

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**Application of target-specific radiopharmaceuticals for the *in vivo*
onco-nuclear imaging of experimental gynaecological tumor
models**

by Tamás Sass, MD

Supervisor: Zita Képes, MD, PhD



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By Tamás Sass, MD

Supervisor: Zita Képes, MD, PhD

Doctoral School of Medical Sciences, University of Debrecen

Head of the Defense Committee:	Prof. Andrea Szegedi, PhD, DSc, MHAS
Reviewers:	Szabolcs Molnár, PhD Zsombor Ritter, PhD
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1 Introduction

The continuous increase of the malignant tumors constitutes an exacerbating global health problem. According to the estimates of the World Health Organization of 2022-2024, in the year 2022 nearly 10 million cancer related deaths and 20 million new cancer cases were registered, and by 2050 this number will increase by 77% to 35 million cases. Among the European countries Hungary occupies an extremely unfavourable position in cancer statistics. According to the data of GLOBOCAN (Global Cancer Observatory) in 2022 in Hungary the annual incidence of malignancies per 100 000 people was approximately 340, while mortality was nearly 140. In our country among the cancer cases gynaecological tumors including breast, ovarian and cervical cancers have an outstandingly high occurrence, and they represent a significant cause of mortality. In the years 2012 and 2017 Hungary recorded the highest mortality rate caused by breast cancer in the European Union and this figure is still the highest (OECD Country Cancer Profile 2025 Hungary). Cervical cancer with 1251 new cases and 482 deaths recorded in 2023 (ICO/IARC), and ovarian cancer with 1012 newly diagnosed cases and 702 mortality represent a high risk in the national statistics (GLOBOCAN, 2022).

Considering the unfavourable data, the early and accurate detection of malignant lesions is crucial in order to start an efficient therapy as early as possible. The timely, targeted therapy does not only improve the patients' chances of survival but it also has favourable social, societal and health repercussions in the long run. One of the most frequently used imaging diagnostic methods in everyday oncology is PET - positron emission tomography - performed with radiopharmaceuticals. This non-invasive, functional imaging technique allows the accurate identification of primary abnormalities and metastases and consequently the determination of the stage of disease, which is essential in the therapy.

The development and progression of tumors is a multi-stage, complex process, in which hypoxia and angiogenesis i.e. the formation of new blood vessels play a crucial role. The formation of new blood vessels ensures the appropriate oxygen and nutrient supply of tumors therefore the neoangiogenesis is inevitable in the survival and growth of malignancies. The hypoxia developing in the tumor microenvironment activates the neoangiogenesis thus promoting the growth of the tumor and the development of metastases. Consequently, hypoxia and angiogenesis are closely related tumor biological factors, which are decisive in carcinogenesis. As a result of this, the development and *in vivo* investigation of specific tracers that are hypoxia selective and others targeting angiogenesis biomarkers represent a promising direction in increasing the efficiency of oncological diagnostics.

Over the recent years prostaglandin E2 (PGE2) - a type of angiogenic biomarker - has become a centre of isotope diagnostic research. This bioactive lipid produced by cyclooxygenase-2 (COX-2) enzyme boosts the tumor-associated angiogenesis through activating several signalling pathways and thereby stimulates the survival, proliferation and systemic dissemination of tumors.

Previous research has demonstrated that glucose-based cyclodextrin molecules such as randomly methylated beta cyclodextrin (RAMEB) bind to PGE2 with high affinity, therefore radiolabelled cyclodextrins may be extraordinary vectors for the non-invasive visualisation of tumor-associated neoangiogenesis. Furthermore, as a consequence of the close relationship between hypoxia and neoangiogenesis, the PET-based examination of reduced oxygenation in tumors is also a valuable tool in *in vivo* detection of angiogenesis. To detect tumor hypoxia 2- $[^{18}\text{F}]$ fluoro-2-deoxy-D-glucose ($[^{18}\text{F}]$ FDG) is used most frequently during oncological PET examinations, however, regarding the non specific character of this tracer the imaging with radiolabelled nitroimidazole derivatives capable of selectively detecting tissue hypoxia - such as $[^{18}\text{F}]$ FAZA (^{18}F -labelled fluoroazomycin arabinoside) - provides a more accurate representation.

By improving diagnostic accuracy, hypoxia and angiogenesis-targeted imaging promotes the correct therapy decisions, which has an unquestionable role in patients' survival. Besides, applying target-specific radiopharmaceuticals provides a deeper insight into molecular mechanisms behind carcinogenesis. All this can lead to identification of further diagnostic and therapeutical targets in the future.

Based on all the considerations above, the purpose of our research is to study the *in vivo* diagnostic capability of angiogenesis-targeting radioactive cyclodextrin derivatives - hydroxypropyl- β -cyclodextrin labelled with gallium-68 ($[^{68}\text{Ga}]$ Ga-NODAGA-HP β CD) and randomly methylated β -cyclodextrin ($[^{68}\text{Ga}]$ Ga-DOTAGA-RAMEB) - as well as hypoxia-specific radiopharmaceuticals, in different types of experimental gynaecological tumors using PET imaging.

2 Objectives

Based on the above findings, we hypothesized that radiolabelled cyclodextrin derivatives may serve as suitable molecular vectors for the *in vivo* detection of tumor-associated angiogenic processes in breast tumors. Therefore, in the first part of our research, we aimed to:

1. evaluate the diagnostic performance of ^{68}Ga -labeled RAMEB and HP β CD molecules (^{68}Ga]-Ga-NODAGA-HP β CD and ^{68}Ga]-Ga-DOTAGA-RAMEB) for the detection of angiogenesis, and
2. compare the diagnostic capability and tumor uptake characteristics of the two cyclodextrin-based radiopharmaceuticals in HER2+ MDA-MB-HER2+ and triple-negative 4T1 breast tumor preclinical models using PET imaging.

In the subsequent part of our study, we investigated whether the combined application of ^{18}F]-FAZA PET and ^{18}F]-FDG PET imaging could reduce the nonspecificity associated with the use of ^{18}F]-FDG alone in the detection of tumor hypoxia. Accordingly, our objectives were to:

3. compare the uptake patterns of ^{18}F]-FAZA and ^{18}F]-FDG tracers in severe combined immunodeficient mouse models bearing human ovarian and cervical carcinoma xenografts with different biological behaviours - multidrug-resistant (P-glycoprotein-positive/Pgp+) and multidrug-sensitive (P-glycoprotein-negative/Pgp-) tumors (ovarian: Pgp- A2780 and Pgp+ A2780AD; cervical: Pgp- KB-3-1 and Pgp+ KB-V-1), and
4. investigate the efficacy of ^{18}F]-FDG for hypoxia identification, as well as examine the effects of oxygen deprivation on glucose metabolism under *in vitro* conditions.

Our preclinical findings may contribute to a more detailed understanding of the biological behaviour of malignant lesions, which, in the long term, may provide a basis for personalized tumor diagnostics and therapeutic decision-making.

3 Materials and methods

3.1 Radiopharmaceuticals

3.1.1 Synthesis of [¹⁸F]FDG

The radiolabelled glucose molecule is synthesised on a daily basis at the Department of Nuclear Medicine and Translational Imaging, Institute of Medical Imaging, Faculty of Medicine, University of Debrecen, in order to perform the [¹⁸F]FDG PET/CT examinations of oncological patients. The ¹⁸F⁻ ion necessary for radiolabelling is created in cyclotron using ¹⁸O-enriched water. Subsequently the ¹⁸F⁻ ion is placed on automated FDG synthesis module, where the precursor (trifluoromethanesulfonyl-β-D-mannose (TATM) dissolved in anhydrous acetonitrile) undergoes radiolabelling through nucleophilic substitution. Nucleophilic substitution takes place at 85 °C producing 1,3,4,6(TA-[¹⁸F]FDG). This molecule carries protective acetyl groups, which are removed with the help of acidic hydrolysis using hydrochloric acid. Hydrolysis is performed at high temperature - 120 °C - under elevated pressure. The final product of the synthesis is 2-[¹⁸F]fluoro-2-deoxy-D-glucose ([¹⁸F]FDG), which is later diluted with physiological saline to the required concentration.

3.1.2 Synthesis of [¹⁸F]FAZA

[¹⁸F]FAZA was produced according to the method described by *Képes et al. (2024)* in our department.

The radionuclide produced in cyclotron was immobilised on a Sep-Pak Accell Plus QMA resin and then put in reaction vessel using aqueous/acetonitrile solution containing 15 mg Kryptofix 2.2.2. and 3.5 mg K₂CO₃. To remove the water, the mixture was dried at reduced pressure and elevated temperature. Later 5 mg precursor (1-(2,3-diacetyl-5-tosyl-α-D-arabinofuranosyl)-2-nitroimidazole) dissolved in 1 mL dimethyl sulfoxide (DMSO) was added. The reactor was heated to 100 °C and was maintained at this temperature for 5 minutes. After that the system was cooled to 40 °C and the reaction mixture was hydrolysed with 1 mL of 0.1 M NaOH solution for 2 minutes (1 mL, 0.1 M) then neutralised with 0.1 M acetic acid. The crude reaction mixture was then passed through a Macherey-Nagel solid-phase extraction (SPE) column series, consisting of a cation-exchange column (PS-H⁺, large), an anion-exchange column (two Chromafix PS-OH⁻, large), a neutral aluminum oxide column (Alox N, large), and a reversed-phase column (two HR-P, large). Prior to this, the column series was activated with 10 mL of EtOH and washed with 25 mL sterile endotoxin free water. Finally [¹⁸F]FAZA was

extracted with the help of 12 mL of 15% ethanol and collected to a vial containing 1.7 mL of 10% NaCl and 0.7 mL of 1 M NaH₂PO₄. The radiochemical purity was checked with a high-performance liquid chromatography (HPLC) combined with a radiodetector.

3.1.3 Synthesis of [⁶⁸Ga]Ga-NODAGA-HPβCD

The precursor compound (NODAGA-HPβCD) was created by Hajdu *et al.* (2019) by conjugation of 6-deoxy-6-monoamino-(2-hydroxypropyl)-β-cyclodextrin (NH₂-HPβCD, (Cyclolab Ltd. Budapest, Hungary)) with p-NCS-benzyl-NODA-GA (NODAGA, (CheMatech, Dijon, France)). For the ⁶⁸Ga radiolabelling of the precursor compound, the ⁶⁸Ge/⁶⁸Ga generator was eluted with ultra-pure HCl solution (5 mL, 0.1 M). Subsequently 1.2 mL of the fraction with the highest content of active ingredients was buffered with sodium acetate (1M, 170 μL, pH = 4). Using NaOH solution (2%, 59 μL) we set the pH value of the sample to 4.2. Then 20 μL precursor stock solution (1 mM) was added to the mixture. Following a 15-minute incubation at 95 °C the sample was applied to the Light C18 Sep-Pak column (Waters, Milford, Massachusetts, USA). In order to remove the buffer, the column was washed with 2 mL of water. The final product ([⁶⁸Ga]Ga-NODAGA-HPβCD) was eluted using a 1:2 mixture of EtOH (96%, 0.5 mL) and isotonic NaCl solution. The product was diluted with 0.9% NaCl solution to reduce the ethanol content below 10%. Prior to the *in vivo* experiments the product was filtered sterile. The radiochemical purity was determined with HPLC.

3.1.4 Synthesis of [⁶⁸Ga]Ga-DOTAGA-RAMEB

The ⁶⁸Ga eluate (1 mL) obtained from ⁶⁸Ge/⁶⁸Ga generator was buffered with 1M 160 μL sodium acetate to achieve the optimal pH value (pH = 4.3-4.5). To this we added the aqueous solution of DOTAGA-RAMEB (5 μL, 3 mM). The sample was incubated at 95 °C for 10 minutes and after that it was placed on the Light C18 Sep-Pak column. The final product radiolabelled with ⁶⁸Ga isotope ([⁶⁸Ga]Ga-DOTAGA-RAMEB) was eluted using a 1:2 mixture of EtOH (96%), and isotonic NaCl solution. The radiochemical purity was determined with the help of RP-HPLC/reversed-phase high-performance liquid chromatography combined with radiodetector. The product was diluted with 0.9% NaCl solution to reduce the ethanol content below 10%. Finally, prior to the *in vivo* application, the final product was sterile filtered.

3.2 *In vitro* experiments

3.2.1 Applied cell lines, cell cultivation

3.2.1.1 MDA-MB-HER2+ cells

The MDA-MB-HER2+ breast cancer cells were bought from American Type Culture Collection (ATCC, Manassas, VA, USA) and they were cultured according to the recommendations of the distributor. The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM + GlutaMAXTM-I (Thermo Fisher Scientific, Waltham, Massachusetts, USA)), which contained 10% heat-inactivated, foetal, bovine serum (FBS, (Thermo Fisher Scientific, Waltham, Massachusetts, USA)) and 1% antibiotic solution. The cell line was stored in T-75 cell culture flasks (12 mL medium) at 37 °C, in an incubator of 5% CO₂ content (ESCO CCI-170B-8 incubator Esco Micro Pte. Ltd., Singapore). After reaching an 80% confluence the cells were passaged three times a week and subsequent to 5 to 8 passages they were used in our *in vivo* tests. Cell transplantation was preceded by a viability examination carried out with trypan blue exclusion test. Following this, the cell concentration needed for the tumor induction was accurately adjusted.

3.2.1.2 4T1 cells

The 4T1 triple-negative breast cancer cell line was also purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultivated following the instructions of the distributor. The cells were cultured in a Roswell Park Memorial Institute 1640 (RPMI Medium 1640 + GlutaMAXTM-I (Thermo Fisher Scientific, Waltham, Massachusetts, USA)) medium supplemented with 10% heat inactivated FBS in T-75 cell culture flasks (12 mL medium/T-75 flask). The cells were incubated at 37 °C with a 5% CO₂ content (ESCO CCI-170B-8 incubator Esco Micro Pte. Ltd., Singapore). Passaging was performed 2-3 times a day upon reaching the 80% confluence. The 4T1 cells were also applied following 5-8 passaging for the biological investigations. Prior to cell transplantation, cell viability was checked with trypan blue exclusion test and then we determined the cell number and cell concentration required for tumor induction.

3.2.1.3 Pgp- A2780 cells

The Pgp- human ovarian cells were obtained from the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen. Cells were cultivated among standard laboratory conditions in DMEM medium supplemented with 10% FBS. As previously

described cell cultures were similarly incubated at 37 °C with 5% CO₂ content and 95% relative humidity. Cell viability was checked with trypan blue exclusion test, and they were passaged every three days.

3.2.1.4 Pgp+ A2780AD cells

The Pgp⁺ A2780AD human ovarian carcinoma cells were also obtained from the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen. In accordance with their Pgp⁻ counterparts, the investigated cell lines were cultured under standard laboratory conditions in DMEM medium supplemented with 10% FBS. In order to maintain the cell line, the cells were cultured in the presence of 28 µM doxorubicin (2 mg/mL doxorubicin hydrochloride, Doxorubicin-Teva solution for injection, Teva Pharmaceutical Industries, Hungary) a chemotherapeutic agent. The cell cultures were stored at 37 °C in an incubator that ensured 5% CO₂ and 95% relative humidity. Cell viability was regularly checked with a trypan blue exclusion method and cells were passaged every three days. The chemotherapeutic agent was removed from the medium during the passaging prior to the radiopharmaceutical injection.

3.2.1.5 Pgp- KB-3-1 cells

The Pgp⁻ KB-3-1 human cervical carcinoma cells were also obtained from the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen. Cells were cultured under standard laboratory conditions in DMEM supplemented with 10% FBS. The cell cultures were stored at 37 °C in an incubator that ensured 5% CO₂ and 95% relative humidity. Cell viability was checked with trypan blue exclusion method and the cultures were passaged every 3 days.

3.2.1.6 Pgp+ KB-V-1 cells

The Pgp⁺ KB-3-1 human cervix tumor cells were provided by the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen. The cells were cultured among standard *in vitro* conditions in DMEM medium supplemented with 10% FBS. To maintain the cell line cultivation was performed in the presence of 20 µM vinblastine (1 mg/mL vinblastine sulfate, Vinko, Kocak Farma Pharmaceutical and Chemical Industry Co. Ltd., Turkey), a chemotherapeutic agent. The cell cultures were stored at 37 °C in an incubator that ensured 5% CO₂ and 95% relative humidity. The viability of the cells was checked with a trypan blue exclusion test and the cultures were passaged every 3 days. The chemotherapeutic

agent was removed from the medium during the passaging prior to the radiopharmaceutical injection.

3.2.2 Induction of chemical hypoxia

To model chemical hypoxia the cells (A2780, KB-3-1) were treated with 250 μ M cobalt dichloride (CoCl_2 , (Sigma-Aldrich St. Louis, Missouri, USA)) during 24 and 48 hours. Based on literature data CoCl_2 is suitable for the *in vitro* representation of hypoxia, since through blocking the activity of HIF-1 α prolyl hydroxylases, it inhibits the degradation of HIF-1 α .

3.2.3 *In vitro* radiopharmaceutical uptake studies

Both the control cells and those treated with CoCl_2 were preincubated in PBS solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a 5 mM D-glucose content in a 1×10^6 cell/mL concentration for 10 minutes at a temperature of 36 °C-on. Following this we added 0.37 MBq/mL of Fluorodeoxyglucose (^{18}F) (^{18}F]FDG) to the untreated cell sample as well as to the cell groups treated with CoCl_2 . After the ^{18}F]FDG-incubation the radiopharmaceutical uptake of the cells was stopped by adding cold PBS solution. Subsequently the cells were washed 3 times with a solution of the same composition. The radioactivity of the samples was measured using a calibrated gamma counter in the energy window of ^{18}F] (Canberra-Packard Cobra II gamma counter, Canberra Packard Central Europe GmbH, Schwadorf, Austria).

The results were decay corrected and the radiopharmaceutical uptake was given as counts per minute⁻¹ (cpm/ 10^6 cell)⁻¹. The radiotracer uptake was also expressed as a percentage of the incubation activity (ID%). It indicates what percentage of the ^{18}F]FDG activity in the incubation medium is taken up by 1×10^6 cells. In view of the fact that ^{18}F]FAZA is able to bind to intracellular macromolecules exclusively in hypoxic conditions and considering that CoCl_2 -induced hypoxia was created under normoxia, the *in vitro* uptake examinations were performed only with ^{18}F]FDG radiopharmaceutical.

3.2.4 Western blot analysis

The HIF-1 α , GLUT1 and HK2 expression of the CoCl_2 treated and untreated control cells was determined with Western blot analysis using total cell lysates. For the analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used, when 100 μ L of $5 \times$ electrophoresis buffer was added to the samples, which were subsequently boiled for 10

minutes. Sixty μg of protein was separated on a 7.5% SDS-PAGE gel. The protein content of the gel was then transferred onto a nitrocellulose membrane. To block non-specific interactions between the membrane and antibodies, we applied 5% fat-free dry milk in PBST (phosphate-buffered saline containing 0.05-0.1% Tween 20). After blocking, the membranes were washed and incubated overnight at 4 °C with the primary antibodies (Abcam Inc., Cambridge, MA, USA) at the dilution recommended by the manufacturer. Following a 30-minute wash with PBST, the membranes were incubated at room temperature for 1 hour with secondary antibodies (anti-rabbit IgG, Bio-Rad Laboratories, Hercules, CA, USA) diluted 1:1000 in PBS containing 1% fat-free dry milk. Protein bands were detected using a chemiluminescent detection system according to the manufacturer's protocol (Pierce, Rockford, IL, USA).

3.3 *In vivo* experiments

3.3.1 Experimental animals, animal housing

To create breast tumor models 12-week-old (approximately 20 gram) CB17 severe combined immunodeficient (SCID) female mice were used ($n = 36$). These were purchased from Innovo Ltd. (Isaszeg, Hungary). The study animals were kept in sterile laboratory conditions in individually ventilated cages (IVC, Techniplast, Buguggiate, Italy) at a temperature of $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ensuring $55\% \pm 10\%$ relative humidity. During this period, we used controlled artificial lighting with 12-hour light-dark cycle. In addition, all the animals were provided with sterile, semi-synthetic rodent feed (Akronom Ltd., Budapest, Hungary) and sterile drinking water *ad libitum*.

For the investigation of human, gynaecological tumors (ovarian, cervix) we also used CB17 SCID mice (12-week-old, female, 18-22 gram, $n = 10$, 5 mice KB-3-1/KB-V-1, 5 mice A2780/A2780AD, Innovo Ltd., Isaszeg, Hungary). The animals were kept in sterile conditions, in IVC cages at controlled temperature ($26^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and humidity ($55\% \pm 10\%$). The artificial lighting was automatically controlled according to a 12-hour circadian rhythm. The animals were provided with *ad libitum* sterile, semi-synthetic feed (Akronom Ltd., Budapest, Hungary) and sterile drinking water.

Animal housing and handling were carried out in accordance with the applicable Hungarian laws and European regulations (directive 2010/63/EU). The animal experiments were conducted with the permission of the Animal Welfare Committee of the University of Debrecen (licence number: 8/2016/DEMÁB, 16/2020/DEMÁB).

3.3.2 Subcutaneous tumor induction

The tumor models were created with subcutaneous tumor cell transplantation. The interventions were carried out in all cases under inhalation anaesthesia. To induce anaesthesia 3% isoflurane (Aerrane/Forane, Baxter International Hungary, Budapest, Hungary/AbbVie Pharmaceutical Trading Ltd., Budapest, Hungary) with 0.4 L/min O₂ and 1.2 L/min N₂O (Linde Healthcare, Budapest, Hungary) was administered. During maintenance the isoflurane concentration was reduced to 1.5%. Anaesthesia was performed using a dedicated small animal anaesthesia system (Tec3 Isoflurane Vaporizer, Eickemeyer Veterinary Equipment, Luton, UK). When creating breast tumor models the left shoulder region of the mice was shaved and disinfected then 5×10^6 MDA-MB-HER2+ or 4T1 tumor cells were injected subcutaneously in a volume of 100-120 μ L in a DMEM:Matrigel 1:1 mixture.

In order to create gynaecological tumor models we applied 3×10^6 of Pgp- A2780 and KB-3-1 tumor cells while from the corresponding Pgp+ cell lines, a cell suspension containing 5×10^6 cells was used in a 150 μ L physiological saline. The Pgp- cells were injected subcutaneously in the left while the Pgp+ cells in the right thigh of the mice.

3.3.3 Tumor growth assessment

We used caliper measurements to assess tumor growth and determined tumor volume on a weekly basis with the following formula:

$$V = (L \times W^2) / 2$$

(V stands for the volume of the tumor (mm³), L stands for the length of the tumor (mm), W stands for its width (mm)).

Based on the size we classified the tumors into small, medium and large categories.

3.3.4 *In vivo* positron emission tomography (PET) imaging

In the first part of our study, when the tumors were clearly detectable, about 20 days following the subcutaneous implantation of the breast tumor cells, with a 40 ± 4.4 mm³ tumor size, *in vivo* microPET images were taken on all experimental animals on a weekly basis using [⁶⁸Ga]Ga-NODAGA-HP β CD and [⁶⁸Ga]Ga-DOTAGA-RAMEB radiotracers (n = 9 4T1, n = 9 MDA-MB-HER2+). The two radiopharmaceuticals were applied on consecutive days. Approximately 8-10 MBq of [⁶⁸Ga]Ga-NODAGA-HP β CD dissolved in 100-150 μ L physiological saline was administered intravenously through the lateral tail vein into the experimental animals. After a 60-minute incubation time, a 20-minute static PET data

acquisition was carried out using the MiniPET-II camera. On the subsequent day, following the same protocol we performed [^{68}Ga]Ga-DOTAGA-RAMEB PET imaging.

The *in vivo* biodistribution investigations of the second part of our research were performed 20 ± 1 days following the tumor induction with a $39.12 \pm 3.34 \text{ mm}^3$ tumor volume. The experimental animals underwent first a [^{18}F]FDG PET then, 24 hours later, a [^{18}F]FAZA PET imaging, which were performed using the MiniPET-II camera. The static data acquisition (20 min) done with the radiolabelled glucose analogue was started about 40 minutes after administering intravenously 7 MBq of [^{18}F]FDG (in 200 μL physiological saline). With the [^{18}F]FAZA radiotracer (7 MBq in 200 μL physiological saline) we performed both static and dynamic imaging. In case of the static imaging following a 120-minute incubation time, data were collected about the tumors for 20 minutes while the dynamic imaging took 140 minutes from the moment the radiotracer was administered (time-point 0 minute).

The *in vivo* tests were executed under isoflurane-induced inhalation anaesthesia and for that 3% isoflurane (Aerrane, Forane) 0.4 L/min O_2 , and 1.2 L/min N_2O were used. During maintenance the isoflurane concentration was reduced to 1.5%. Anaesthesia was delivered with the help of a dedicated small animal anaesthesia system (Tec3 Isoflurane Vaporizer, Eickemeyer Veterinary Equipment, Luton, UK).

3.3.4.1 MiniPET-II camera

The *in vivo* tests were conducted using preclinical PET camera (MiniPET-II). This camera was created as a result of a common research and development cooperation between the former DEOEC Institute of Nuclear Medicine, University of Debrecen (currently University of Debrecen, Faculty of Medicine, Institute of Medical Imaging, Department of Nuclear Medicine and Translational Imaging), the Institute for Nuclear Research of the Hungarian Academy of Sciences, and MEDISO Ltd.

The camera consists of 12 detector units, which contain cerium-doped lutetium-yttrium orthosilicate (LYSO) scintillation crystals and position sensitive Hamamatsu H9500 photomultipliers. The crystal matrices are made up of 35×35 crystals where the individual crystals have a size of $1,27 \times 1,27 \times 12 \text{ mm}^3$. The crystals are separated with reflective material. The digitalisation of the detector signals is performed with 4-channel data acquisition cards, while events are collected via optical link using data acquisition server (Cisco Catalyst switch). The system stores the primary data in a list mode, which allows the retrospective application of different, advanced image reconstruction methods. The system has an axial field of view of 48 mm and a transaxial field of view of 100 mm. Its spatial resolution is 1.1 mm while its

sensitivity is 11.4%. Due to its compact size and high resolution, the MiniPET-II is suitable for *in vivo* PET imaging of radiopharmaceutical distribution in laboratory rodents.

3.3.4.2 Assessment of the positron emission tomography (PET) images

Following the *in vivo* PET studies, the images were assessed both visually and quantitatively. During the quantitative analysis we placed volumes of interests (VOI) on the tumors in the coronal PET images with the help of the Brain CAD image analysing software. Then quantitative PET parameters were established. Using the formula below, we registered the radiopharmaceutical concentration within the VOI and determined it in standardized uptake values (SUV) as maximum (SUVmax) and mean (SUVmean):

$$\text{SUV} = \frac{\text{radiopharmaceutical concentration within the VOI (MBq/mL)}}{\text{injected activity (MBq) / body weight (g)}}$$

SUVmax represents the voxel with the highest radioactivity, whereas SUVmean reflects the average activity of all the voxels of the given region. Besides, tumor-to-muscle ratio (T/M) was calculated in order to compare the SUV values measured in the tumor as well as in the muscle (background) tissue.

$$\text{T/M} = \text{tumor SUVmean} / \text{muscle SUVmean}$$

3.3.5 Ex vivo biodistribution studies

Ex vivo biodistribution measures were conducted to examine the changes of cyclodextrin uptake connected to tumor development at the same time as the PET imaging. Similarly to the previous experiments about 8-10 MBq of [⁶⁸Ga]Ga-NODAGA-HPβCD and [⁶⁸Ga]Ga-DOTAGA-RAMEB radiopharmaceuticals in 100-150 μL physiological saline were injected to the lateral tail veins of the mice. Sixty minutes following the injection of the radiotracers the animals were euthanised and dissected. The tumors were completely removed and were weighed using analytical balance. The next step was to determine the radioactivity of the tumors with the help of calibrated gamma counter (Perkin-Elmer Packard Cobra, Waltham, MA, USA). The radiopharmaceutical uptake of the tumor tissue was assessed following decay correction, and the results were expressed as percentage of the injected dose per gram (%ID/g).

3.4 Statistical analysis

The statistical analysis was carried out with the help of MedCalc 18.5 statistical software package (MedCalc Software, Mariakerke, Belgium). To evaluate the differences between the

groups two sample t-tests, two-way analysis of variance (ANOVA), and Mann-Whitney U-tests were applied depending on data distribution. The threshold for statistical significance was set at $p < 0.05$ and the results were presented as mean $\pm$ standard deviation (SD).

4 Results

4.1 *In vivo* monitoring of PGE2 production of breast tumor xenografts with cyclodextrin based PET radiopharmaceuticals

4.1.1 *In vivo* PET imaging

The diagnostic potential of ^{68}Ga -labelled cyclodextrin derivatives was evaluated in HER2+ MDA-MB-HER2+ and triple negative 4T1 breast tumor xenografts using PET imaging. The subcutaneous tumor induction proved to be successful in case of every injected animal as all the mice developed a palpable tumor in the region of the left shoulder on about the 20th day following the transplantation. Consequently, all the animals were involved in the *in vivo* biological tests (MDA-MB-HER2+ = 18 and 4T1 n = 18).

4.1.1.1 MDA-MB-HER2+ tumors

Based on the visual assessment of the PET images taken of the MDA-MB-HER2+ tumor-bearing mice, we could establish that both radiotracers exhibited a pronounced tumor accumulation at all time points. The radiopharmaceutical uptake of the tumors gradually increased with the passing of time, which was particularly significant in case of the RAMEB molecule between days 50 and 65. Furthermore, considerable background activity could be observed on the [^{68}Ga]Ga-DOTAGA-RAMEB PET images, whereas on the HP β CD images only minimal, non-specific radioactivity was detected in the thoracic and abdominal regions, which enabled the accurate delineation of the tumors. Apart from the subcutaneous accumulation of the tumors, on both PET image series significant tracer accumulation was displayed in the kidneys, which refers to the renal excretion of the radiotracers.

Our visual observations were confirmed by the quantitative analysis of the PET scans. Both cyclodextrin tracers bound with high affinity to the HER2+ tumors. The SUV value analysis - in accordance with the qualitative analysis - exhibited a continuously raising tracer concentration in case of both radiopharmaceuticals during the entire period of the experiment ([^{68}Ga]Ga-NODAGA-HP β CD: 0.04 ± 0.006 , 0.06 ± 0.015 and 0.07 ± 0.013 on days 30, 50 and 65 of the experiment, [^{68}Ga]Ga-DOTAGA-RAMEB: 0.02 ± 0.003 , 0.03 ± 0.005 and 0.06 ± 0.009 30, 50 and 65 days following the injection). During the comparison of the tumor uptake values of the HP β CD and RAMEB derivatives, on days 35 and 50, the HP β CD molecule showed a significantly higher accumulation compared to the RAMEB compound, however, on the last day (day 65) this difference did not turn out to be statistically significant ($p \leq 0.05$).

4.1.1.2 4T1 tumors

The subcutaneously growing triple negative 4T1 tumors, similarly to the MDA-MB HER2+ tumors, could be clearly identified using both radiotracers at all the three time points. At the same time, the tumor accumulation was found to be higher in case of the radiolabelled RAMEB derivative, especially on day 20. In line with our previous observations a continuously increasing tracer accumulation was noticed in case of both radiopharmaceuticals during the experimental period. This accumulation proved to be more pronounced in case of RAMEB.

The results of the visual assessment were confirmed with quantitative PET data in case of the 4T1 tumors, too. In accordance with this, both the [^{68}Ga]Ga-NODAGA-HP β CD, and the [^{68}Ga]Ga-DOTAGA-RAMEB quantitative PET analyses confirmed an intensive and specific accumulation in the 4T1 tumors with increasing signal intensity over time. The SUV values measured on days 10, 15 and 20 following the tumor cells transplantation were 0.02 ± 0.003 , 0.03 ± 0.006 and 0.035 ± 0.004 in case of [^{68}Ga]Ga-NODAGA-HP β CD, whereas in case of [^{68}Ga]Ga-DOTAGA-RAMEB these figures were 0.025 ± 0.002 , 0.034 ± 0.004 and 0.04 ± 0.005 . Comparing the two tracers we could see that the compound RAMEB exhibited a slightly higher tumor accumulation at both time points, however these differences were not statistically significant in either case.

4.1.2 *Ex vivo* organ distribution studies

To support the *in vivo* PET results, we performed *ex vivo* organ distribution studies with necropsy.

In the 4T1 tumors both tracers demonstrated a gradually increasing accumulation between days 10 and 15 following the tumor induction. The peak accumulation registered on day 15 ([^{68}Ga]Ga-DOTAGA-RAMEB: $3.10\% \pm 0.45$ %ID/g; [^{68}Ga]Ga-NODAGA-HP β CD: $2.65\% \pm 0.51$ %ID/g) was followed by a slight decrease on day 20 ([^{68}Ga]Ga-DOTAGA-RAMEB: $2.35\% \pm 0.38$ %ID/g; [^{68}Ga]Ga-NODAGA-HP β CD: $2.11\% \pm 0.40$ %ID/g). Besides, the concentration of [^{68}Ga]Ga-DOTAGA-RAMEB measured in the 4T1 tumors slightly exceeded the values of [^{68}Ga]Ga-NODAGA-HP β CD at all time points, nevertheless these differences were not statistically significant.

In case of MDA-MB-HER2+ tumors, the HP β CD molecule exhibited a considerably higher tumor accumulation ($p \leq 0.05$) compared to RAMEB at the first two time points (on day 35: [^{68}Ga]Ga-DOTAGA-RAMEB: $2.727\% \pm 0.48$ %ID/g, [^{68}Ga]Ga-NODAGA-HP β CD: $5.779\% \pm 1.06$ %ID/g; on day 50: [^{68}Ga]Ga-DOTAGA-RAMEB: $4.929\% \pm 1.06$ %ID/g,

[⁶⁸Ga]Ga-NODAGA-HPβCD: 10.039% ± 2.09 %ID/g). On day 65 following the development of tumors, the tumor concentration of [⁶⁸Ga]Ga-NODAGA-HPβCD continued to be slightly higher than that of the RAMEB derivative, however, this difference failed to reach the statistical threshold.

The temporal evolution of the radiopharmaceutical uptake of MDA-MB-HER2+ tumors followed the pattern observed in 4T1 tumors: the %ID/g values showed a rise till the second time point and then declined till the end of the experiment.

4.1.3 Comparison of [⁶⁸Ga]Ga-NODAGA-HPβCD and [⁶⁸Ga]Ga-DOTAGA-RAMEB uptake in MDA-MB-HER2+ and 4T1 tumors

Subsequent to the biological examinations, the [⁶⁸Ga]Ga-NODAGA-HPβCD and [⁶⁸Ga]Ga-DOTAGA-RAMEB *in vivo* uptakes were also compared according to the tumor sizes (small, medium and large).

The analysis of the SUV data indicated that the uptake of the radiolabelled HPβCD molecule was significantly higher in the small sized MDA-MB-HER2+ tumors than in the 4T1 tumors of the same size ($p \leq 0.05$). This tendency persists in the medium and large categories, where the radioactivity of the MDA-MB-HER2+ tumors surpassed the one of 4T1 tumors, moreover, these differences remained statistically significant ($p \leq 0.05$). In addition, the gradual increase of the HPβCD concentration parallely with the growth of the tumor was also apparent.

With regard to the RAMEB derivative in the small and medium sized tumor categories the radiopharmaceutical uptake of the 4T1 tumors exceeded the activity of the MDA-MB-HER2+ tumors. As opposed to this, in case of the large tumor size, RAMEB uptake was higher in the HER2+ lesions. It is important to note, however, that the differences in the activities did not reach the statistical threshold in any of the tumor size categories.

4.1.4 Assessment of tumor growth dynamics

Alongside the *in vivo* studies we also assessed the intrinsic growth potential of the two different breast cancers. The growth curves of triple negative 4T1 breast tumors exhibited rapid cell proliferation and exponentially growing tumor size. The corresponding tumor volumes were consistently $75 \pm 13 \text{ mm}^3$, $212 \pm 24 \text{ mm}^3$ and $342 \pm 30 \text{ mm}^3$ on days 10, 15 and 20 following the tumor cell transplantation, respectively. In contrast, MDA-MB-HER2+ tumors grew more slowly, and the tumors reached $30 \pm 4 \text{ mm}^3$, $79 \pm 11 \text{ mm}^3$, and $128 \pm 22 \text{ mm}^3$ volumes on days 35, 50, 65 post tumor transplantation, respectively.

4.2 Investigation of tumor-associated hypoxia in preclinical gynaecological tumor models using PET imaging

4.2.1 Findings of the *in vitro* studies

Hypoxia induced in KB-3-1 and A2780 cells incubated with CoCl₂ and the related cellular processes were investigated both with radiopharmaceutical uptake ([¹⁸F]FDG) studies and molecular biology (Western blot) methods. These tests showed that the CoCl₂-treatment provoked the increased expressions of GLUT1 glucose transporter and HK2 enzyme.

4.2.1.1 The *in vitro* study of KB-3-1 cells

4.2.1.1.1 Cellular [¹⁸F]FDG uptake

The [¹⁸F]FDG uptake of KB-3-1 cells increased continually in all the 3 cell groups (control, cells incubated with CoCl₂ for 24 and 48 hours) till the end of the test (60 min). The highest radiopharmaceutical uptake was detected in those cells which were treated with CoCl₂ for 24 hours. These cells showed a considerably higher radioactivity at all the 3 time points (15, 30, 60 min) compared to the tracer uptake of both the control cells and KB-3-1 cells incubated with CoCl₂ for 48 hours. ($p \leq 0.01$).

4.2.1.1.2 Western blot analysis

We used Western blot analysis to determine the expression levels of HIF-1 α , GLUT1 and HK2 proteins. Depending on the duration of CoCl₂ treatment the cells exhibited a different level of protein accumulation. Nevertheless, the CoCl₂ raised the amount of all the proteins examined in the cell groups treated, independently of the duration of the treatment. This is in accordance with the elevated [¹⁸F]FDG uptake experienced in the treated cells. In the detailed analysis we could detect a significantly higher level of HIF-1 α and GLUT1 transporters in the treated cells following the 24-hour CoCl₂ incubation compared to the control cells and those treated for 48 hours. In contrast, the highest amount of HK2 enzyme was registered after the 48-hour incubation.

4.2.1.2 *In vitro* examination of 2780 cells

4.2.1.2.1 Cellular [¹⁸F]FDG uptake

While analysing the [¹⁸F]FDG uptake of 2780 cells we experienced that the radiopharmaceutical accumulation in the cells treated with CoCl₂ surpassed the values measured in the control cells at all the time points. On the other hand, as a function of incubation time, considerable differences were detected in the [¹⁸F]FDG accumulation of the treated cells.

At the first time point (15 min), no significant difference in radioactivity was registered between cells treated with CoCl₂ for 24 and 48 hours. However, at 30 and 60 min, [¹⁸F]FDG uptake in the 24-hour incubation group significantly exceeded that of the 48-hour treated cells (30 min: $p \leq 0.05$, 60 min: $p \leq 0.01$).

4.2.1.2.2 Western blot analysis

The results of the Western blot analysis of the 2780 cells revealed that the hypoxia-associated HIF-1 α level in the cells treated with CoCl₂ grew considerably compared to the untreated cells, particularly in case of the 24-hour long incubated cells. In a similar fashion the amount of GLUT1 transporter responsible for [¹⁸F]FDG transport as well as the HK2 enzyme level responsible for its phosphorylation were also more elevated in the treated cells, especially after the 24-hour long treatment.

4.2.2 In vivo PET results

Using the MiniPET-II small animal PET camera, static and dynamic PET studies were performed on the hypoxic tumors.

4.2.2.1 Static PET studies with [¹⁸F]FAZA radiopharmaceutical

About 20 days (20 ± 5 days) following the tumor induction with a 400 ± 80 mm³ average tumor volume, PET studies were conducted on the gynaecological tumor bearing experimental animals using [¹⁸F]FAZA radiotracer. Based on the current recommendations 20-minute static PET images were acquired on the tumors (Pgp- A2780, Pgp+ A2780AD, Pgp- KB-3-1 and Pgp+ KB-V-1) 120 minutes after the intravenous administration of the tracer. Following the analysis of the quantitative PET data no significant difference was found between the SUVmean values of the tumors (Pgp- A2780: 0.48 ± 0.095 , Pgp+ A2780AD: 0.60 ± 0.015 , Pgp- KB-3-1: 0.37 ± 0.05 and Pgp+ KB-V-1: 0.39 ± 0.03). In view of the T/M ratios the [¹⁸F]FAZA-accumulation turned out to be twice - four times as high in all the examined tumors as in the background tissues. Despite this the differences between the certain groups were not statistically different (average T/M ratios: Pgp- A2780: 2.6 ± 1.29 , Pgp+ A2780AD: 2.8 ± 1.4 , Pgp- KB-3-1: 3.85 ± 0.84 , Pgp+ KB-V-1: 4.25 ± 0.75).

4.2.2.2 Dynamic PET studies with [¹⁸F]FAZA radiopharmaceutical

With the purpose of investigating the temporal kinetics of [¹⁸F]FAZA uptake, apart from the static images, dynamic images were also acquired about the mice. The evaluation of the

time-activity curve revealed that the SUVmean values of the hypoxic tumor regions increased exponentially up to minute 120 and subsequently reached a plateau (SUVmean: 0.67 ± 0.07). As opposed to this, in the non-hypoxic background tissue the radiopharmaceutical uptake exhibited a moderate growth till 30 minutes (SUVmean: 0.15 ± 0.05). Afterwards, in the absence of hypoxia, the tracer was simply washed out from the cells, which resulted in the continuous reduction of the tracer concentration until 120 minutes (SUVmean: 0.11 ± 0.05). Another observation was that the uptake ratio (T/M) between the hypoxic tumor region and the background reached its peak (6.2 ± 0.1) at 110-120 minutes after administering the radiopharmaceutical.

4.2.2.3 PET studies with [^{18}F]FDG and [^{18}F]FAZA radiopharmaceuticals

In order to describe the viability and glucose metabolism of the tumors, the PET studies performed with the hypoxia-specific [^{18}F]FAZA were complemented with [^{18}F]FDG PET imaging. The imaging tests with the two different radiopharmaceuticals were carried out within one day. The 20-minute long static [^{18}F]FDG PET data acquisition took place 40 minutes following the intravenous injection of the radiolabelled glucose analogue. The main purpose of [^{18}F]FDG PET imaging was to assess the viability and the metabolic activity of the tumors. Besides, we also strived to identify the intratumoral regions which showed an elevated [^{18}F]FDG uptake and proved to be [^{18}F]FAZA positive at the same time.

The [^{18}F]FDG exhibited a markedly heterogeneous distribution in all the examined tumor xenografts, which refers to an extremely high glucose metabolism of the certain regions within the tumor. A considerable distinction was observed between the SUVmean values of the regions with intensive [^{18}F]FDG accumulation (2.72 ± 0.23) and the regions with low accumulation (1.2 ± 0.36). Even more remarkable differences were noted in the intratumoral images in case of the [^{18}F]FAZA tracer, where the radioactivity of the strongly [^{18}F]FAZA positive regions (SUVmean: 0.86 ± 0.06) was found to be 6-7 times as high as in the [^{18}F]FAZA negative regions (SUVmean: 0.11 ± 0.05). A further observation demonstrated that the intratumoral regions with intensive [^{18}F]FDG uptake show overlap with the hypoxic, [^{18}F]FAZA accumulating tumor regions.

5 Novel scientific findings

1. Based on the results of the experiments performed with radiolabelled cyclodextrins we established that the tumor biological characteristics of the heterotopic gynaecological xenograft models created by subcutaneous transplantation can be monitored with *in vivo* PET imaging and tumor selective tracers.
2. Using *in vivo* PET imaging and radiolabelled cyclodextrin derivatives ($[^{68}\text{Ga}]\text{Ga-DOTAGA-RAMEB}$ and $[^{68}\text{Ga}]\text{Ga-NODAGA-HP}\beta\text{CD}$) we demonstrated the PGE2 expression of the subcutaneously transplanted HER2+ and triple negative breast tumors.
3. With our research we were the first to verify that the ^{68}Ga -labelled cyclodextrin derivatives ($[^{68}\text{Ga}]\text{Ga-DOTAGA-RAMEB}$ and $[^{68}\text{Ga}]\text{Ga-NODAGA-HP}\beta\text{CD}$) can be successfully applied for the PET based detection of subcutaneous preclinical models of HER2+ and triple negative breast cancers.
4. Based on our findings we concluded that the $[^{68}\text{Ga}]\text{Ga-DOTAGA-RAMEB}$ and $[^{68}\text{Ga}]\text{Ga-NODAGA-HP}\beta\text{CD}$ radiopharmaceuticals can be promising molecular vectors in the future in diagnosing breast cancers, specifically the receptor-negative lesions. Moreover, the investigated cyclodextrin tracers proved to be suitable starting molecules for developing further carbohydrate based, angiogenesis-targeting diagnostic agents to detect breast cancer.
5. The spacial overlaps between the $[^{18}\text{F}]\text{FDG}$ and $[^{18}\text{F}]\text{FAZA}$ PET images of the gynaecological tumor models revealed that the tumor areas with high $[^{18}\text{F}]\text{FDG}$ uptake are $[^{18}\text{F}]\text{FAZA}$ positive, which verifies the hypoxia in the regions with elevated glucose metabolism. These results suggest that the complementary application of $[^{18}\text{F}]\text{FDG}$ and $[^{18}\text{F}]\text{FAZA}$ allows the more precise identification of the hypoxic tumor regions, which may not only increase the diagnostic value of imaging, but may also have significant clinical relevance. The accurate detection of the hypoxic tumor regions can promote the discovery of the so far unknown aspects of tumor biological processes, which, in turn, can lead to the identification of further diagnostic and therapeutical targets. Besides, the $[^{18}\text{F}]\text{FDG}/[^{18}\text{F}]\text{FAZA}$ PET-based hypoxia can be regarded as a potential biomarker in the prediction of therapeutic response as well as clinical outcome. In addition, our

findings show that the common application of [^{18}F]FDG/[^{18}F]FAZA PET seems promising in monitoring the efficacy of the therapy and as a result in identifying those patient groups, who can benefit from the application of hypoxia-specific treatment strategies.

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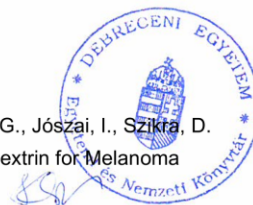
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1. **Sass, T.**, Péli-Szabó, J., Dienes, R. A., Árkosy, P., Opposits, G., Józai, I., Fekete, A., Fenyvesi, F., Hajdu, I., Trencsényi, G., Képes, Z.: Radiolabelled Cyclodextrins for the Positron Emission Tomography Imaging of Breast Cancer: Preclinical Perspectives.
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3. Barkóczi, A., Képes, Z., Péli-Szabó, J., Dienes, R. A., Károlyi, P. K., Papp, T., Kálmán-Szabó, I., **Sass, T.**, Opposits, G., Kertész, I., Hajdu, I., Trencsényi, G., Deák, Á.: Metastasis and angiogenesis: preclinical PET study on hepatocellular carcinoma (He/De) tumor models.
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