body, the muscle layer was sutured and the skin layer was held together with wound clips. Animals were monitored until awakening and then given a suspension of non-steroidal anti-inflammatory ibuprofen (Nurofen syrup 10mg/kg) for pain relief.

2.4 Parathymic lymph node transplantation

Animals bearing Ne/De tumours under the kidneys were exterminated 14 days after implantation of Ne/De cells using 5% isoflurane. Subsequently, the thorax of the rats was opened, the metastatic cervical lymph node was removed and cut into small pieces. These pieces were implanted under the kidneys of new animals using SRCA surgery in the same way as before.

2.5 Subcutaneous induction of tumours

Mice were anaesthetised for the intervention by inhalation anaesthesia. For anaesthesia, 3% Aerrane inhalation vapour and 0.4 L/min of oxygen and 1.2 L/min of nitrous oxide were used as carrier gas, and the amount of Forane vapour was reduced to 1.5% to maintain anaesthesia. The area above the left shoulder blade of the animals was shaved, disinfected, and then with the skin lifted using forceps, 100-120 µl of physiological saline cell suspension containing $5x10^6$ cells was injected directly under the skin using a syringe.

2.6 Used radipharmacuticals

2.6.1 2-[^{18}F]FDG

The production of 2-[^{18}F]FDG is routinely performed at the Institute of Nuclear Medicine, primarily for the diagnostic testing of oncology patients. The required $^{18}F^-$ ion for production is generated in a cyclotron using ^{18}O-enriched water. The resulting $^{18}F^-$ ion is transferred to an automated FDG production panel where the precursor (trifluoromethanesulphonyl-β-D-mannose (TATM) dissolved in anhydrous acetonitrile) is labelled by nucleophilic substitution. The reaction of [^{18} F]fluoride with TATM takes place at 85ºC. The product of the nucleophilic substitution is 1,3,4,6(TA-[^{18}F]FDG). The molecule still contains protective acetyl groups, which are removed by hydrolysis in an acid medium using hydrochloric acid. Hydrolysis is carried out at elevated temperature (120ºC) and under pressure (closed reaction vessel). The end product of the reaction is 2-[^{18}F]fluoro-β-D-deoxyglucose, which is diluted with

physiological saline to the desired concentration. In the diagnostic assays, 2-[^{18}F]FDG is involved indirectly in the glucose metabolism of the body and helps to visualise angiogenic processes.

2.6.2 [^{68}Ga]Ga-NOTA-cNGR

The c[KNGRE]-NH_2 peptide was prepared and provided by the Peptide Chemistry Research Group of the Hungarian Academy of Sciences (MTA ELTE). Conjugation of the peptide with a NOTA chelator was performed in our institute. Of the c[KNGRE]-NH_2 peptide, 11.7 mg (20 μmol) was dissolved in 0.9 mL 0.1 M $NaHCO_3$ buffer (pH 9.5) and 12.3 mg (22 μmol) of p-SCN-Bn-NOTA (Macrocycles Inc., Dallas, TX, USA) was added dissolved in 0.1 mL DMSO. .After stirring for 2 h at room temperature, the NOTA-conjugated NGR analogue (NOTA-c(NGR)) was obtained by semi-preparative HPLC purification. The resulting peptide chelator was used as a precursor in the labeling reaction. ^{68}Ga for labeling was obtained by fractional elution of ^{68}Ge/^{68}Ga generator with 0.1M HCl solution. The fraction with the highest activity was then buffered (with sodium acetate solution) to adjust the pH to ~4.1. NOTA-c(NGR) solution (5 μL 3 mM) was added to this solution and the mixture was kept at 95 °C for 5 min. The reaction mixture was then transferred to an Oasis HLB 30 mg loading volume extraction column, which had been previously activated (5 mL of 96% ethanol followed by 10 mL of water). The column was washed with 5 mL of water and the radiolabelled derivative was eluted with 0.5 mL of a 1:1 solution of 96% EtOH and isotonic saline. The final product was filtered through a sterile filter and further diluted with isotonic saline to reduce the ethanol concentration below 10 % before use. The radiochemical purity of the labeled peptide was checked by HPLC.

2.6.3 [^{68}Ga]Ga -NODAGA-RGD

For labelling, we used a NODAGA-[c$(RGD)_2$] peptide chelator conjugate, purchased from ABX GmbH (Radeberg, Germany). ^{68}Ga was obtained by fractional elution of the ^{68}Ge/^{68}Ga generator with 0.1M HCl solution. The fraction containing most of the radioactive material was buffered with NaOAc solution (160 μL, 1M ultra pure water) and 5 μL of 3 mM NODAGA-[c$(RGD)_2$] solution was added. The resulting mixture was incubated for 5 min at 95 °C (pH = 4.0). From the reaction mixture, the radiolabeled derivative was bound on the surface of an Oasis HLB 30 mg loading volume extraction column, then washed with 1 mL of water. The [^{68}Ga]Ga-NODAGA-[c$(RGD)_2$]bound on the column surface was eluted with a 1:1 mixture of 200 μL EtOH/0.9 % NaCl aqueous solution, filtered to sterile and further diluted with

isotonic saline to reduce the ethanol concentration below 10 % before use. The radiochemical purity of the labeled peptide was checked by HPLC.

2.6.4 [^{68}Ga]Ga-NODAGA-HPBCD

The NODAGA-HPBCD compound used as a precursor was prepared by conjugation of 6-deoxy-6-monoamino-(2-hydroxypropyl)-β-cyclodextrin (NH_2-HPBCD) produced by Cyclolab Kft. (Budapest, Hungary) and p-NCS benzyl-NODA-GA (NODAGA) in our institute. For the labeling of NODAGA-HPBCD with ^{68}Ga, 5 mL of 0.1 M ultra-pure HCl solution was eluted from the ^{68}Ge/^{68}Ga generator, followed by buffering 1.2 mL of the fraction containing most of the active species with sodium acetate solution (1M, pH=4, 170 µl) and adjusting the pH of the mixture to ~4.2 using NaOH (2%, 59 µl). This was followed by the addition of 20 µl of precursor stock solution (1 mM) to the buffered mixture and the reaction mixture was kept at 95 °C. After 15 min, the solution was transferred to a Light C18 Sep-Pak column and the column was washed with 2 ml water to remove the buffer. The resulting [^{68}Ga]Ga-NODAGA-HPBCD was eluted from the column using 0,5 ml of a 1:2 mixture of 96 % EtOH and isotonic saline. For biological use, the eluted final product solution was diluted with isotonic salt solution to bring the ethanol content below 10 % and filtered to sterile. The radiochemical purity of the product was checked by HPLC.

2.6.5 [^{68}Ga]Ga-NODAGA-RAMEB

The NODAGA-RAMEB compound used as a precursor was prepared by conjugation of 6-monodeoxy-6-monoamino-randomly-methylated-beta-cyclodextrin hydrochloride (NH_2-RAMEB) produced by Cyclolab Kft. (Budapest, Hungary) and p-NCS benzyl-NODA-GA (NODAGA) in our institute. The ^{68}Ga used for labelling was obtained by fractional elution of the ^{68}Ge/^{68}Ga generator with 0.1 M HCl solution, then the highest activity fraction was selected from the fractions and 1 ml of this was buffered with sodium acetate solution (1 M, 160 µl) to pH 4.3-4.5. Then NODAGA-RAMEB aqueous solution (10 µl, 1 mM) was added to the buffered fraction and the reaction mixture was incubated for 10 min at 95 °C. After the reaction time, the product was fixed on a Light C18 Sep-Pak column and the column surface was washed with 2 ml water to remove the buffer solution. The pure final product, [^{68}Ga]Ga-NODAGA-RAMEB, was eluted from the column using 0.5 ml of a 1:2 mixture of 96% EtOH and isotonic saline. For biological use, the eluted final product solution was diluted with isotonic salt solution to bring the ethanol content below 10 % and filtered to sterile. The radiochemical purity of the product was checked by HPLC.

2.7 *In vivo* studies

In vivo biodistribution studies were performed 10±2 days after subcutaneous injection of tumour cells at a tumour volume of 95±8 mm^3 and 8±2 days after SRCA implantation. Tumour-bearing animals were anaesthetized with isoflurane (Aerrane) using a small animal inhalation anaesthesia device. The anesthetized animals were injected with approximately 8-10 MBq of activity of the pharmacuticals (2-[^{18}F]FDG or [^{68}Ga]Ga-NODAGA-RAMEB or [^{68}Ga]Ga-NODAGA-HPβCD, [^{68}Ga]Ga-NODAGA-[c(RGD)] or [^{68}Ga]Ga-NOTA-c(NGR)) required to perform the mapping via the lateral tail vein, was injected in 100 - 150 ul physiological saline. Following transplantation of tumor cells or tumor lymph node fragments into the kidneys, we monitored the growth of primary tumours in experimental animals from day 8 using 2-[^{18}F]FDG. After an incubation period of 50 min, during which the animal was awake and at rest in its own cage, and after injection of 2-[^{18}F]FDG into the farmacutical, static collection (scan time: 20 min) was performed from the thoracic region and the tumor-affected kidney tissue using the MiniPET-II camera.

When the primary tumor and the parathymic lymph node were visually well detectable on PET images using 2-[^{18}F]FDG, PET studies using [^{68}Ga]Ga-NODAGA-[c(RGD)], [^{68}Ga]Ga-NOTA-c(NGR) and [^{68}Ga]Ga-NODAGA-RAMEB and [^{68}Ga]Ga-NODAGA-HPβCD were performed. For the ^{68}Ga-labeled pharmacuticals, an incubation period of 90 min from injection was followed by 20-20 min of static scan from the cervical and renal regions of the animals.

For subcutaneous growing tumours, PET studies were performed using 2-[^{18}F]FDG, [^{68}Ga]Ga-NODAGA-RAMEB and [^{68}Ga]Ga-NODAGA-HPβCD pharmacuticals.

2.8 MiniPET-II and data processing

In our work, both mice and rats were examined using a small animal PET (MiniPET-II). The MiniPET-II camera is an institute-developed device containing 12 detectors in a ring with a total diameter of 211 mm. The camera has an axial field of view of 48 mm and a trans-axial field of view of 100 mm. The examination bed of the MiniPET-II camera is equipped with a motor allowing continuous movement in four directions, thus ensuring accurate and precise positioning.

The detectors are equipped with LYSO (lutetium yttrium orthosilicate mixed with cerium) scintillation crystals and position sensitive Hamamatsu H9500 photoelectron multipliers. The crystal matrices are made of 35 x 35 crystals, each measuring 1.27 x 1.27 x 12 mm^3 . Each crystal is attached to the other with an adhesive with appropriate reflective properties. Four-

channel data acquisition cards are used to digitise the signals from the detectors. Individual events are collected by the data acquisition server via an optical link (Cisco Catalyst switch). The primary data is stored in the system in the so-called list mode, in order to be able to apply multiple advanced image reconstruction techniques later on. For researchers, 2D/3D FBP, ML-EM, OSEM, ART and MAP reconstruction methods are available. As a result of the reconstruction, Bq/mL and SUV scaled images are available in DICOM, MINC and NifTI-1 formats. Of these formats, the first can be used for basic image processing, while the others allow the use of software used worldwide (Matlab, SPM, FSL, etc.) and proprietary software (BrainCAD, BrainREG, BrainTrace, BrainLOC). All kinds of methods for post-processing of MiniPET images are available, including ROI analysis based on simple CT/PET or MRI/PET fusion and brain atlas-based regional tracer kinetic evaluation.

In our studies, we used the image analysis software BrainCAD to evaluate the reconstructed images. During the evaluation of the reconstructed images, the radiofrequency overaccumulations were compared using the Standardized Uptake Value (SUV), including SUVmax, SUVmean and T/M.

The SUV value characterises how the concentration of the radiopharmaceutical within the area of interest, the VOI (Volume Of Interest), is proportional to the ratio of the administered dose to the body weight of the animal.

$$\text{SUV} = \frac{\text{concentration in VOI } [\frac{\text{MBq}}{\text{mL}}]}{\text{injected dose [MBq]/weight [g]}}$$

The drawing of the VOI was based on a visual assessment using the image analysis software BrainCAD. The individual volume elements within the VOI are the voxels.

The SUVmean is the average of the SUV values of the voxels within a given VOI.

The T/M (tumour/muscle) value gives the difference between the SUV values in tumour and muscle tissues, thus providing information on the difference between the amounts of radiofrequency accumulated in the different tissues.

$$\text{T/M} = \frac{\text{tumour SUVmean}}{\text{muscle SUVmean}}$$

2.9 *Ex vivo* studies

Ex vivo studies were performed to compare the accumulation of ^{68}Ga-labelled cyclodextrin variants in tumours. Tumour-bearing animals were injected with 8-10 MBq of

[^{68}Ga]Ga-NODAGA-RAMEB or [^{68}Ga]Ga-NODAGA-HPβCD in 100-150µl physiological saline via the lateral tail vein. Ninety minutes after intravenous injection of the radiopharmaceuticals, the animals were hyperventilated with 5% isoflurane. Tumors were removed, weighed using an analytical balance, and the radioactivity of the samples was measured using a calibrated gamma counter (Perkin-Elmer Packard Cobra, Waltham, MA, USA). Using decay corrected activity values, radiotracer uptake was expressed as %ID/g tissue.

2.10 Immunohistochemical studies

After *in vivo* studies were performed, the experimental animals were over anaesthetised with 5% isoflurane, tumours were removed and tumour samples were fixed in 10% formadehyde solution. The experimental tumours were then embedded in paraffin and 4µm thick sections were made. The prepared sections were deparaffinized, rehydrated and used after anitgenic recovery (pH 6.0) as usual in routine. Samples were labeled with a rabbit monoclonal anti-prostaglandinE receptor (EP2/PTGER2) antibody (Abcam, USA; cat. no. ab167171) applied at a dilution of 1:1000. HRP-labelled anti-rabbit polymeric antibody (Mach2, BioCare Medical, USA) and Envision DAB detection kit (DAKO-Agilent Technologies, USA) were used to visualise specific antibody binding, followed by hematoxylin counterstaining. For melanin-producing melanoma (B16F10), VIP-peroxidase (HRP) (ImmPACT® VIP Substrate, Peroxidase (HRP); Vector Laboratories, Newark, USA) was used to visualize the primary antibody. For imaging, a research microscope equipped with a DFC495 digital camera was used in combination with LAS imaging software (Leica Microsystems, BioMarker Kft., Gödöllő, Hungary).

2.11 Western blot analysis

To perform Western blot analysis, samples were taken from healthy kidney and lymph node tissue from SRCA rats, as well as from primary tumours and lymph node metastases, and stored frozen until Western blot analysis. For the assay, the tumour samples were homogenised in 1 ml PBS using a ball homogeniser, and the supernatant was extracted and processed further. For lysis of samples, RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% TritonX 100, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM Na3VO4, 1 mM PSMF, 1 mM NaF, protease inhibitor cocktail) was used. Pierce BCA reagent (Thermo Fisher Scientific, Waltham, MA, USA, 23225) was used to determine the protein content of each sample. Molecular weight separation of 10µg of protein lysate from the samples was performed on a 10% SDS polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA, USA, 26619) with a molecular weight marker, followed by blotting of protein bands onto nitrocellulose membranes (Bio-Rad,

Hercules, CA, USA, 1620097). To block the free binding sites on the membrane, the membrane was soaked in TBS-Tween buffer solution containing 5% BSA at room temperature for 1 h, and labelling was performed overnight at 4 °C with a primary antibody (Santa Cruz sc-136484, dilution 1:1000) raised against the protein of interest. The membranes were washed in TBS-Tween solution for 1 h at room temperature and labeled with peroxidase-conjugated anti-mouse secondary antibody (1:2000, Cell Signaling Technology, Inc., Beverly, MA, USA, 7074). Finally, the membranes were washed twice more for 10-10 min in TBS-Tween buffer and once more for 10 min in TBS. Antibody-labelled bands were detected by chemiluminescence reaction (SuperSignal West Pico Solutions, Thermo Fisher Scientific Inc., Rockford, IL, USA, 35060) and ChemiDoc Touch Imaging gel documentation system (BioRad, Hercules, CA, USA). Band intensities were determined using Image Lab 5.2.1 (BioRad, Hercules, CA, USA) software.

2.12 Statistical analysis

The data shown in the graphs are the results of at least three independent series of measurements, mean ± SD. Student's two-sample t-test, two-way ANOVA and Mann-Whitney rank sum test were used to determine the significance level. The significance level was set at $p<0.05$ and all statistical analyses were performed using the commercial software package (MedCalc 18.5, MedCalc Software, Mariakerke, Belgium).

3 Results

3.1 Study of metastatic tumours and their metastases in the rat renal tissue

3.1.1 Investigation of primary Ne/De tumour and metastases with PET radiopharmacons

In the initial step of the study, $1x10^6$ cells from our maintained Ne/De cell culture were seeded on Gelaspon discs under the left kidney of 16-week-old female F344 rats, and the tumorigenicity and parathymic lymph node metastasis of Ne/De cells were monitored by *in vivo* PET imaging. Primary tumor growth was assessed by 2-[^{18}F]FDG radiotracer. In addition, we used [^{68}Ga]Ga-NODAGA-RGD labeled peptide to assess the expression of $\alpha_v\beta_3$ integrin and [^{68}Ga]Ga-NOTA-cNGR radioligand to assess the expression of APN/CD13 in both primary tumors and metastases. Qualitative analysis of the acquired decay-corrected PET images showed that primary Ne/De tumors developing under kidney metastases were well evaluable

with all three compounds, although slightly lower tracer uptake was observed when using [^{68}Ga]Ga-NODAGA-RGD. Paratympanic lymph node metastases in the thorax were adequately identifiable with 2-[^{18}F]FDG and [^{68}Ga]Ga-NOTA-cNGR, whereas unfortunately these metastases were more difficult to detect with [^{68}Ga]Ga-NODAGA-RGD. Qualitative observations were confirmed by quantitative SUV data analysis of PET images. At 8±2 days after implantation of Ne/De cells, the uptake of 2-[^{18}F]FDG by primary tumors growing under the kidney was the highest (SUVmean: 7.25±2.62; SUVmax: 14.82±3.21), followed by the accumulation of [^{68}Ga]Ga-NOTA-cNGR indicative of APN/CD13 expression (SUVmean: 4.12±0.56; SUVmax: 10.72±1.85), and then the accumulation of $\alpha_v\beta_3$ integrin-specific [^{68}Ga]Ga-NODAGA-RGD (SUVmean: 2.05±0.45; SUVmax: 5.77±1.08). When analyzing radiotracer accumulation in thoracic paratesticular lymph nodes containing metastatic cells, similar, albeit more moderate uptake values were found. The uptake of 2-[^{18}F]FDG (SUVmean: 4.53±1.58; SUVmax: 13.58±2.89) was significantly higher ($p \leq 0.01$) than the accumulation of ^{68}Ga-labelled radiotracers. The amount of [^{68}Ga]Ga-NOTA-cNGR (SUVmean: 0.72±0.12; SUVmax: 1.92±0.58) in paratesticular lymph nodes was significantly ($p \leq 0.01$) higher than the activity of [^{68}Ga]Ga-NODAGA-RGD (SUVmean: 0.11±0.08; SUVmax: 0.46±0.15). The evaluability of PET images is strongly influenced by the level of difference in pharmacological accumulation between the tumour and its surroundings, the so-called tumour-to-muscle ratio (T/M ratio), which is particularly important for the assessment of a diagnostic radio-pharmacological image. When calculating the T/M ratio, we found that primary Ne/De malignancies showed significantly ($p \leq 0.05$) higher [^{68}Ga]Ga-NOTA-cNGR and [^{68}Ga]Ga-NODAGA-RGD tracer accumulation in comparison to background when compared to 2-[^{18}F]FDG (Figure 2E). However, for metastatic lymph node metastases, the use of 2-[^{18}F]FDG and [^{68}Ga]Ga-NOTA-cNGR resulted in higher contrast PET images.

3.1.2 Investigation of secondary Ne/De tumours and their metastases with radiotracers

In the second series of experiments, a piece of thoracic parathymic lymph node metastases formed by malignant tumours derived from Ne/De cells previously transplanted into the kidney of rats was transplanted into the kidney of new F344 rats. *In vivo* PET imaging was used to evaluate the tumor-forming and metastatic capacity of metastases transplanted during SRCA surgery, as well as the expression of $\alpha_v\beta_3$ integrin and APN/CD13 in the developed "secondary" tumors and their metastases 8±2 days after surgery. In this study phase, we also observed, by evaluating the acquired decay-corrected PET images, that Ne/De tumours growing under the kidney tissue were well separated from the surrounding tissue when using each radiotracer. Although [^{68}Ga]Ga-NODAGA-RGD appeared to accumulate in smaller amounts in the

tumours, primary tumours that developed from the implanted metastasis were clearly identifiable using all three pharmakons used. Also, in this part of the experiments, thoracic parathymic lymph node metastases were clearly identifiable by 2-[^{18}F]FDG and [^{68}Ga]Ga-NOTA-cNGR, whereas [^{68}Ga]Ga-NODAGA-RGD did not show a clear picture. The conclusions of the visual assessment were confirmed by quantitative SUV data analysis of PET images taken 8±2 days after metastatic lymph node transplantation. The 2-[^{18}F]FDG uptake of tumors growing below the kidney was the highest (SUVmean value: 8.56±2.58; SUVmax: 16.25±3.41). This was followed by the APN/CD13 specific [^{68}Ga]Ga-NOTA-cNGR (SUVmean: 5.23±0.89; SUVmax: 11.41±2.21) and finally the lowest accumulation was shown by [^{68}Ga]Ga-NODAGA-RGD targeting $\alpha_v\beta_3$-integrin (SUVmean: 2.85±0.52; SUVmax: 6.49±1.12). Lower SUV values were found in thoracic parathymic lymph node metastases, where 2-[^{18}F]FDG accumulation was also highest (SUVmean and SUVmax were 5.36±1.69 and 14.75±3.08, respectively). When [^{68}Ga]Ga-NOTA-cNGR was used, the uptake (SUVmean: 0.99±0.15; SUVmax: 2.09±0.49) was significantly higher ($p \leq 0.01$) than that of [^{68}Ga]Ga-NODAGA-RGD (SUVmean: 0.23±0.14; SUVmax: 0.63±0.15). Consistent with the previous series of experiments, we also demonstrated a higher tumor-to-muscle ratio using [^{68}Ga]Ga-NOTA-cNGR and [^{68}Ga]Ga-NODAGA-RGD compared to 2-[^{18}F]FDG for Ne/De tumors arising from transplanted metastases under kidney (Figure 3E). In this case, the thoracic metastatic lymph nodes were also better visualized in the PET images obtained with 2-[^{18}F]FDG and [^{68}Ga]Ga-NOTA-cNGR.

3.1.3 Investigation of tertiary Ne/De tumours and metastases with radiotracers

In the third part of our study, metastatic thoracic parathymic lymph node sections containing Ne/De cancer cells from the second series of the study were implanted under the rat kidney and the development of the growing tumour was monitored by *in vivo* PET imaging. In addition, tumour metastasis and expression of $\alpha_v\beta_3$ integrin and APN/CD13 were also investigated. *In vivo* PET imaging was also performed 8±2 days after SRCA surgery. In this series of experiments, we observed similar results as before, i.e. we were able to clearly identify the growing Ne/De tumor under the kidney by all three radiotracers used. As in the previous two experiments, the tumors growing under the kidneys showed the lowest accumulation for [^{68}Ga]Ga-NODAGA-RGD. In this part of the study, thoracic parathymic lymph node metastases have been clearly identified with all of the radiopharmaceuticals used. Renal tumor 2-[^{18}F]FDG accumulation was the highest (SUVmean: 9.63±2.66; SUVmax: 17.56±3.52), followed by APN/CD13 specific [^{68}Ga]Ga-NOTA-cNGR uptake (SUVmean: 6.35±1.09; SUVmax: 12.45±2.36), followed by $\alpha_v\beta_3$ integrin-specific [^{68}Ga]Ga-NODAGA-RGD uptake

(SUVmean: 3.35±0.63; SUVmax: 7.09±1.35). Also in this part of the experiment, lower SUV values were measured in thoracic parathymic lymph node metastases. The 2-[^{18}F]FDG uptake proved to be the highest with SUVmean and SUVmax values of 6.33±1.70 and 15.23±3.21, respectively. Comparing the uptake of [^{68}Ga]Ga-NOTA-cNGR (SUVmean: 1.56±0.20; SUVmax: 2.78±0.51) and [^{68}Ga]Ga-NODAGA-RGD (SUVmean: 0.56±0.12; SUVmax: 0.88±0.14), the former showed higher uptake. For Ne/De tumors developing from metastasis, the tumor-to-muscle ratio was significantly ($p \leq 0.05$) higher for [^{68}Ga]Ga-NOTA-cNGR and [^{68}Ga]Ga-NODAGA-RGD than for 2-[^{18}F]FDG. However, the thoracic metastatic lymph nodes were better isolated from the background using 2-[^{18}F]FDG and [^{68}Ga]Ga-NOTA-cNGR.

3.1.4 Results of autopsies

Animals were exterminated 14±1 days after implantation of tumour cells or metastatic lymph node sections. In all cases, post-overexposure necropsies showed a clearly visible large primary tumour on the left kidney and both left and right parathymic lymph node involvement were clearly identified. The mean size of the primary tumours infiltrating the renal tissue was 15-17 mm, while the removed lymph nodes were 4-6 mm. Western blot studies were performed on the removed primary tumors and excised lymph nodes, and lymph node pieces were transplanted into the kidney tissue of additional rats during SRCA surgery.

3.1.5 Quantitative PET and Western blot analysis of parathymic lymph nodes.

Given that our group was specifically interested in the changes in the expression of the APN/CD13 neo-angiogenic molecule in metastatic lymph nodes, we focused our protein level analysis - in the first instance - on this area only. The results of *in vivo* PET scans showed a steady increase in [^{68}Ga]Ga-NOTA-cNGR accumulation in thoracic metastases during serial transplantation. This is consistent with the results of Western blot analyses performed on metastatic lymph nodes. The amount of APN/CD13 protein in parathymic lymph nodes also showed a steadily increasing trend over successive transplants.

3.2 Investigation of the PGE selectivity of cyclodextrin derivatives

Tumour angiogenesis can be indirectly monitored by *in vivo* imaging. 2-[^{18}F]FDG, which can signal "angiogenesis" through increased glucose metabolism, and the PGE2-specific ^{68}Ga-labelled cyclodextrin derivatives may be suitable for this purpose. In our next study, we compared the PET imaging properties of [^{68}Ga]Ga-NODAGA-HPβCD and [^{68}Ga]Ga-NODAGA-RAMEB radiopharmaceuticals in different animal models.

3.2.1 *In vivo* PET imaging using SRCA tumour model

To evaluate the primary tumor- and metastasis-targeting properties of ^{68}Ga-labelled cyclodextrin derivatives, we used rats with subrenal transplanted Ne/De tumors and parathmic lymph node metastases 8±2 days after implantation of Ne/De tumor cells. The presence of a primary Ne/De tumor growing under the renal tumor of the left kidney and thoracic parathymic lymph node (PTLN) metastases was confirmed by 2-[^{18}F]FDG PET imaging. Following qualitative image analysis, we found that primary tumors growing under the kidneys could be well identified using both [^{68}Ga]Ga-NODAGA-HPβCD and [^{68}Ga]Ga-NODAGA-RAMEB, but metastatic parathymic lymph node metastases were only detectable with the [^{68}Ga]Ga-NODAGA-conjugated HPβCD cyclodextrin derivative. These visual observations were confirmed by quantitative evaluation of the SUV data. Accumulation of 2-[^{18}F]FDG in both tumor and metastatic lymph node was approximately twice as high as that of ^{68}Ga-labeled molecules, and this difference was significant at $p \leq 0.01$. However, no significant difference ($p \leq 0.05$) was found between the 2-[^{18}F]FDG and ^{68}Ga-labelled cyclodextrin derivatives in terms of tumour-to-background ratio, which affects the evaluability of PET images. Comparing the accumulation of the two radiolabeled cyclodextrin derivatives in primary Ne/De tumors, we found higher SUV values for [^{68}Ga]Ga-NODAGA-HPβCD (SUVmean: 3.52±0.23; SUVmax: 4.80±0.21) than for [^{68}Ga]Ga-NODAGA-RAMEB, where SUVmean was 2.51±0.19 and 3.21±0.35, respectively. This lower [^{68}Ga]Ga-NODAGA-RAMEB accumulation was also observed in metastatic paratesticular lymph nodes, where SUV values were found to be approximately 2-fold higher for [^{68}Ga]Ga-NODAGA-HPβCD. In contrast to the primary tumour, 2-[^{18}F]FDG-T/M ratios were significantly ($p \leq 0.01$) higher in paratymphatic lymph nodes compared to ^{68}Ga-labelled radiopharmaceuticals. When comparing the radiolabeled cyclodextrin derivative uptake in primary and secondary tumors, significantly ($p \leq 0.01$) lower uptake was observed in metastases. Consistent with these results, immunohistochemical staining also showed lower PGE2 receptor expression in the parathymic lymph node.

3.2.2 *In vivo* PET imaging using subcutaneous tumour models

In addition to investigating SRCA tumor models, we also examined the tumor targeting properties and PGE2 selectivity of [^{68}Ga]Ga-NODAGA-HPβCD and [^{68}Ga]Ga-NODAGA-RAMEB in subcutaneous tumors by *in vivo* PET imaging. In the experiments, a suspension containing $5x10^6$ cells was injected under the skin of the animals and imaging was performed 10±2 days after tumor cell inoculation, focusing on the formed subcutaneous growing experimental tumors. Qualitative analysis of the PET images obtained showed that all the subcutaneously grafted tumors examined were clearly identifiable with both [^{68}Ga]Ga-

NODAGA-HPβCD and [^{68}Ga]Ga-NODAGA-RAMEB. However, there were significant differences in the extent of pharmacon accumulation between tumour types. We also observed that for some tumours, a very large difference was observed between the accumulation of the two ^{68}Ga-labelled radiopharmaceuticals in the same tumour (HT1080, A20, B16-F10). The tumour targeting ability of [^{68}Ga]Ga-NODAGA-HPβCD and [^{68}Ga]Ga-NODAGA-RAMEB was compared with that of the radiopharmaceutical 2-[^{18}F]FDG. When analyzing the 2-[^{18}F]FDG images, we found that HT1080, PancTu-1 and BxPC3 tumors showed strikingly low radiopharmaceutical accumulation without 2-[^{18}F]FDG avid regions. These visual observations were confirmed by quantitative SUV data analysis of decay-corrected PET images.

3.2.3 *Ex vivo* radioactive uptake of experimental tumours

To evaluate the tumor targeting ability of [^{68}Ga]Ga-NODAGA-HPβCD and [^{68}Ga]Ga-NODAGA-RAMEB, ex vivo biodistribution studies were performed 90 min after intravenous injection of the radiopharmaceutical. The *ex vivo* %ID/g data for [^{68}Ga]Ga-NODAGA-conjugated cyclodextrin molecules correlate well with the SUV values obtained from *in vivo* imaging. Similar to the *in vivo* PET scan results, PGE2 positive BxPC3, A20, Ne/De and He/De tumors showed the highest accumulation using both ^{68}Ga-labeled cyclodextrin derivatives. For Ne/De tumors, a significant difference ($p \leq 0.01$) was observed between subcutaneous and SRCA transplanted tumors in radiopharmaceutical uptake.

3.2.4 Immunohistochemical studies

The expression of prostaglandin E receptor (EP2) in subcutaneously growing experimental tumours was investigated by immunohistochemistry. Consistent with *in vivo* and *ex vivo* radiotracer uptake results, strong EP2 receptor positivity was observed in the membranes of A20, BxPC3, B16-F10, Ne/De and He/De tumor cells, whereas lower signal intensity was detected in HT1080 and PancTu-1 tumors with low prostaglandin E2 receptor expression.

4 New results and conclusions

1. In an animal model of syngeneic metastasis created by the SRCA surgical technique, we describe that the biological behaviour of both primary tumours (sub-renal) and metastases can be described and followed by non-invasive imaging techniques using target-specific PET radiopharmaceuticals.
2. 2. We have established a novel *in vivo* experimental animal model system for oncology in which the tumorigenicity of lymph node metastases can be monitored by serial transplantation of metastatic lymph nodes.
3. In our preclinical PET studies, the expression of neo-angiogenesis-related $\alpha_v\beta_3$ integrin and APN/CD13 in Ne/De chemically induced mesoblastic nephrotumors was identifiable by [^{68}Ga]Ga-NODAGA-RGD and [^{68}Ga]Ga-NOTA-cNGR radiotracers.
4. Successive metastatic lymph node transplantations resulted in a steady increase in the accumulation of 2-[^{18}F]FDG, [^{68}Ga]Ga-NODAGA-RGD and [^{68}Ga]Ga-NOTA-cNGR in the tumors growing under the kidneys and in thoracic parathyroid metastases, as confirmed by Western blot analyses.
5. Using PET imaging, we confirmed that the increased glucose metabolism observed in Ne/De tumors and their metastases as a result of serial lymph node transplantation, as well as the increased expression of neo-angiogenesis-related $\alpha_v\beta_3$ integrin and APN/CD13 molecules, are indicative of the increasing malignancy of metastases in this experimental system.
6. *In vivo* preclinical PET imaging has demonstrated the applicability of ^{68}Ga-labelled HPβCD and RAMEB cyclodextrin derivatives in the diagnosis of PGE2 receptor-positive subcutaneous tumours.
7. We found that using [^{68}Ga]Ga-NODAGA-HPβCD and [^{68}Ga]Ga-NODAGA-RAMEB, both PGE2 positive orthotopically and heterotopically transplanted primary tumors and their developing metastases can be identified.

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6 List of publications

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List of publications related to the dissertation

1. **Péli-Szabó, J.**, Csige, K., Kálmán-Szabó, I., Arató, V. Z., Opposits, G., Jószai, I., Kertész, I., Képes, Z., Méhes, G., Fenyvesi, F., Hajdu, I., Trencsényi, G.: In vivo assessment of tumor targeting potential of 68Ga-labelled randomly methylated beta-cyclodextrin (RAMEB) and 2-hydroxypropyl-[béta]-cyclodextrin (HPβCD) using positron emission tomography.
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Total IF of journals (all publications): 128,491
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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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