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In vivo investigation of angiogenesis in experimental tumors using PET radiotracers

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1. Introduction

In 2019, according to WHO's estimation 31700 patient death caused by malignant cell proliferation (neoplasia), which a little bit less than the number of patient (36800) died in ischaemic heart disease in Hungary. The total cardiovascular system concerned death number was 62100, which approximately twice as much as people died in malignant cancer. The wrong lifestyle, such as sedentary lifestyle, obesity, smoking, alcoholism, malnutrition and stress plays an important role in the development of the previously mentioned cause of deaths. The issue of prevention cannot be neglected, which may delay or prevent the incidence of cardiovascular or cancerous diseases. The importance of screening should be emphasized since with the help of these examinations the pathological lesions are recognizable at an early stage, thus improving the patient's chances of survival and contribution to complete recovery. In Hungary, the screening for breast and lung lesions based on the use of traditional imaging equipments, such as US or X-Ray, and not negligible the importance of these devices in diagnosis and monitoring the effectiveness of therapies. The tools of nuclear medicine are the cross-sectional SPECT and PET modalities which play a major role in the diagnosis of diseases and the follow-up of therapies. The most often used radioisotopes in PET technique are ^{11}C ($T_{1/2}$=20 minutes) and ^{18}F ($T_{1/2}$=110 minutes). In recent decades the biomedical researches focused on the use of other positron emitting isotopes, which have more ideal half-time or energy, and perhaps for their production is not require the presence of cyclotron. In the field of tumor diagnosis, staging and therapy follow-up the most frequently used PET radiopharmacon is ^{18}FDG. However, it is also important to mention that ^{18}FDG is not tumor specific, as the uptake of ^{18}FDG is increased in organs

which requires more glucose or the glucose uptake is elevated (such as inflammatory areas, brain, heart, muscle) or in the kidneys. Moreover, there are tumors such as well differentiated thyroid carcinomas or neuroendocrine tumors that do not accumulate ^{18}FDG. For this reason, the development of radiotracers that targets tumor specific molecules (e.g. APN/CD13) is justified in biomedical research. Aminopeptidase N (APN/CD13) is a key marker in the process of new vessel development, which called angiogenesis. APN/CD13 is consisting of 100 amino acids, type II membrane bounded metalloproteinase which can be characterized with 3 functions: enzyme, receptor and signaling molecule. It is expressed on the epithel cells of the kidney and small intestine, nervous system and myeloid cells in a normal level. However, the APN positivity was proved on several clinical samples such as breast-, prostate-, ovarium-, thyroid-, colon-, lung- and pancreas cancers and melanomas. To identify tumor-homing molecules phage-display technique was used and NGR peptide sequence was revealed, which contains asparagine-glycine-arginine amino acids and specifically binds to APN/CD13. NGR sequencies can help to diagnose APN/CD13 positive cancers with PET imaging and can provide a more effective and less toxic treatment if it conjugated with anticancer agents. The linear and cyclic form of NGR can be characterized with different imaging properties, the cyclic form is more stable and specific to tumor-homing, than the linear form. In our previous study, the cyclic form of NGR (^{68}Ga-NOTA-c(NGR)) was investigated and APN/CD13 specificity was confirmed on subcutan Ne/De tumor-bearing rats with *in vivo* and *ex vivo* studies. The applied ^{68}Ga radioisotope has ideal physical properties for PET imaging and does not require the presence of cyclotron, as the ^{68}Ga isotope can be extracted cost- effectively from a simple ^{68}Ge/^{68}Ga generator.

2. Aim of work

As mentioned earlier, we aimed to investigate the radiopharmaceuticals -that produced by our radiochemical group- applicability for diagnosis and monitoring therapy efficiency:

- We postulated to investigate the relationship between hypoxia and angiogenesis with *in vivo* and *ex vivo* methods on subcutan He/De tumors using ^{68}Ga-labeled hypoxia and angiogenesis specific radiotracers.

- We wanted to confirm the APN/CD13 specificity of the four c(NGR) derivatives -produced by our radiochemical group- for which we performed *in vivo* and *ex vivo* studies on subcutan He/De and Ne/De tumor-bearing animals.

- We planned to investigate the applicability of one APN/CD13 specific c(NGR) derivative to monitor the efficacy of antitumor therapy. For this, subcutan HT1080 and B16F10 bearing mice were treated with anti-APN/CD13 inhibitory agents (actinonin and bestatin) and the efficacy of the treatment was followed by *in vivo* PET imaging.

- We wanted to prove the APN/CD13 positivity of the used tumors with immunohistochemistry and western blot analysis.

3. Materials and methods

3.1 Production of the investigated radiotracers

The members of the radiochemical group produced $\alpha_V\beta_3$, APN/CD13 and hypoxia specific radiotracers. The first step of production was the different c(NGR) peptide or nitroimidazole conjugation to the chelator.

The chelator conjugated (NODAGA-[c(RGD)]$_2$) form of the $\alpha_V\beta_3$ receptor binding RGD peptide was obtained from commercial sources and used without further purification for ^{68}Ga-labeling.

The c[KNGRE]-NH_2 peptide was conjugated with p-SCN-Bn-NOTA and NODAGA-NHS-esther chelators. The c[CH_2-CO-Lys-Asn-Gly-Arg-Cys]-NH_2 and c[CH_2-CO-Lys-Asn-N(Me)Gly-Arg-Cys]-NH_2 peptides were conjugated with p-SCN-Bn-NODAGA chelator and the nitroimidazole was conjugated with DOTA chelator.

The created NODAGA-[c(RGD)]$_2$, NOTA-c(NGR), NODAGA-c(NGR), NODAGA-c[CH_2-CO-Lys-Asn-Gly-Arg-Cys]-NH_2, NODAGA-c[CH_2-CO-Lys-Asn-N(Me)Gly-Arg-Cys]-NH_2 and DOTA-Nitroimidazole precursors were purified with semi-preparative HPLC (Supelco Discovery® Bio Wide Pore C18 column with 4 mL/min flow rate was used in case of peptides and in case of nitroimidazole Luna C18 10 µm column with 5 mL/min flow rate was used), then the precursors were lyophilized and their structure was determinated with ESI-MS.

The next step was the ^{68}Ga labeling of the purified precursors. For ^{68}Ge/^{68}Ga generator eluating HCl was used then it was buffered. The peptide precursors

were added to the buffered $^{68}GaCl_3$ and it was incubated without mixture at 95°C, for 5 min. In case of nitroimidazole the incubation was also 95°C, but the time was 15 min. During the purifying and formulating procedure the peptide reaction mixture was pipetted on HLB column, the nitroimidazole reaction mixture was put on SPE column, then it was resumed with physiological saline, after all it was filtrated to sterile form.

For the quality control of the completed radiolabeled compounds HPLC method was used. In case of peptides Supelco Discovery® Bio Wide Pore C18 column with 1.2 mL/min flow rate, moreover in case of nitroimidazole Luna C18 column was used.

In last step the LogP and serum stability were determined. The partition coefficient was defined with shaken method using octanol-PBS 1:1 mixture. For this, 10 µL aqueous radioactive compound was added to 0.5 mL octanol containing test tube, then 0.5 mL PBS was added. To reach equilibrium state the mixture was shaked strongly, then it was centrifuged at 20.000 rpm for 5 min. 3 times, 100 µL samples from each layer were introduced into the test tubes and the radioactivity was determined with a gamma counter (HEWLETT PACKARD Cobra II Autogama Gamma Counter).

The ^{68}Ga-labeled compounds chemical stability was determined in PBS at 95°C. At different time points (0, 30, 60, 90, 120 min) 25 µL sample was injected to reverse phased HPLC.

For the determination of enzymatic stability, a stock solution was made with 100 µL radioactive solution and 1 mL PBS. 50 µL stock solution was pipetted to 450 µL rat or mouse serum, then it was incubated at 37°C. 50 µL abs. EtOH

was taken to 50 µL sample containing Eppendorf tube at different time points (0, 30, 60, 90, 120 min). To remove the precipitations the samples were centrifuged at 20.000 rpm for 5 min. The supernatant was removed and diluted with the eluent of HPLC, and then the analytical measurement was prepared with the standard protocol.

The preparation and the quality control of the radiotracers were performed by Noémi Stéfánné Dénes and Dániel Szücs PharmD.

3.2 Cell culturing

Ne/De cells (rat originated mesoblastic nephroma) were cultured in DMEM cell culturing media enriched with 10% FBS and 1% antibiotic-antimycotic solution using T75 cell culturing flasks.

He/De cells (rat originated hepatocellular carcinoma) were cultured in IMDM cell culturing media enriched with 10% FBS and 1% antibiotic-antimycotic solution using T75 cell culturing flasks.

HT1080 cells (human originated fibrosarcoma purchased from ATCC) were cultured in GlutaMAX ™ DMEM cell culturing media enriched with 10% FBS and 1% antibiotic-antimycotic solution using T75 cell culturing flasks.

B16F10 cells (mouse originated melanotic melanoma purchased from ATCC) were cultured in GlutaMAX ™ DMEM cell culturing media enriched with 10% FBS and 1% antibiotic-antimycotic solution, 1% MEM non-essential amino acid solution and 1% MEM vitamin solution, using T75 cell culturing flasks.

The cells were cultured at 37°C, 5% CO_2 atmosphere and 95% humidity in a cell culturing incubator (ESCO CCL-170B-8) and every two days the cells were seeded. At passage 5th the cells were removed from T75 flasks, counted, centrifuged and prepared to the tumor cell inoculation in a demanded volume. The cell viability was verified with trypan blue exclusion test using microscope (Olympus CH-2).

3.3 Experimental animals

16 weeks old, male F-344 rats were used in the first (tumorous n=36; control n=9) and second (tumorous n=64; control n=12) part of our experiments and 10-12 weeks old CB17 SCID mice (treated- tumor-bearing n=20; untreated- control, tumor-bearing n=10) and C57BL/6 mice (treated- tumor-bearing n=20; untreated- control, tumor-bearing n=10) were used in the third part of our experiments. All the animals were kept in an air- conditioned conventional animal house room at a temperature of 26±2°C with 50±10% humidity and artificial lighting with a circadian cycle of 12 h. (Animal house permission number: III/6- KÁT- 2015). The F-344 rats were kept in an IVC-ZJ3 IVC system with LTE E-004 abele bedding supplied IVC cages and the C57BL/6 and CB17 SCID mice were kept in a Sealsafe® Blue line IVC system with a sterilized REHOFIX® granulated corn- cob bedding. Sterile tap water and sterilized SDS-VRF1 semi synthetic rodent diet were available ad libitum to all the animals. All of the experiments were registered (8/2016/DEMÁB) by the Occupational Committee for Animal Welfare, University of Debrecen, Hungary in accordance with the relevant European Union and Hungarian Laws. If moderate and short-term pain was observed ibuprofen containing oral Motrin narcotics was used in 15 mg/kg/day dose ad libitum. The animals were

monitored continuously and, if their distress increased, the above dose was increased to 30 mg/kg/day.

3.4 Tumor induction

Male F-344 rats and male CB17 SCID mice or C57BL/6 mice were anaesthetized with a dedicated small animal anaesthesia device (induction: 3% isoflurane, maintain: 1.5% isoflurane + 0.4 L/min O_2 and 1.4 L/min N_2O). After depilation and decontamination of the left shoulder area of the animals $5x10^6$ He/De or Ne/De cells (in 150 µl sterile saline) were injected subcutaneously into F-344 rats. CB17 SCID mice were injected subcutaneously with $1.5x10^6$ HT1080 (human fibrosarcoma) cells in 150 µl (1/3 part of Matrigel and 2/3 part of saline), and C57BL/6 mice were injected subcutaneously with $3x10^6$ B16F10 cells in 150 µl of sterile saline.

3.5 Animal treatment with APN/CD13 inhibitors

Four days after tumor induction HT1080 (n=30) and B16F10 (n=30) tumor-bearing animals were randomly distributed into 3-3 groups as follows: control-untreated group (n=10/tumor type), bestatin-treated group (n=10/tumor type) and actiononin-treated group (n=10/tumor type). For anticancer therapy, the mice of the bestatin-treated groups were injected intraperitoneally daily with 15 mg/kg bestatin dissolved in 150 µL Humaqua for 7 days. For the actinonin-treated groups, 5 mg/kg actinonin (dissolved in 10 µL abs. EtOH, and diluted with 140 µL Humaqua) was administrated daily by intraperitoneal injection for 7 days.

3.6 *In vivo* PET studies

The small animal PET/MRI studies were taken place at Scanomed Ltd. Translational Center's Preclinical Laboratory. In the first part of our experiments subcutan He/De (n=27) tumor-bearing and control (n=9) animals were injected and investigated with 10.2±1.1 MBq ^{68}Ga-labeled angiogenesis (^{68}Ga-NODAGA-[c(RGD)]$_2$, ^{68}Ga-NOTA-c(NGR)) and hypoxia (^{68}Ga-DOTA-Nitroimidazol) specific radiotracers. For monitoring tumor growth, 10 days after tumor cell inoculation He/De tumor-bearing rats were examined daily and alternately with ^{68}Ga-NOTA-c(NGR) and ^{68}Ga-DOTA-Nitroimidazole.

Two radiotracers -produced by our radiochemical workgroup- names were changed from c[CH_2-CO-Lys-Asn-Gly-Arg-Cys]-NH_2 to c(NGR) (MG1) and from c[CH_2-CO-Lys-Asn-N(Me)Gly-Arg-Cys]-NH_2 to c(NGR) (MG2) in *in vivo* studies, where the MG abbreviation refers to our honour for Prof. Gábor Mező. In the second part of our investigation control (n=12) and subcutan He/De or Ne/De tumor-bearing (n=40) male F-344 rats were injected via the lateral tail vein with 12.3±1.2 MBq of ^{68}Ga-NOTA-c(NGR) or ^{68}Ga-NODAGA-c(NGR) or ^{68}Ga-NODAGA-c(NGR) (MG1) or ^{68}Ga-NODAGA-c(NGR) (MG2) in 150 µL physiological saline and 11±1 day after tumor cell inoculation.

The animals were anaesthetized with inhalation method (induction: 3% isoflurane, maintain: 1.5% isoflurane + 0.4 L/min O_2 and 1.4 L/min N_2O) using dedicated small animal anaesthesia device during the injection and the imaging procedure. 90 min after radiotracer injection whole body PET scans (10 minutes static scans) were taken with preclinical nanoScan PET/MRI 1T

device. Special rat imaging chamber (MultiCell Imaging Chamber, Mediso Ltd., Hungary) was used to eliminate the movement of rats and to maintain a permanent body temperature. For the anatomical localization of tissues and organs T1 weighted MRI scans (Material map) were performed.

The small animal PET studies were taken place at University of Debrecen, Faculty of Medicine, Department of Medical Imaging, Division of Nuclear Medicine and Translational Imaging using miniPET device. In the third part of our experiments six and eleven days after HT1080 and B16F10 tumor cell implantation control-untreated tumor-bearing and treated tumor-bearing animals were anaesthetized with 3% isoflurane and were injected with 5.5±0.2 MBq APN/CD13 specific ^{68}Ga-NODAGA-c(NGR) in 150 µl saline via the lateral tail vein. After 90 min incubation time 20 min static PET scans were performed from the tumorous area using the preclinical miniPET device.

In PET/MRI studies for the image reconstruction (3D-OSEM) and analysis of the PET images Nucline and InterView™ FUSION software (Mediso Ltd., Hungary) were used. In miniPET device after 2D ML-EM iterative image reconstruction, volumes of interest (VOIs) were manually drawn around the examined regions using the BrainCAD image analysis.

After image analysis to determine the tumors and background organs radiotracer uptake SUVmean, SUVmax and T/M SUVmean, T/M SUVmax values were defined. Tumor-to-muscle (T/M) ratios were calculated from the activity of tumor and background (muscle).

3.7 Blocking experiments

For blocking experiments in the first part 200 µg unlabeled NOTA-c(NGR) or in the second part c(NGR) was injected in 100 µL saline via the lateral tail vein to He/De (n=9 in the first part of our experiments and n=12 in the second part of our experiments) and/or Ne/De (n=12) tumor-bearing rats five minutes before the injection of radiolabeled compounds. After 90 minutes incubation time *in vivo* PET imaging and *ex vivo* studies were performed using the ^{68}Ga-labeled tracers, as described above

3.8 *Ex vivo* studies

In first part of experiments after the *in vivo* PET/MRI imaging with ^{68}Ga-NODAGA-[c(RGD)]$_2$, ^{68}Ga-NOTA-c(NGR) and ^{68}Ga-DOTA-Nitroimidazole and 9±1 day (small tumor, n=3), 12±1 day (medium tumor, n=3) and 15±1 day (big tumor, n=3) after tumor cell inoculation subcutan He/De tumor-bearing and control animals were euthanized. In the second part of experiments after the *in vivo* PET/MRI imaging with ^{68}Ga-NOTA-c(NGR) or ^{68}Ga-NODAGA-c(NGR) or ^{68}Ga-NODAGA-c(NGR) (MG1) or ^{68}Ga-NODAGA-c(NGR) (MG2) and 11±1 day after tumor cell injection of He/De and Ne/De tumor-bearing (n=64) and control (n=12) animals were euthanized. In the third part of experiments after the *in vivo* PET imaging with ^{68}Ga-NODAGA-c(NGR) and 12±1 day after tumor cell inoculation of HT1080 (n=30) and B16F10 (n=30) tumor-bearing mice were euthanized. All the animals were dissected and blood, urine and samples were taken from liver, spleen, kidneys, small intestines, large intestines, stomach, heart, lungs, tumors, muscles and fat. The weight and the radioactivity of the selected organs and tissues were measured with analytical scale and calibrated gamma counter (HEWLETT PACKARD

Cobra II Autogama Gamma Counter). The uptake of the APN/CD13, $\alpha_V\beta_3$ and hypoxia specific radiotracers were expressed as %ID/g tissue using Microsoft Excel Software.

3.9 Immunohistochemistry and western blot studies

The first step in immunohistochemistry assay was that after the dissection the tumor samples was coated to gauze dressing saturated with physiological saline to avoid exsiccation. Then samples were freezed in liquid nitrogen (195,8°C) and 6 μm thick sections were made with cryostat. The sections were fixed in 10% neutral formaline then it was washed out and excavated with pH 6 citrate buffer. The sections were exposed to the mouse anti-rat CD13 antibody at a dilution of 1:50 for 35 min. The detection system based on a HRP polymer and DAB chromogen. During this procedure the slices were incubated with HRP for 15 min, then washed with PBS. For the visualisation DAB chromogen was used under microscope control. The reaction was stopped with tap water washing, followed with seed-dyeing, dehydration and covering. As positive control kidney, as negative control reaction without primer antibody was used. For evaluation Olympus B72 microscope equipped with Olympus DP72 camera and Twain Driver Ver 03.01 Image Analyzer software were used in 20x enlargement. The immunohistochemical analysis was performed by Szilvia Rácz.

For western blot analysis frozen tissue samples were pulverized under liquid nitrogen and tissue homogenization was performed with TissueLyser II (QIAGEN). Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% TritonX 100, 0.5% sodium deoxycolate, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 1 mM PMSF, protease inhibitor cocktail). After tissue

homogenization, the samples were subjected to protein isolation. Protein samples (10-40 μg) were separated on 10% SDS polyacrylamide gels and electrotransferred onto nitrocellulose membranes. After blocking for 1 h with TBST containing 5% BSA, the membranes were incubated with primary mouse anti-rat CD13 and mouse-anti-human $\alpha_V\beta_3$ integrin antibody at the dilution of 1:1000 overnight at 4°C. After washing with 1x TBST solution, the membranes were probed with IgG HRP conjugated secondary antibody (dilution: 1:2000). Bands were visualized by enhanced chemiluminescence reaction. Beta-actin was used as a loading control, and mouse kidney was used as positive control.

3.10 Statistical analysis

Experimental data was presented as mean±SD of at least three independent experiments. The significance was calculated by Student's t-test (two-tailed), two-way ANOVA and Mann-Whitney U-test. The significance level was set at $p \leq 0.05$ and $p \leq 0.01$.

4. Results

4.1 Results of the radiochemical labeling

The synthesis of the chelator conjugated peptides and nitroimidazole were successful in all cases. After the formulation step, the radiochemical purity was determined and it was more than 95% in all cases. The molar activities of the radiotracers were between 5.49 and 7.36 GBq/ μmol.

The LogP values of the investigated radiolabeled compounds were between -4.07 and -2.29, which refer to the hydrophilic character of the compounds.

All analogues were stabile (<90%) after 120 min in PBS at elevated temperature (95°C) and pH 7.4. The original structure decomposition was marked in rat serum in case of ^{68}Ga-NODAGA-c[CH_2-CO-Lys-Asn-Gly-Arg-Cys]-NH_2 and ^{68}Ga-NODAGA-c[CH_2-CO-Lys-Asn-N(Me)Gly-Arg-Cys]-NH_2 which refers to the moderate serum stability of these radiotracers.

4.2 Results of the *in vivo* and *ex vivo* investigation of hypoxia and angiogenesis

Our experiments were started with the *in vivo* and *ex vivo* investigation of control, healthy rats. 90 min after intravenous injection of ^{68}Ga-NODAGA-[c(RGD)]$_2$, ^{68}Ga-NOTA-c(NGR) or ^{68}Ga-DOTA-Nitroimidazole whole body PET/MRI scans were taken about healthy, not tumor-bearing animals. Based on the PET/MRI scans we found that all three radiotracers excreted through the urinary system. The *ex vivo* studies correlated well with *in vivo* studies.

90 min after intravenous injection of $\alpha_V\beta_3$ receptor expression related ^{68}Ga-NODAGA-[c(RGD)]$_2$, APN/CD13 receptor expression related ^{68}Ga-NOTA-

c(NGR) or hypoxia related ^{68}Ga-DOTA-Nitroimidazole, He/De tumor-bearing rats were examined with PET/MRI equipment. The subcutaneously growing He/De tumors were identified well with all three radiotracers. During qualitative and quantitative analysis of PET scans, heterogenous tumor structure and radiotracer uptake was found in He/De tumors. Significant ($p \leq 0.01$) differences were noticed among avid (radiotracer accumulative) and non-avid (radiotracer not accumulative) regions.

The APN/CD13 specificity of ^{68}Ga-NOTA-c(NGR) was confirmed by blocking experiments using *in vivo* PET imaging. Quantitative SUV data analysis showed that significantly ($p \leq 0.01$) lower SUV values were observed using the unlabeled NOTA-c(NGR) (blocking) than that of the basic studies. These results verified the APN/CD13 specificity of ^{68}Ga-NOTA-c(NGR).

Subcutan transplanted He/De tumors were inspected day by day by turns using ^{68}Ga-DOTA-Nitroimidazole for imaging hypoxia and ^{68}Ga-NOTA-c(NGR) for imaging angiogenesis. Qualitative PET/MRI image analysis showed increased level of hypoxia and angiogenesis related to tumor volume enlargement. Changes in the size of hypoxic and angiogenic regions were correlated with the tumor volume which was confirmed by the quantitative SUV data. The uptake of ^{68}Ga-NOTA-c(NGR) and ^{68}Ga-DOTA-Nitroimidazole increased with the enlargement of tumor volume. ^{68}Ga-NOTA-c(NGR) uptake correlated (R^2=0.9953) with the tumor growth. Similar results were observed when ^{68}Ga-DOTA-Nitroimidazole was used, where the increasing of tumor volume also correlated well (R^2=0.9692) with the elevated uptake of ^{68}Ga-DOTA-Nitroimidazole. By analyzing the accumulation of the two radiopharmaceuticals during tumor growth, we found that they show an

increasing uptake in close correlation with each other. Data obtained from *in vivo* PET observations was confirmed by *ex vivo* measurements.

4.3 Results of the *in vivo* and *ex vivo* comparative investigation of different c(NGR) derivatives

In the second part of our experiments a comparative study was taken with four different c(NGR) derivatives. In the first step, *in vivo* and *ex vivo* biodistribution studies were performed with ^{68}Ga-NOTA-c(NGR), ^{68}Ga-NODAGA-c(NGR), ^{68}Ga-NODAGA-c(NGR) (MG1) and ^{68}Ga-NODAGA-c(NGR) (MG2). After the qualitative and quantitative analysis of the PET images, we found low radiotracer accumulation in the abdominal and thoracic organs using the four radiotracers, however, remarkable uptake was seen in the urinary system. None of the investigated radiotracers no significant differences (at $p \leq 0.05$) were found between the uptake of the investigated organs and tissues. These *in vivo* PET/MRI results correlated well with the *ex vivo* data.

For the *in vivo* assessment of the APN/CD13 specificity of the ^{68}Ga-labeled cNGR-based tracers subcutan He/De and Ne/De tumor-bearing rats were injected intravenously with ^{68}Ga-NOTA-c(NGR), ^{68}Ga-NODAGA-c(NGR), ^{68}Ga-NODAGA-c(NGR) (MG1) or ^{68}Ga-NODAGA-c(NGR) (MG2) and after 90 min incubation time whole-body PET/MRI scans were acquired. The subcutaneously growing tumors were clearly visualized using ^{68}Ga-NOTA-c(NGR), ^{68}Ga-NODAGA-c(NGR) and ^{68}Ga-NODAGA-c(NGR) (MG1) 90 min after the tracer injection, however, ^{68}Ga-NODAGA-c(NGR) (MG2) radiopharmaceutical showed the lowest accumulation in both tumors which was confirmed by quantitative SUV analysis. Significantly ($p \leq 0.05$ and $p \leq 0.01$) lower uptake was noticed in He/De and Ne/De tumors after the

injection of ^{68}Ga-NODAGA-c(NGR) (MG2) than that of the three other radiotracers, where the SUVmean and SUVmax data were approximately 2-5-fold higher in He/De tumors and approximately 1.5-3-fold higher in Ne/De tumors. A similar tendency was observed in the tumor-to-background (muscle) ratios in both investigated tumors, where the ^{68}Ga-NODAGA-c(NGR) (MG2) radiotracer showed the lowest accumulation. All in all, it was found that NOTA- and NODAGA-conjugated c(NGR) molecules showed higher accumulation in He/De and Ne/De tumors, than the NODAGA conjugated MG1 and MG2 probes.

For the investigation of APN/CD13 specificity of ^{68}Ga-NOTA-c(NGR), ^{68}Ga-NODAGA-c(NGR), ^{68}Ga-NODAGA-c(NGR) (MG1) or ^{68}Ga-NODAGA-c(NGR) (MG2) *in vivo* blocking studies were performed. During the visual evaluation of PET scans reduced accumulation was seen in He/De and Ne/De tumors in proportion to the basic conditions. Moreover, in the course of the quantitative SUV analysis reduced uptake was observed during the blocking experiments, than that of the basic circumstances. On the whole, by the qualitative and quantitative analysis of PET images we can state the APN/CD13 specificity of the four investigated radiotracers.

The *in vivo* results are correlated well with the *ex vivo* data in both basic and blocking experiments.

4.4 Results of the investigation of the efficiency of actinonin and bestatin, as APN/CD13 inhibitors

In the last part of our experiments two naturally occurring APN/CD13 inhibitor, the actinonin and bestatin therapeutic efficiency was followed with

^{68}Ga-NODAGA-c(NGR) radiotracer on subcutan HT1080 and B16F10 tumor-bearing animals. Four days after tumor induction intraperitoneally actinonin (5 mg/kg) or bestatin (15 mg/kg) was administered for 7 days. 6 and 11 days after tumor induction and 90 min after the intravenous injection of ^{68}Ga-NODAGA-c(NGR) 20 min static PET scans were taken from the tumorous area of control-untreated and actinonin- or bestatin- treated mice.

After the qualitative PET image analysis of subcutan HT1080 tumors, we found that the untreated-control tumors and actinonin-treated tumors were well identifiable at day 6 and 11. In contrast, in the bestatin-treated group it was difficult to identify the HT1080 tumors due to the low ^{68}Ga-NODAGA-c(NGR) accumulation. After the quantitative analysis of the PET images, we found that the SUV values of the bestatin-treated tumors were significantly ($p \leq 0.05$ and $p \leq 0.01$) lower than that of the control-untreated groups or the actinonin-treated groups on day 6th and 11th. We identified the contrast of the PET images, namely the T/M SUVmean values, which indicates the efficacy of the treatments. Compared to control group's T/M SUVmean values on day 6th and 11th, significantly ($p \leq 0{,}01$) lower values were seen in the bestatin-treated group in each time point and significantly higher ($p \leq 0{,}05$) values were measured on day 11th in actinonin-treated group.

The *ex vivo* biodistribution data were correlated well with *in vivo* results. High %ID/g values were observed in kidneys and low values were detected in the abdominal and thoracic organs. No significant differences (at $p \leq 0.05$) were found between the ^{68}Ga-NODAGA-c(NGR) uptake of healthy organs and tissues when the untreated and treated animals were compared.

We determined the tumor- to- muscle (background) %ID/g ratios and we found that the T/M %ID/g values correlated well with *in vivo* SUVmean values. Compared to T/M values of control-untreated animals, significantly ($p \leq 0.05$) higher radiopharmacon uptake was measured in actinonin-treated group and significantly ($p \leq 0,05$) lower values were noticed in bestatin-treated group.

For the assessment of the effect of the actinonin and bestatin treatment on B16F10 tumor-bearing mice, PET scans were performed with ^{68}Ga-NODAGA-c(NGR). During the visual evaluation of PET images high ^{68}Ga-NODAGA-c(NGR) accumulation was observed in control-untreated tumors and moderate uptake was seen in actinonin- and bestatin-treated groups. In relation to the quantitative analysis of PET images, corresponding to the previous observations, homologous tendencies were noticed. Significantly ($p \leq 0.01$) higher SUVmean values were observed in the control-untreated group than that of the actinonin-treated and bestatin-treated groups. In the control-untreated group remarkable increasing of SUVmean values were seen between the 6th and the 11th days, while negligible elevation was observed in the bestatin-treated group and actinonin-treated group. Similarly, to HT1080 tumors the T/M SUVmean values were compared in case of B16F10 tumors. Significantly ($p \leq 0.01$) lower T/M SUVmean values were seen on day 6th and 11th in the actinonin- and bestatin-treated groups compared to the untreated-control group.

The *ex vivo* biodistribution studies were also prepared in case of B16F10 tumor-bearing animals and the results correlated well with *in vivo* results. Low uptake was seen in the thoracic and abdominal organs and high uptake was noticed in kidneys. No significant differences (at $p \leq 0.05$) were found between

the %ID/g values of healthy organs and tissues when the untreated and treated animals were compared. However, appreciably lower T/M %ID/g values were seen in the treated groups compared to the control group.

Besides of the ^{68}Ga-NODAGA-c(NGR) uptake, tumor growing were followed up. We found that the size of the control-untreated and actinonin-treated HT1080 tumors were continuously grown from the 4th day of tumor induction to the 11th day. However, significantly ($p \leq 0.01$) lower tumor volumes were measured on day 4th, 6th and 11th in bestatin-treated groups compared to the control group. In case of subcutan B16F10 tumors, the actinonin- and bestatin-treated tumors volume growing were negligible. On the other hand, the untreated-control tumors are got large volume to the 11th day.

5. Discussion

Tumor hypoxia and angiogenesis are an intensively researched area in the field of preclinical PET imaging and radiotracer development. The most frequently used radiotracer for the imaging of cancers and metastases is ^{18}FDG, however, this radiopharmacon is not specific for the imaging of hypoxia or angiogenesis, moreover less suitable for tracking the efficacy of anticancer (e.g.: anti-angiogenesis therapy) treatment. For this reason, the claim to develop more specific radiotracers were increased. In the first part of our research work the relationship between hypoxia and angiogenesis were investigated with $\alpha_v\beta_3$ specific ^{68}Ga-NODAGA-[c(RGD)]$_2$, APN/CD13 specific ^{68}Ga-NOTA-c(NGR) and hypoxia specific ^{68}Ga-DOTA-Nitroimidazole. *In vivo* and *ex vivo* studies of control, healthy animals showed that the three mentioned radiotracers are eliminated through the urinary system, which refers to these radiotracers hydrophilic character. After the transplantation of He/De tumors alternately hypoxia and angiogenesis specific radiotracers were used to examine the subcutan tumors and intriguingly 10 days after tumor cell inoculation, high ^{68}Ga-DOTA-Nitroimidazole uptake was already observed in subcutan transplanted He/De tumors at the tumor size of 125 mm^3. Moreover, from 11 days after the implantation of He/De cells hypoxic (^{68}Ga-DOTA-Nitroimidazole avid) and normoxic (^{68}Ga-DOTA-Nitroimidazole non avid) regions were clearly separated, which was confirmed by the significantly ($p \leq 0.01$) elevated SUV values in the avid regions. This heterogeneity in the tumor mass was also described by other research groups and they found that its rate can be 50-60% of the whole tumor. It is well known from the literature that NGR peptides specifically bind to APN/CD13 and RGD molecules are the ligands of $\alpha v\beta 3$ integrin receptors and they are suitable

ligands for the investigation of these receptors. Our research group previously reported that the uptake of ^{68}Ga-NOTA-c(NGR) of the primary tumors was significantly higher than that of the accumulation of the commercially available ^{68}Ga-NODAGA-[c(RGD)]$_2$ in the same tumor, when experimental renal (Ne/De) tumors were investigated. In our hepatocellular carcinoma model, this difference between the uptake of ^{68}Ga-NODAGA-[c(RGD)]$_2$ and ^{68}Ga-NOTA-c(NGR) in He/De tumors was also found. This observation is due to the higher expression of APN/CD13 in He/De tumors which was confirmed by western blot analysis. In the investigated He/De tumors heterogenous tumor structures were identified with both hypoxia and angiogenesis tracers. We hypothesized that the hypoxic and angiogenic areas increase in size with tumor volume. This assumption was confirmed since ^{68}Ga-NOTA-c(NGR) and ^{68}Ga-DOTA-Nitroimidazole uptake increased and showed strong correlation with the tumor volume enlargement. Furthermore, we hypothesized that elevation of hypoxia will induce increasingly strong angiogenesis with tumor growth. This was confirmed by indirect evidence that the increasing uptake of ^{68}Ga-DOTA-Nitroimidazole was strongly correlated with the accumulation of ^{68}Ga-NOTA-c(NGR). As our research group previously demonstrated for Ne/De (mesoblastic nephroma) tumors, ^{68}Ga-NOTA-c(NGR) is a suitable diagnostic agent for the detection of APN/CD13 expression in hepatocellular carcinoma (He/De) also. APN/CD13 and hypoxia specific radiopharmaceuticals may contribute significantly to the better understanding of the relationship between hypoxia and angiogenesis in tumors.

In the second part of our experiments comparative investigation was prepared with four different c(NGR) derivatives on control and tumor (He/De and Ne/De) bearing animals. It is well known from the literature that asparagine-

glycine- arginine (NGR) peptide sequences are specifically bind to APN/CD13 molecule, therefore from results of our and other research groups is known that ^{68}Ga-NOTA-c(NGR) is a suitable tracer for imaging of APN/CD13 expression of tumors. c[KNGRE]-NH_2 was characterized as an extraordinarily good chemically inert peptide, which was conjugated with NOTA chelator in the first step. The next step was the c(NGR) peptide conjugation with NODAGA chelator, where we hypothesised the very similar chemical properties to the NOTA chelated peptide. Furthermore, two thioether cyclised c(NGR) were conjugated with NODAGA chelator, which resulted the remaining hydrophile properties of the analogues due to the originated thiourea bond and the extra aromatic ring. The MG1 and MG2 analogues only differ in a methyl group from each other. The *in vitro* lipophilicity experiments demonstrated that newly synthesized ^{68}Ga-NODAGA-c(NGR) derivatives are highly hydrophilic (logP values were between approximately -2.2 and -4.1). This observation was confirmed by *in vivo* PET imaging and *ex vivo* biodistribution studies where it was revealed that ^{68}Ga-labeled c(NGR) derivatives were mainly excreted through the urinary system, due to their hydrophilic properties, furthermore, abdominal and thoracic organs showed relatively low radiotracer uptake. The relatively higher liver spleen and lung uptake of ^{68}Ga-NODAGA-c(NGR) (MG2) may be attributed to its lower hydrophilicity. The low radiotracer accumulation in healthy tissues also improve the quality of the images, as low background activity results high T/M ratios which greatly facilitates image evaluation and tumor identification when preparing the diagnostic report. In the second part of this comparative study, subcutan He/De and Ne/De tumor-bearing F-344 rats were used to the investigation of the APN/CD13 specificity of the four c(NGR) derivatives. Although He/De and Ne/De tumors were well

identifiable using all the four radiopharmaceuticals, different accumulation was observed in the investigated tumors. Overall, it was found that NOTA- and NODAGA-conjugated c(NGR) molecules showed higher accumulation in He/De and Ne/De tumors than the NODAGA conjugated MG1 and MG2 probes. This difference between the four APN/CD13 probes can be due to the chemical properties described above (lower stability of MG1 and probably the lower binding efficiency of N-methylated version), furthermore, - as a limitation - it is important to note that although studies have been performed at the same tumor size, it is known that the expression of angiogenic markers changes with tumor growth, can be influenced by tumor type, and is highly dependent on the presence and degree of hypoxia. The APN/CD13 specificity of the ^{68}Ga labeled c(NGR) derivatives were verified where significantly lower radiotracer uptake was observed in tumors during blocking experiments than that of basic results. Among markers of tumor angiogenesis, APN/CD13 is a very promising target in positron emission tomography imaging, however, the selection of the appropriate ^{68}Ga-labeled NGR-based radiopharmaceutical (e.g.: ^{68}Ga-NOTA- and NODAGA-c(NGR) with the highest binding affinity in this study) is critical for the precise detection of tumors angiogenesis and for monitoring the efficacy of anticancer therapy.

In the last part of our investigation the effect of actinonin and bestatin, as APN/CD13 inhibitors were examined with ^{68}Ga-NODAGA-c(NGR). In this study the effect of bestatin (originates from *Streptomyces olivoreticuli*) and actinonin (isolated from *Streptomyces Cutter C/2)* treatment on APN/CD13 expression of HT1080 and B16F10 tumors were investigated. Previous studies have shown that these cell lines showed APN/CD13 positivity and it was confirmed by our research group's results, where the control-untreated

HT1080 and B16F10 tumors were clearly visualized by the APN/CD13 specific ^{68}Ga-NODAGA-c(NGR) radiopharmacon. The next step was the investigation of the actinonin and bestatin treatment efficiency on subcutan HT1080 and B16F10 tumors using ^{68}Ga-NODAGA-c(NGR). The antitumor effect of actinonin was investigated by other research groups. In these studies, actinonin blocked the tumor growth of APN/CD13-positive and negative leukemias and lymphomas too. In *in vivo* studies actinonin generated full antitumor effect in syngeneic leukemias, however, this action was not seen in subcutan human lymphoma bearing nude mice. Our results correlated well with these previously mentioned results, in our syngeneic melanoma (B16F10) significant tracer uptake and tumor size reduction were observed after actinonin treatment, however, this dosage was ineffective in human fibrosarcoma (HT1080) tumor-bearing mice. One possible explanation for this phenomenon that APN/CD13 inhibitors may act as an immunomodulator. Hence, we cannot expect for this effect in a T and B cell deficient, HT1080 tumor-bearing CB17 SCID mice. Another possible explanation for the insufficient antitumor effect in subcutan HT1080 tumors is the dosage of actinonin. Presumably, the dose sensitivity of various types of tumors is different, dose dependent studies are required. In our studies when the efficacy of bestatin was investigated we found that this competitive inhibitor of APN/CD13 exerted its antitumor effect in both investigated tumors. The bestatin treated HT1080 and B16F10 tumor's ^{68}Ga-NODAGA-c(NGR) tracer uptake and tumor size were significantly reduced after the treatment. Bestatin can directly inhibit tumor growth and angiogenesis by blocking APN/CD13 on the tumorous cell surface, furthermore, bestatin can modulate the antitumoral immune response by enhance cell mediated immunity and induce blastogenic

and mitogenic effect. As previously observed, bestatin may augment or reduce NK cell activity which depends on the dose and the administration route. Presumably, bestatin can exerts its inhibitory effect by NK cell modulation in NK cell possessed HT1080 tumor-bearing CB17 SCID and B16F10 tumor-bearing C57BL/6 mice. In conclusion ^{68}Ga-NODAGA-c(NGR) might be suitable radiotracer for the *in vivo* monitoring of the efficacy of actinonin and bestatin- as APN/CD13 inhibitors- in subcutan HT1080 and B16F10 tumor-bearing mice.

The APN/CD13 positivity of He/De, Ne/De, B16F10 and HT1080 tumors were verified by western blot and immunohistochemistry analysis.

New findings obtained during the research work:

1. ^{68}Ga-NOTA-c(NGR) is a suitable radiotracer for the imaging of APN/CD13 expression not just in subcutan Ne/De tumors, but also in subcutan He/De tumors.

2. Based on the *in vivo* PET investigation of angiogenesis and hypoxia of subcutan He/De tumors it was established, that the fields of hypoxia and angiogenesis are grow together with the tumor volume, and it was found that with the augmentation of hypoxia, the angiogenesis intensify too. APN/CD13 and hypoxia specific radiotracers may help to better understand the relationship between hypoxia and angiogenesis.

3. *In vivo* and *ex vivo* investigations of control and He/De and Ne/De tumor-bearing animals were revealed that the four ^{68}Ga labeled c(NGR) derivatives (^{68}Ga-NOTA-c(NGR), ^{68}Ga-NODAGA-c(NGR), ^{68}Ga-NODAGA-c(NGR) (MG1), ^{68}Ga-NODAGA-c(NGR) (MG2)) excreted

through the urinary system due to their hydrophilic properties and the radiotracer uptakes were relatively low in the thoracic and abdominal organs.

4. Higher accumulation was observed in He/De and Ne/De tumors with NOTA and NODAGA conjugated c(NGR) molecules, than that of MG1 and MG2 analogues, therefore ^{68}Ga-NOTA-c(NGR) and ^{68}Ga-NODAGA-c(NGR) are promising radiotracers for the imaging of tumor angiogenesis.

5. Among the investigated APN/CD13 inhibitors, actinonin exerted it's APN/CD13 inhibitory effect on subcutan B16F10 tumors, while bestatin took effect on B16F10 and HT1080 tumors too.

6. ^{68}Ga-NODAGA-c(NGR) is a suitable radiotracer to follow acinonin's and bestatin's- as APN/CD13 inhibitors- therapeutic effect on subcutan B16F10 and HT1080 tumor-bearing animals.

6. Summary

Nowadays in the field of tumor PET diagnostics the default used radiotracer is ^{18}FDG, which can be used in a large scale of malignancies, but it’s not tumor specific and a few tumor does not accumulate this type of radiotracer. Hence, a part of radiobiological researches focused to develop new tumorspecific radiotracers. One possible tumorspecific target is the APN/CD13 cell surface molecule which is an important marker in the angiogenic process. Radiolabeled NGR peptide sequences are give an opportunity to examine the APN/CD13 expression *in vivo*.

In the first part of my thesis, the correspondence between hypoxia and angiogenesis was investigated with specific radiotracers using tumor-bearing animal models. It was noted, that the hypoxic and angiogenic areas increase in size with tumor volume, which was confirmed since ^{68}Ga-NOTA-c(NGR) and ^{68}Ga-DOTA-Nitroimidazole uptake increased and showed strong correlation with the tumor volume enlargement. Furthermore, the increasing uptake of ^{68}Ga-NOTA-c(NGR) and ^{68}Ga-DOTA-Nitroimidazole in He/De tumors were correlated well with each other, namely, with increasing hypoxia the rate of angiogenesis is also augmented. The used APN/CD13 and hypoxia specific radiopharmaceuticals may contribute significantly to the better understanding of the relationship between hypoxia and angiogenesis in tumors.

In the second part of this research work four different c(NGR) derivatives were compared and investigated in control and tumor-bearing (He/De and Ne/De) animal models. All of the investigated radiotracers have hydrophilic properties, which was confirmed by the observation that these compounds mainly excreted through the urinary system. He/De and Ne/De tumors were

well identifiable using all the four radiopharmaceuticals, however, different accumulation was observed in the investigated tumors. ^{68}Ga-NODAGA-c(NGR) (MG1) was characterized with lower serum stability and ^{68}Ga-NODAGA-c(NGR) (MG2) was marked with lower binding capacity. For tumor diagnostics the most suitable radiotracers were ^{68}Ga-NOTA-c(NGR) and ^{68}Ga-NODAGA-c(NGR).

The last step in my research work was the investigation of the suitability of ^{68}Ga-NODAGA-c(NGR) to follow the efficiency of APN/CD13 inhibitors on HT1080 and B16F10 tumor-bearing animal models. The control HT1080 and B16F10 tumors were clearly visualized by the APN/CD13 specific ^{68}Ga-NODAGA-c(NGR) radiopharmaceutical. Due to actinonin treatment B16F10, while as a result of bestatin treatment HT1080 and B16F10 tumors radiotracer uptakes and tumor volumes are decreased. Consequently, ^{68}Ga-NODAGA-c(NGR) is suitable radiotracer not just for tumor diagnosis, but also appropriate radiotracer for monitoring the therapeutic effect of APN/CD13 inhibitor treatment.

Therefore, in the field of PET imaging APN/CD13 is a promising target, which can be examined with various ^{68}Ga labeled c(NGR) derivatives both in diagnosis of tumors and in monitoring the efficacy of anti-APN/CD13 therapies.

7. Appendix

Work performed independently:

- Study planning
- He/De, Ne/De, HT1080 and B16F10 cell culturing and freezing
- Tumor induction in F-344 rats and C57BL/6 or CB17 SCID mice
- Doing tasks at animal house
- Anaesthetizing with Isoflurane
- Injecting radiopharmacons
- Performing PET scans
- Performing *ex vivo* studies
- Animal treatment with APN/CD13 inhibitors
- Processing and evaluating of the results of PET scans and *ex vivo* biodistribution studies
- Statistical analysis

Work performed by collaborators:

- Producing of radiotracers (Noémi Stéfánné Dénes and Dániel Szücs PharmD)
- Implementing of western blot analysis (Orsolya Matolay MedD)
- Implementing of immunohistochemistry assays (Szilvia Rácz and Lívia Beke)

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List of publications related to the dissertation

1. **Kis, A.**, Dénes, N., Péli-Szabó, J., Arató, V. Z., Beke, L., Matolay, O., Enyedi, K. N., Méhes, G., Mező, G., Bai, P., Kertész, I., Trencsényi, G.: In Vivo Molecular Imaging of the Efficacy of Aminopeptidase N (APN/CD13) Receptor Inhibitor Treatment on Experimental Tumors Using 68Ga-NODAGA-c(NGR) Peptide.
   BioMed Res. Inter. 2021, 1-11, 2021.
   IF: 3.411 (2020)
2. **Kis, A.**, Dénes, N., Péli-Szabó, J., Arató, V. Z., Jószai, I., Enyedi, K. N., Rácz, S., Garai, I., Mező, G., Kertész, I., Trencsényi, G.: In vivo assessment of aminopeptidase N (APN/CD13) specificity of different 68 Ga-labelled NGR derivatives using PET/MRI imaging.
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