SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

Biodistribution examinations of tumor specific ^{44}Sc- and ^{68}Ga-labeled radiotracers with preclinical imaging technics

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In vivo biodistribution examinations of tumor specific ^{44}Sc- and ^{68}Ga-labeled radiotracers with preclinical imaging technics

Dissertation in order to obtain a PhD degree in the field of nuclear medicine

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Place and date of the examination:
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1. Introduction

Over the past 30 years positron emission tomography (PET) has grown into one of the most important *in vivo* diagnostic imaging technic. Therefore continuous researches are in progress to find new, useful and non-conventional PET radioisotopes (e.g.: ^{68}Ga and ^{44}Sc).

^{68}Ga has been used since the early 2000s in numerous preclinical and clinical trials while ^{44}Sc has been in the focus of attention in the last 5 years. In the case of ^{68}Ga(III) a very wide range of chelators is in practice, but at the start of my work the same could not be said in connection with ^{44}Sc. Beside the conventional chelators AAZTA - which has been already used as Gd-contrast agents in MRI - seems to be promising. The number of other complexes specialized for scandium is very low and none of them was labeled with ^{44}Sc at the beginning of my Ph.D. work.

This may be due to - among other reasons - the fact that a new radiometal-chelator system requires a lot of time to map the optimal labeling conditions. The solution could be the microfluid and capillary systems which operates fast, automatically and works with low liquid volume and amount of samples. Moreover the system can be upgraded with a "scale-up" step making it possible to produce radiopharmaceuticals in sufficient volume and activity to perform preclinical examinations.

The overwhelming majority of the mentioned radiopharmaceuticals plays an important role in numerous type of tumor diagnosis and therapy. In the case of cancerous diseases the formation of metastases leads to the death of a large proportion of the patients. For this

reason the understanding of the metastatic processes become more and more critical nowadays. Hypoxia plays an important role in metastatic progression which occur in every tumor exceeding a certain size. This is closely related to the angiogenic process that occurs near the hypoxic areas, enabling to observe it indirectly. The expression of $\alpha_V\beta_3$ integrin receptor plays an important role in the tumor neo-angiogenesis. This molecule is overexpressed in the blood vessels of tumorous tissues, and can be targeted with RGD peptides

Malignant melanoma is a very aggressive type of cancer with high mortality due to its metastatic potential. Therefore early detection is vital to increase patient-survival rates. Many radioisotope-labeled α-melanocyte stimulating hormone (α-MSH) analogue peptides, antibodies and melanin specific molecules were tested. NAPamide and its derivatives specifically bind to melanin positive tumors and to more than 80 % of melanoma metastases expressing melanocortin-1 receptors (MC1-R).

2. Aim of our work

In my doctoral work we have focused on the ^{44}Sc radioisotope. The aim of our studies were the optimization of ^{44}Sc production, its purification and evaluation of the chemical and biological properties of ^{44}Sc-labeled radiotracers. Based on the described information, we have set the following goals:

Examine and compare ^{44}Sc production methods

Our first objective was to construct a ^{44}Ti/^{44}Sc generator which produces sufficient amount of ^{44}Sc for radiochemical measurements and biological examinations. Moreover it was important to compare the labeling efficiency of the generator- and cyclotron produced ^{44}Sc.

Use of a capillary system for optimization and production

Our next goal was the development of a capillary system for optimization of radiochemical labeling processes and reactions of the radiotracer production. These improvements make the radiolabeling process faster and more reproducible than that of manual methods.

It was planned to use the system for labeling different chelators with ^{44}Sc(III) and ^{68}Ga(III) under controlled conditions.

Chemical and biological examination of ^{44}Sc-AAZTA

We wanted to carry out labeling reactions, performing kinetic measurements and create quality check methods using AAZTA chelator and ^{44}Sc radioisotope. Then it was planned to use the chelator-

radionuclide system to map biological distribution of c(RGDfK) peptide and BSA protein with *in vivo* PET/MRI.

Comparison of ^{68}Ga- and ^{44}Sc-labeled melanoma markers

In addition we planned to investigate melanoma tumors with high metastasis potential using ^{68}Ga- and ^{44}Sc-labelled DOTA-NAPamide. Through the *in vivo* PET/MRI examinations we wanted to see the biological differences caused by the change of the radionuclide in the DOTA-NAPamide tracer.

3. Materials and methods

3.1. Methods for the production of ^{44}Sc and their comparison

When we constructed the generator Filosofov et al. were the only ones who have a ^{44}Ti/^{44}Sc radioisotope generator reported in the literature. The main difference between our and their generator was the activity of the ^{44}Ti adsorbed on the solid phase of the generator. Our system contained 3 MBq while their generator contained 185 MBq.

First, the ^{44}Ti parent nuclide was purified on a column filled with an AG 50W-X8 cation exchange resin washed by water, increasing concentrations of u.p. HCl and oxalic acid. Fractions were collected and the ones containing the most activity were separated for the second purification step. The fractions containing ^{44}Ti(IV) radioisotope were evaporated to dryness and dissolved in 0.1 M oxalic acid solution which was loaded onto a conditioned AG 1-X8 anion exchange column.

^{44}Sc(III) was eluted from the prepared system with 0.07 M u.p. HCl/0.005 M oxalic acid, which was also the final eluent. The leakage of ^{44}Ti(IV) was continuously monitored thus after approximately 60 elutions it was seen that a significant amount of parent nuclide has left the generator. Therefore it was necessary to refill the system. The whole amount of ^{44}Ti(IV) was washed out of the generator and united with the fractions collected during the elutions. The mixture was lyophilized giving us a low volume of ^{44}Ti(IV) solution which could be applied in the new AG 1X-8 anion exchange resin. At the end of the process, the column was filled with the residual inactive resin and closed. From that point desorption of the ^{44}Ti(IV) was followed by SPECT/CT.

During the cyclotron production of ^{44}Sc (200-400 MBq) we have been collaborated with the Medical Imaging Institute of University of Debrecen. Calcium (natural isotope abundance) was used to irradiate which was pressed with a hydraulic press into a pastille. The irradiation was carried out with 30 μA for 30 to 60 minutes at atmospheric pressure, then the pressed metal Ca (^{44}Sc) was dissolved in 3 M HCl solution. To purify the solution from the Ca and other metal impurities DGA resin was used with various concentrations of u.p. HCl solution, HNO_3 solution and water. The final fractions containing the highest activity were obtained in 0.1 M u.p. HCl solution.

The labeling efficiency of the generator- and cyclotron produced ^{44}Sc were compared which gave us information in connection with the relative amount of metal ion impurities. To do this we used decreasing concentrations (30 to 0.01 μM) of DOTA chelator and labelled it using

the following conditions: NH_4OAc buffer (pH = 4), 15 min reaction time and 95 °C.

3.2. ^{44}Sc and ^{68}Ga labeling optimization with the capillary system

The heart of the system is three HPLC valves which are connected via teflon capillaries. An Arduino Mega card is responsible for the control while the fluid movement is carried out by a double syringe pump. After mixing step the sample is pumped into a PEEK reactor. From the reactor the reaction mixture is transferred directly (on-line) to an analytical column where the analytical separation takes place. For the on-line analysis an UPLC system and a radioactivity detector were used.

Acetophenone and phenyl ethanol solutions were used to optimize the injection reproducibility and to synchronize fluid movement. During consecutive injections the scattering and the ratio of peaks have been examined in the UV chromatograms (220 nm). Reduction of cross-contamination between two injections was achieved by changing the composition of the washing fluid and increasing the time of the wash cycle.

Using the configured system ^{44}Sc(III) and unfunctionalized (in the following: free) chelator optimization measurements were performed. For the optimization reactions ^{44}Ti/^{44}Sc generator was used. The eluent was purified and concentrated using an AG 50W-X8 cation exchange resin. During the labeling reactions, the system withdrew 20-20 µl of buffered

^{44}Sc(III) solution and free chelator solution (DOTA, NOTA and NOPO) (200-0.6 µM). Then 10-10 µl from them were injected to a 25 µl PEEK reactor, where the mixture incubated for 5 minutes at 95 °C. After a reaction the sample pumped from the reactor to a column for analytical separation.

The labeling efficiency of the free and functionalized chelators are often different due to chemical changes. Therefore optimization was also performed with four maleimide derivatives (DOTA/DOTA-GA/NOTA/NODA-GA-Maleimide). During labeling reactions, radiochemical purity and radiochemical yields have been investigated at different pH and chelator concentrations.

We chose the ideal conditions from the optimization experiments and used them on scale-up production to reach sufficient activity and quantity of product for biological examinations. For this purpose the reactor volume was increased to 200 µl and the volume of the withdrawn reagents increased to 150-150 µl. Reaction conditions: NH_4OAc buffered radioisotope solution and 0.002 mg/ml NODA-GA-c(RGDfK) peptide solution were used. The reaction mixture was incubated also at 95 °C for 15 minutes in the reactor and after the reaction was completed the sample was injected onto an analytical column. Fractions were taken in every 30 seconds, so that the product was prepared in a small volume, which was evaporated to dryness (60 °C under N_2 gas stream) and then dissolved in 200 µl of PBS. Manual labeling was also performed under the same conditions in order to compare the results gotten with the capillary system.

3.3. Experiments with AAZTA and its derivatives

First the ^{44}Sc-AAZTA labeling conditions were optimized and compared to the parallel ^{44}Sc-DOTA results. During the optimization the effect of the following conditions on the labeling efficiency were investigated: pH, chelator concentration, reaction temperature, reaction time. First of all the effect of pH (1-9) was mapped at different chelator concentrations (0.1-100 μM). For optimization examinations 10 μl of chelator solution (1-1000 μM) was added to 90 μl buffered ^{44}Sc(III) solution (from the ^{44}Ti/^{44}Sc generator) and incubated for 5 minutes at 95 °C. To examine the effect of temperature 0.1 μM chelator concentration was used at a temperature of 25-90 °C range. To see the time dependence during the labeling reactions 0.1 μM chelator concentration and room temperature has been used.

For the kinetic examinations the ^{44}Sc-AAZTA was produced the same way as described above with 10 μM chelator concentration. The quality checked product was mixed in 1:1 ratio with 100 μl mouse plasma and incubated at 37 °C. RCP was examined after 0, 1, 2, 4, 8 and 12 hours with the above described HPLC method.

After the free chelator studies the next step was the investigation of peptide (c(RGDfK) and protein (BSA) derivatives. First it was necessary to conjugate the c(RGDfK) and the BSA with AAZTA. For the preparation of AAZTA-C9-c(RGDfK) we had to synthetize the linkable form of the chelator (AAZTA-C9-$(tBu)_4$). For this the reagents were dissolved in DMF and mixed with HBTU and DIPEA. After purification, the protected form of the product (AAZTA-C9-$(tBu)_4$-c(RGDfK)) was

treated with TFA to deprotect the molecule. Before the purification with preparative HPLC the product was washed three times with acetic-acid and evaporated to dryness.

The production of AAZTA-BSA was accomplished in three steps. First, the AAZTA-$(tBu)_4$-TFP was prepared with the addition of DCC to the mixture of TFP and AAZTA-$(tBu)_4$ in dry DCM. During the mixing step the solution have been kept at 0 °C and then reacted at room temperature for one day. After filtration, the concentrated filtrate was purified by gravity chromatography on a silica gel column. In the second step the protecting groups were removed by stirring the solution with TFA at room temperature for 2 days. Then the purified AAZTA-TFP was dissolved in DMF and then added to borate-buffered 0.9 % NaCl solution of BSA. After stirring for one day at room temperature G25 gel filtration was obtained giving us the AAZTA-BSA product.

During the next step the chelator-conjugated derivatives were labeled and examined *in vivo* with PET/MRI. To prepare ^{44}Sc-AAZTA-C9-c(RGDfK) 200 µl NH_4OAc buffered ^{44}Sc(III) solution was added to the solution of AAZTA-C9-c(RGDfK) (10 µl; 1 mg/ml), and incubated at 95 °C for 15 min. The crude product was purified on a conditioned Strata X column, eluted with ethanol and evaporated to dryness. The product was dissolved in 200 µl of PBS and used for *in vivo* examinations.

During the preparation of ^{44}Sc-AAZTA-C9-BSA the conjugated protein was also mixed with 200 µl of NH_4OAc buffered ^{44}Sc(III) solution. The reaction was incubated at 40 °C for 60 minutes and the crude product was purified by gel filtration.

3.4. Experiments with $^{44}Sc/^{68}Ga$-DOTA-NAPamide

To prepare ^{68}Ga-DOTA-NAPamide for biological investigations $^{68}Ge/^{68}Ga$ isotope generator was eluted in fractions. A portion of the most active fraction was buffered with NH_4OAc (0.5 M, pH = 4) and mixed with 5 µl DOTA-NAPamide then incubated for 15 minutes at 95 °C. After the end of the reaction the mixture was purified on a conditioned Strata X and the product was dissolved in 200 µl of PBS.

We used cyclotron produced ^{44}Sc for the preparation of ^{44}Sc-DOTA-NAPamid. The ^{44}Sc(III) solution obtained after the purification was reacted and processed the same way described in the case of ^{68}Ga-DOTA-NAPamide.

For the determination of logP, $^{68}Ga/^{44}Sc$-DOTA-NAPamide product solution was prepared as described above and then fixed volumes added into a mixture of 1-octanol and water (1:1). The biphasic system was shaken for 10 minutes and centrifuged for 5 minutes, samples were taken from the separated phases and their activity determined using a gamma counter.

For kinetic investigation $^{68}Ga/^{44}Sc$-DOTA-NAPamide product solutions were added to mouse plasma, Na_2EDTA solution and oxalic acid solution. The resulting solutions were incubated at 37 °C and the RCP of the samples were checked at 0, 30, 60, and 120 minutes.

3.5. Materials and methods used during *in vitro* examinations

For the examinations targeting melanoma tumors B16-F10 and A375 cells were used. The cells were kept in DMEM supplemented with 10 % FBS serum, 1 % non-essential amino acids and 1 % MEM vitamin. For the angiogenic investigation 4T1 cells were used and cultured in RPMI-1640 + 10 % FBS. During culturing of each cell line, 5 % CO_2 and 37 °C conditions were used. For *in vitro* experiments and induction of tumors the cells were used after 6-8 passage. Cell viability was above 90 % in all cases, which was checked by trypan blue exclusion test.

For *in vitro* binding assays of melanoma B16-F10 cells were cultured on a 24-well plate for 24 hours and then $^{44}Sc/^{68}Ga$-DOTA-NAPamid added to them at different concentrations (20-3200 nM). After 60 minutes of incubation the medium was removed, the cells were washed with PBS and glycine and then lysed with NaOH for 10 minutes at 37 °C. The radioactivity of the samples was measured by a gamma counter.

For uptake studies B16-F10 and A375 cells were taken up with trypsin, centrifuged, resuspended in glucose-containing PBS and incubated at 37 °C for 10 minutes. Subsequently $^{44}Sc/^{68}Ga$-DOTA-NAPamide was added to the cells and incubated at 37 °C for 30, 60 and 90 minutes. After this it was washed with PBS, suspended, and the radioactivity of the samples was measured using a gamma counter. The decay corrected radiotracer uptake was given in ID% value. All of the results contain ± SD data of at least three independent experiments.

3.6. Materials and methods used during *in vivo* examinations

For animal experiments 10-12 weeks old adult female C57BL/6, BALB/c and CB17 SCID mice were used. Mice were kept under sterile conditions in an IVC cage system at constant temperature, humidity and automated artificial illumination. Experimental animals were fed *ad libitum* with VRF1 and autoclaved tap water. The experiments were carried out with the permission of the Animal Experiment Committee of the University of Debrecen (8/2016/DEMÁB) in according to the European and Hungarian law.

The tumor cell injection to the animals was carried out under inhaled anesthesia. For the syngeneic melanoma model, B16-F10 tumor cells were injected under the skin on the left shoulder area of C57BL/6 mice. To create the xenograft melanoma model, Melur tumor cells were injected under the skin on the left shoulder area of SCID mice. To produce the synthetic breast tumor model, 4T1 cells were injected under the skin on the left shoulder area of BALB/c mice. *In vivo* experiments were performed after 20 ± 2 days after the injection of tumor cells.

PET imaging were performed in the Preclinical Laboratory of the Translation Research Center of Scanomed Ltd.. Control and tumor-bearing mice were under inhalation anesthesia during the injection and the imaging. For PET assays approximately 15 MBq radiotracers were injected via the lateral tail vein.

Whole body static PET imaging were performed with a preclinical PET/MRI device at 60, 90 and 240 minutes after the injection of the

radiotracers. T1 weighted MRI records were made to determine the anatomical localization of tissues and organs.

Reconstruction of the PET data was performed with three dimensional iterative algorithms. Determination of the activity concentration of test areas was made by manually drawing VOIs. After it the software automatically calculated the SUVmin, SUVmax and SUVmean values based on 3D VOIs.

The control and tumor-bearing experimental animals were euthanized after the *in vivo* imaging examination. The abdominal and the chest were opened then blood was taken from the heart then a piece was removed from specified organs. During the *ex vivo* investigation mass and radioactivity of the samples were measured and the final result was expressed as % ID/g tissue.

During *in vivo* melanoma blocking studies B16-F10 tumor (high MC1-R expression) bearing mice injected with α-MSH blocker agent and with ^{44}Sc/^{68}Ga-DOTA-NAPamide radiotracer intravenously. One hour after injection in vivo PET/MRI and *ex vivo* biodistribution studies were performed based on the methods described above.

4. Discussion of results

My work consisted of four main series of experiments that can be evaluated together and separately, but their common point is the ^{44}Sc radioisotope, which has been tested for different ways of productions, labeling reactions with chelators, peptides and a BSA protein. The labeled derivatives also examined *in vitro, ex vivo and in vivo*. The following conclusions were made during these tests:

1. In the first step, a ^{44}Ti/^{44}Sc isotope generator was constructed to test the elution methods, the labeling efficiency of the eluted ^{44}Sc(III), and the process of refilling. Our results were compared with a similar ^{44}Ti/^{44}Sc isotope generator already has been described in the literature. Examining the labeling efficiency gave us the same results, while in connection with the method of elution, we came to the opposite conclusion with the reference. In addition, we have shown that the ^{44}Sc produced in a cyclotron can be used with lower chelator concentration than that of the ^{44}Sc obtained from the ^{44}Ti/^{44}Sc generator.

2. We have successfully developed a capillary synthesis system that allows to done optimization labeling reactions in a short period of time with small amounts of material using ^{68}Ga(III) and ^{44}Sc(III) radiometal ions and various chelators. In our optimization measurements, it was found that the DOTA-GA and NODA-GA chelators were the most suitable for the coordination of ^{68}Ga(III) and ^{44}Sc(III).

3. In the next step, we performed experiments with AAZTA which is a new chelating agent in the field of nuclear medicine. It soon became apparent that the ^{44}Sc-AAZTA system surpassed the results of the chelators mentioned in the previous point. At 95 °C and pH = 3-4 with 5 minutes reaction time and using very low AAZTA concentration (0.1 μM) a high labeling efficiency (>80%) could be reached. In addition, one of our most valuable results was the evidence that the ^{44}Sc-AAZTA radiometal-chelator system allows to label at neutral pH and room temperature, which can open a new field of diagnostic and therapy with pH- and thermosensitive molecules, antibodies and proteins.

4. As a final series of measurements DOTA-NAPamide melanoma marker peptide derivative was labeled with ^{68}Ga(III) and ^{44}Sc(III) to compare the effect of radiometal on *in vivo* biodistribution and kinetics. It has been found that the ^{44}Sc-labeled product is more kinetically inert in mouse plasma, it has a higher rate of tumor accumulation. The result of this experiment also confirmed the hypothesis that radiotracers labeled with ^{44}Sc(III) could have more favorable properties than that of the ^{68}Ga-labeled ones.

4.1. Interpreting and discussing the result of ^{44}Sc production

Our initial goal has been fully implemented so in the Radiochemistry Laboratory of Scanomed Ltd. a ^{44}Ti/^{44}Sc isotope generator was constructed. The established system provided enough activity to perform radiochemical optimization reactions and preliminary biological experiments. At the beginning of my work there was only one published ^{44}Ti/^{44}Sc generator (Filosofov et al.). The most significant difference between the two isotope generators is the activity since the Filosofov-generator provides 180 MBq while ours produces 3 MBq of ^{44}Sc. On our side this was a deliberate decision since we wanted to demonstrate that a sixty times "smaller" (and cheaper) generator can produce sufficient amount of radioisotope for radiochemical optimization and preliminary biological examinations.

Our result with the generator was the same as that of the Filosofov-generator, except the ^{44}Ti(IV) breakthrough. First we used the recommended "direct-reverse" elution method but it was seen that approximately 0.3 MBq of ^{44}Ti parent nuclide left the generator at the revers step. For this reason during the other approximately 60 elutions just the direct step was used. This is probably due to the localization of the parent nuclide since the ^{44}Ti(IV) was adsorbed onto the top of the resin-column resulting higher leakage at the top side than that of the bottom. The activity of the eluates were measured each time so we could follow the leakage and the ^{44}Ti(IV) level of the generator. If we plotted the generator activity against the time (through approx. half a year) it could be seen from the slope of the curve that the activity is decreasing. The

theoretical half-life calculated from the slope was lower than that of the half-life of the ^{44}Ti. It proved that the decrease of the whole eluent activity was due to the continuous desorption of the parent nuclide. Thanks to the SPCET/CT we could observe the unfavorable motion of the ^{44}Ti(IV) on the resin which made it easier to define the exact point when it was necessary to refill the generator. We performed the refill after 60 elutions and in the case of the new system the parent nuclide took place in the middle of the resin column. The "direct-revers" elution method was also tested but soon it was visible that we couldn't reduce the "tailing effect" of the ^{44}Ti(IV) in the longitudinal direction. It might be caused by the increased volume of eluent since in the case of the first setup 10 ml of eluent was used directly but in the second case during the "direct-revers" elution 10-10 ml (summarized: 20 ml) was used.

When comparing the ^{44}Sc produced in the generator and in the cyclotron, it is worth mentioning first the radionuclide purity. In the case of the generator, the only radionuclide impurity can be ^{44}Ti, which can get into the eluate by desorption from the stationary phase. This was approximately 0.03-0.21%, resulting in 99.97-99.79% radionuclide purity for ^{44}Sc. This value is the same as Filosofov et al. experience. In contrast, in the cyclotron production, 3 other scandium isotopes were produced (^{44m}Sc, ^{47}Sc, ^{48}Sc) which cannot be separated by the same element with chemical methods. Radionuclide purity of the samples obtained with cyclotron was 94.19±1.35%, which correlated to the value (95.7%) in the literature. Based on these, we can state that the radionuclide purity of the ^{44}Sc from the generator is nearly 5% higher than that of the cyclotron produced.

To get more information in connection with the metal ion impurities we performed labeling reactions with the isotope generator of Scanomed Ltd. and the ^{44}Sc produced by the Institute of Medical Imaging of University of Debrecen. The results were well correlated with our expectations and the data can be found in publications. Therefore it can be stated that ^{44}Sc produced in cyclotron labels with approximately one order of magnitude lower chelator concentration (near the inflexion point) than that of the ^{44}Sc produced in generator. This is probably due to the fact that the solution eluted from the generator contains more metal impurities causing a negative effect on the labeling. Often the lowest measurable amounts of metal impurities are present in the solution at orders of magnitude higher concentration than the radioisotope. That's why "competitive" processes can take place which may put the complexion of the radioactive isotope into the background. Depending on the type of radiometal and chelator the labeling efficiency is mostly affected by Zn^{2+}, Fe^{3+} and Cu^{2+}. The eluent typically contains metal ions only at ppm concentration range but due to the multiple elution of the isotope generator, these impurities can accumulate and subsequently contaminate the eluate. In addition the elution profile of the ^{44}Ti/^{44}Sc isotope generator shows that ^{44}Sc(III) can be eluted in relatively large volume which can cause varying impurity levels in different fractions. The effect of metal ion impurities on labeling was consistent with the data reported in the literature

Compared to the cyclotron produced ^{44}Sc the advantage of the constructed isotope generator is the easy handling, the cost-effectiveness and the activity supply over a long period of time. The main disadvantages

of the generator is the amount of metal impurities accumulated and washed out from the system causing worse labeling efficiency than in the case of the ^{44}Sc produced in cyclotron.

4.2. Interpreting and discussing the results obtained with the capillary system

During the construction of the capillary system our main goal was to create an apparatus for quick labeling optimization with good reproducibility and on-line analytical separation while the instrument works with a minimum amount of ligand. Compared to commercially available synthesis panels and modules one of the most important difference is the reaction volume. Modules generally work with 5-20 ml of liquid movement, 10-20 ml final product volume and significant ligand excess. The reproducibility of injection was optimized with 10-10 µl of precursors while the preparative production was carried out with 100-100 µl of total injection. Thanks to this we could reach high activity concentration and high specific activity with consumption of small amount of ligand.

After the appropriate adjustments and fine-tuning we started the investigation of reproducibility with the labeling reactions. It has been found that the relative standard deviation (RSD) was 1-3 % at the inflection point of the RCP-chelator concentration curves. In the previous case same ^{44}Sc(III) solutions were used but if we compared the labeling yields using solutions from two different elution the RSD increased to 10-

30 %. This effect could also be explained with the varying amount of metal impurities dissolving from the generator during the different elutions. Furthermore it is important to note if we perform examinations not so close to the inflexion point the RSD drops under 2 % in every case.

Then non-functionalized, "free" chelators were labeled using ^{44}Sc(III). The three selected cheaters were DOTA, NOTA and NOPO. Comparing the obtained results it was found that NOTA and DOTA behaves similar and could reach a yield of approximately 90 % using 3 μM chelator concentration. For NOPO only 20 % yield was observed at the same chelator concentration, which did not increase to the addition of extra chelator. For comparison we used ^{68}Ga labeling data found in the literature. It showed that ^{68}Ga-DOTA and ^{68}Ga-NOTA can be produced with a yield of 90 % at about 5 μM, while the same value can be reached with ^{68}Ga-NOPO at 0.1 μM. Compared to our results it can be stated that the DOTA and the NOTA chelators showed similar affinity to ^{44}Sc(III) and ^{68}Ga(III). In addition we have also seen that ^{44}Sc-NOPO has worse affinity to ^{44}Sc(III) using the similar circumstances. The low labeling yield of NOPO can be explained by the formation of a non-beneficial coordinative bond between the phosphinate arms and the metal ion.

Free chelator studies needed to be supplemented, as in the field of radiopharmaceuticals typically one of the carboxyl groups of a bifunctional chelators is functionalized and unable to form coordinating bonds. Therefore we used mono-functionalized DOTA and NOTA derivatives. Results of ^{68}Ga(III) were similar in the case of each four chelator derivatives that were correspond to the data found in literature. With ^{44}Sc(III) DOTA-GA-maleimide proved to be the best, while NOTA-

maleimide was the worst. Unlike the expectations, the second best was NODA-GA-maleimide, not the suspected DOTA-maleimide. These data are contradicted by K. A. Domnanich's result, which described the mono-functionalized DOTA better than the mono-functionalized NODA-GA. The experiment was repeated on the next day but the same curves were obtained. The difference between K. A. Domnanich et al. and our results can be explained in two ways. The first is the ligand effect, where the molecules (simple groups, peptides, proteins, etc.) conjugated to the chelator have a significant effect on complexing properties through the change in steric relationship and electron distribution. We studied small-molecule (maleimide) functionalized chelators while K. A. Domnanich et al. investigated chelators conjugated with peptides like RGD and NOC. Such changes in the structure of the linked ligands can already influence the complex formation so much to see these type of differences. Another possible explanation is the variable amount of metal impurities. K. A. Domnanich et al. has also shown that the ^{44}Sc-NODA-GA-RDG/NOC system is much more sensitive to Cu^{2+} and Fe^{3+} contaminants than that of ^{44}Sc-DOTA-RDG/NOC. It would mean that we could grant lower levels of impurities therefore labeling efficiency of NODA-GA-maleimide was increased more than that of DOTA-maleimide.

Due to the above-described chelator order the preparative labeling with the capillary system was performed with ^{68}Ga-NODA-GA-c(RGDfK). For this purpose the withdrawn liquid volumes were increased from 10-10 µl to 100-100 µl to reach a final volume what can be directly used for biological examinations. Since there is a rather large literary

background of ^{68}Ga-NODA-GA-c(RGDfK) we have compared our results with other research groups. Based on these we can say that our capillary system can operate with 1-2 orders of magnitude less ligand than some synthesis modules. During a manual labeling or labeling with a synthesis module up to 10-20 μg of peptide is used, while in our case this value can reach 0.6 μg. Moreover the reaction time is less than the general 15-20 minutes since typically 5-10 minutes is enough thanks to the good heat transfer and high surface-volume ratio. In our case the total production takes about 30 minutes which includes the liquid movement, the reaction itself and the preparative separation.

4.3. Interpretation and discussion of the results obtained with ^{44}Sc-AAZTA and its derivatives

AAZTA chelator was provided by Bracco pharmaceutical manufacturer company. First it is important to point out that the labeling reactions in this section were performed manually to get totally literature-comparable result. To make our comparison full we decided to fulfill the labeling experiments not only with AAZTA but with a reference chelator: DOTA. Optimization surfaces were made and these revealed that AAZTA is an outstanding chelator for the ^{44}Sc(III) as it can provide high yield at low chelator concentrations on a very wide pH range. Although DOTA is a suitable complexing agent for ^{44}Sc(III) the pH-range of the ideal labeling reaction is quite narrower (2-4). It is important to mention that it is possible to perform good yields with AAZTA at room temperature and around pH = 7 which is promising in the field of heat and pH sensitive

compounds (certain peptides, proteins and antibodies). The labeling with good yield at neutral pH is mainly due to the favorable hydrolytic properties of Sc(III), whereby hydrolysis under physiological conditions begins at a higher pH than in the case of Ga(III). Radioisotope-chelator can be used at room temperature on pH = 7 are the followings: ^{64}Cu-TETA, ^{111}In-HBED and SHBED, ^{89}Zr-DFO, and ^{68}Ga-AAZTA and DATA. It could be seen that there are not too much of them which meaning that with ^{44}Sc-AAZTA system the spectrum of these pairs has been expanded.

Based on the results described above PET/MRI was used to determine *in vivo* biodistribution of ^{44}Sc(III), ^{44}Sc-AAZTA and derivatives of peptide and protein in healthy animals. ^{44}Sc(III) was in the liver predominantly while ^{44}Sc-AAZTA was excreted very quickly through the kidneys to the urine as it was expected. ^{44}Sc-AAZTA-C9-c(RGDfK) was also found in the gall bladder, intestine, and bladder as described in the literature with other RGD-based radiotracers. This may be due to the higher expression of $\alpha_v\beta_3$ integrin in the given areas, the more lipophilic property of the substance or the hepatobiliary pathway. After the injection of the 4T1 tumor-bearing BALB/c mouse, there was a significantly higher accumulation in the tumor than in the surrounding organs. The level of accumulation was given in the ratio of tumor/muscle with a value of approximately 25, which is remarkably high compared to the same value (1.8-12.8) of other RGD-based radiotracers.

With the aid of these result we can prove that the produced ^{44}Sc-AAZTA-C9-c(RGDfK) has similar or better biodistribution

properties than other derivatives already published in the literature. Thanks to high tumor/muscle ratio, the low ligand consumption, and the favorable 4-hour half-life of the ^{44}Sc it can provide a promising alternative in the future.

In addition to the disclosures the effectiveness of labeling at room temperature and on pH = 7 was proved through AAZTA-C9-BSA labeling. Following the conjugation of BSA with AAZTA-C9 it was labeled with ^{44}Sc(III) under the described conditions, then injected into a healthy mouse for PET/MRI imaging. Healthy mouse was used because our primary goal was to investigate the *in vivo* kinetic behavior and biodistribution of ^{44}Sc-AAZTA-C9-BSA. As expected 90 minutes after injection most of the activity was observed in the heart and liver.

4.4. Interpretation and discussion of results obtained in ^{44}Sc/^{68}Ga-DOTA-NAPamide experiments

For the following experiment we used ^{44}Sc produced in cyclotron. The purpose of this part of my work was to map the MC1-R specificity of a newly prepared and used ^{44}Sc-DOTA-NAPamide radiotracer. ^{44}Sc-DOTA-NAPamide labeling was developed based on the ^{68}Ga-labeled derivative. As a result we could produce the radiotracers with 99 % of RCP, each having a specific activity of over 15 GBq/μM. When compared to ^{68}Ga-labeled derivative, the first deviation can be seen between the decay-corrected radiochemical yield (^{44}Sc-DOTA-NAPamide: 60-70 %; ^{68}Ga-DOTA-NAPamide: 80-86 %). The lower yield in the case of ^{44}Sc(III) was caused by the extra

purification and concentration-step we had to use due to the cyclotron production. Based on logP assays (^{44}Sc-DOTA-NAPamide: -3.30; ^{68}Ga-DOTA-NAPamide: -3.50), both radiotracers were hydrophilic so they were expected. It should be noted that - based on logP values - ^{44}Sc-labeled molecule showed a bit higher hydrophobic character than that of the ^{68}Ga-labeled. This is probably due to the fact that Ga^{3+} typically forms octahedral structures through 6 coordinate bonds. Therefore only 6 of the 7 donor atoms of DOTA-NAPamid may be coordinated (4 ring-N and 2 deprotonated carboxyl arms), which can result a carboxyl arm remaining uncoordinated and the complex will not be charge-neutral. ^{44}Sc(III) tends to create 7 or even 8 coordinate bonds which can also coordinate the third carboxyl arm to make the entire molecule charge-neutral, causing differences in logP values.

Kinetic measurements carried out in Na_2EDTA, oxalic acid and mouse plasma showed that ^{44}Sc-DOTA-NAPamide was more kinetic inert since no RCP change was observed after 2 hours. In the case of ^{68}Ga(III) the RCP value was decreased by 5% in mouse plasma after 1 hour. As described, it can be stated that the similar or better chemical properties of ^{44}Sc-DOTA-NAPamide and the approximately four times longer half-life of ^{44}Sc(III) have an advantage over ^{68}Ga-DOTA-NAPamide.

The MC1-R specificity was first tested on receptor-positive B16-F10 tumor cells *in vitro*. The binding studies showed that the total binding value (mol/cell × 10-18) increased quickly to approximately 1600 nM of (^{44}Sc/^{68}Ga-)DOTA-NAPamide concentration and after it reached 3600 nM the system became steady state. We have confirmed

that it has been published in the literature that among other DOTA-α-MSH analogs the DOTA-NAPamide is a very suitable radiotracer for imaging MC1-R. In addition, the low accumulation of radioactivity in MC1-R negative A375 melanoma cells and the approximately 5-6 fold higher %ID uptake in MC1-R positive B16-F10 cells also demonstrated the DOTA-NAPamide specificity.

After *in vitro* assays, we examined the biodistribution of both substances using healthy control and tumor-bearing mice. PET/MRI imaging and *ex vivo* biodistribution studies were used to confirm the renal excretion by the high kidney uptake. In addition, low uptake was observed in the chest and abdominal regions. For melanoma specificity examinations –with α-MSH analogs - we used B10-F19 tumors with high uptake and A375 tumors as negative reference. In addition in the case of subcutaneous injection of B16-F10 tumors - thanks to the MC1-R expression - high contrast can be reached. It can be stated that - although slightly - higher SUV and tumor/muscle values were obtained with ^{44}Sc-DOTA-NAPamide than that of ^{68}Ga-DOTA-NAPamide. Similar observations were described by Domnanich et al. when they compared ^{68}Ga- and ^{44}Sc-labeled DOTA- and NODA-GA-RGD derivatives. They published that in the case of the ^{44}Sc-labeled complex the tumor uptake and tumor/background ratio was higher than for the ^{68}Ga(III) complex. The difference observed by us can be explained by differences from logP assays and from kinetic measurements. The best tumor/tissue contrast was obtained within one hour from the injection with ^{68}Ga-labeled DOTA-NAPamide. For ^{44}Sc-DOTA-NAPamide, it was found that 4 hours after injection, the tumor/muscle ratio was higher than that of the 1-hour.

This is due to the near 4-hour half-life of ^{44}Sc, its kinetic inertness under *in vivo* conditions, and the slow elimination from tumor.

Despite the very promising results, blocking assays were also performed to fully demonstrate the MC1-R receptor specificity of ^{44}Sc-DOTA-NAPamid. After injecting a sample containing α-MSH and ^{68}Ga/^{44}Sc-DOTA-NAPamide, the uptake of labeled peptides significantly decreased in MC1-R positive B16-F10 tumors, confirming the specificity.

In the light of the foregoing, it can be seen that the results gained during ^{68}Ga-DOTA-NAPamide examinations correlated with the published data, and gave us a reference point to compare the ^{44}Sc-DOTA-NAPamide to. It can be stated that the ^{44}Sc-labeled peptide has the same MC1-R specificity compared to ^{68}Ga-labeled derivative and other compounds in the literature. In addition, the *in vivo* kinetic inertness and the rate of tumor/muscle in PET/MRI imaging is outstanding. Important to mention that ^{44}Sc-DOTA-NAPamide is the first ^{44}Sc-labeled radiotracer for melanoma detection.

5. Summary

^{68}Ga is one of the most widely used PET radionuclide produced in isotope-generator. In other hand its use and delivery is limited by the relatively short half-life of 1 hour. An alternative solution can be provided by ^{44}Sc(III) which has similar complexation properties than that of ^{68}Ga(III), but a half-life of 4 hours. Therefore our aim was to map the production, and the chemical and biological behavior of the labeled derivatives and compare the gained results.

We successfully constructed a ^{44}Ti/^{44}Sc isotope-generator using ^{44}Ti radioisotope as parent nuclide. Beyond that the operation, the elution and the recharging effect of the generator were tested. It was found that the referred and applied direct-revers elution method resulted a considerable ^{44}Ti(IV) loss with every case of milking. In addition, we tested the generator and cyclotron produced ^{44}Sc with the aim of labeling reactions and compered with results found in the literature, which suggest a high correlation to our data.

A capillary system was developed for radiochemical labeling optimization that quickly and reproducibly provide information in connection with affinity between ^{44}Sc(III) and different chelators. It has been shown that free NOPO and mono-functionalized NOTA was not suitable for ^{44}Sc(III) complexation, but the mono-functionalized DOTA, DOTA-GA, and NODA-GA seemed to be extremely promising.

We had the opportunity to collect information in connection with the labeling properties of the ^{44}Sc-AAZTA complex. First it has been found that high radiochemical yields can be achieved with extremely low

amount of chelator in the pH-range of 3-7. For this reason, a peptide (^{44}Sc-AAZTA-C9-c(RGDfK)) and a protein (^{44}Sc-AAZTA-C9-BSA) were also labeled and *in vivo* tested, where the expected results were obtained.

To get a wider view about biological behavior, ^{44}Sc-DOTA-NAPamide was prepared as an analog of the MC1-R specific ^{68}Ga-DOTA-NAPamide, which was characterized and compared with other melanoma markers. As a result, the ^{44}Sc-DOTA-NAPamide has an outstanding chemical, kinetical and biodistribution properties compared to other MC1-R specific α-MSH analogues. In addition, it should be highlighted that ^{44}Sc-DOTA-NAPamide is the first ^{44}Sc-labeled biomarker for melanoma imaging.

6. Attachment

Work done independently

- Construction, testing and use of the $^{44}Ti/^{44}Sc$ generator in comparative and labeling experiments
- Processing, purification, concentration and use of cyclotron-produced ^{44}Sc in comparative and labeling experiments
- Inactive testing and adjustment of capillary system, then use for chelator comparative reactions
- Design and implementation of optimization labeling reactions with AAZTA chelator
- Synthesis and ^{44}Sc-labeling of AAZTA-C9-c(RGDfK) peptide and AAZTA-BSA
- Labeling reactions with $^{68}Ga(III)/^{44}Sc(III)$ with DOTA-NAPamid
- Experimental design, processing and evaluation of results

Collaborative work:

- ^{44}Sc production in cyclotron (Dezső Szikra Ph.D.)
- Adjust ^{44}Sc-channel on dose calibrator (Sándor Szabó Ph.D., Attila Forgács Ph.D.)
- Construction of capillary synthesis system (Dezső Szikra Ph.D.)
- Making AAZTA available to us (Zsolt Baranyai Ph.D., Bracco)
- Synthesis of AAZTA-$(tBu)_4$-TFP (Anikó Fekete Ph.D.)
- *In vitro*, *ex vivo* and *in vivo* biological examinations (György Trencsényi Ph.D., Tamás Nagy Ph.D.)

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List of publications related to the dissertation

1. **Nagy, G.**, Szikra, D. P., Trencsényi, G., Fekete, A., Garai, I., Giani, A. M., Negri, R., Masciocchi, N., Maiocchi, A., Uggeri, F., Tóth, I., Aime, S., Giovenzana, G. B., Baranyai, Z.: AAZTA: an Ideal Chelating Agent for the Development of 44Sc PET Imaging Agents.
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List of other publications

3. Trencsényi, G., Dénes, N., **Nagy, G.**, Kis, A., Vida, A., Farkas, F., Péli-Szabó, J., Kovács, T., Berényi, E., Garai, I., Bai, P., Hunyadi, J., Kertész, I.: Comparative preclinical evaluation of 68Ga-NODAGA and 68Ga-HBED-CC conjugated procainamide in melanoma imaging.
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