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Evaluation of a PACAP peptide analogue labeled with ^{68}Ga using two different chelating agents.

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ABSTRACT

Objective: The authors have conjugated chelating agents (DOTA and NODAGA) with a peptide (PACAP analogue) which has a high affinity for VPAC1 receptors expressed on cancer cells. In order to determine a suitable chelating agent for labeling with ^{68}Ga, we have compared the labeling kinetics and stability of these peptide conjugates.
Methods: For labeling, $^{68}GaCl_3$ was eluted in 0.1 M HCl from a [^{68}Ge-^{68}Ga] generator. The influences of peptide concentration, pH and temperature on the radiolabeling efficiency were studied. The stability was evaluated in saline, human serum, DTPA, Transferrin and metallic ions ($FeCl_3$, $CaCl_2$ and $ZnCl_2$). Cell binding assay was performed using human breast cancer cells (T47D). Tissue biodistribution was studied in normal athymic nude mice.
Results: Optimal radiolabeling (> 95.0%) of the DOTA-peptide conjugates required higher (50°C–90ºC) temperature and 10 min incubation at pH 2–5. The NODAGA-peptide conjugate needed incubation only at 25°C for 10 min. Both radio-complexes were stable in saline, serum as well as against transchelation and transmetallation. Cell binding at 37°C for 15 min incubation with ^{68}Ga-NODAGA-peptide was 34.0% as compared to 24.5% for ^{68}Ga-DOTA-peptide. Tissue biodistribution at 1 hr post-injection of both ^{68}Ga labeled peptide conjugates showed clearance through the kidneys.
Conclusions: NODAGA-peptide showed more convenient radiolabeling features than that of DOTA-peptide.
Key words: Breast cancer, Prostate Cancer, chelating agent, peptides, radiopharmaceuticals.

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Introduction

Positron emission tomography (PET) has become a prominent imaging modality in the field of oncology.[1] PET has several technical advantages over the single photon emission computed tomography (SPECT) like attenuation correction, and higher spatial resolution.[2-3] Small molecules such as sugars, amino acids, nucleic acids, or receptor-binding ligands are labeled with positron-emitting radionuclides for PET imaging to study *in vivo* visualization of physiological processes on a molecular level.[4-7] There are several positron emitting radionuclides like fluorine-18 [^{18}F] ($t_{1/2} \approx$ 110 min), copper-64 [^{64}Cu] ($t_{1/2} \approx$12.7 hr), carbon-11 [^{11}C] ($t_{1/2} \approx$ 20 min), and oxygen-15 [^{15}O] ($t_{1/2} \approx$ 2 min). However, their production requires in-house cyclotron, not available at every academic institution and hospital.

In recent years, Gallium-68 [^{68}Ga] - a positron emitting radionuclide conveniently available in the form of Germanium-68 ($t_{1/2} \approx$ 270 days)-Gallium-68 ($t_{1/2} \approx$ 68 min) [^{68}Ge → ^{68}Ga] generator has drawn considerable attention for oncologic diagnostic applications. The short physical half-life of ^{68}Ga induces low radiation burden on patients and makes it an ideal radionuclide for diagnostic use.[8-10] The labeling of biomolecules with ^{68}Ga is achieved by using bi-functional chelators (BFC) such as DTPA (diethylene triamine pentaacetic acid), DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid), or NODAGA (1,4,7-triazacyclononane-1-glutamic acid-4,7-diacetic acid). BFCs differ from each other in their physio-chemical properties which can influence radiolabeling kinetics. They can also influence overall charge of the complex, biodistribution and more importantly uptake in the tumor site.[11-13]

In recent years, there has been increasing interest in the receptor targeted scintigraphy in which peptides have emerged as promising biomolecules.[14] Their properties such as fast clearance, rapid tissue penetration, low antigenicity, and relatively easy synthesis have added to their applicability.[15] In past few years, our laboratory has designed, synthesized and radiolabeled peptide conjugates which have high affinity for VPAC-1 receptors, over-expressed in many malignant tumors including those of the breast and prostate.[16-20] The peptides are analogues of Pituitary adenylate cyclase-activating peptide (PACAP) - a 27 amino acid peptide and vasoactive intestinal peptide (VIP)- 28 amino acid. Both have similar biochemical properties and bind to VPAC1 receptors expressed in high density on the surface of certain cancers such as of the breast, prostate and urinary bladder (100%), colon (96%), pancreas (65%), lung (58%), stomach (54%), and liver (49%).[21-24]

The present study was aimed to investigate the influence of these two BFC agents on the PACAP analogue, which differs in terms of their radiolabeling kinetics, biological activity and stability of the resultant product.

Methods and materials

Reagents

Fmoc amino acid, solvents and reagents for peptide chelator synthesis, was purchased from Fluka Chemicals (St. Louis, MO). The BFC DOTA was purchased from Macrocyclics (Dallas, TX) and NODAGA was purchased from Chematech (Dijon, France). Sodium acetate, diethylenetriamine pentaacetic acid (DTPA), calcium chloride (CaCl2), zinc chloride (ZnCl2), Ferric chloride (FeCl3), hydrochloric acid solutions were purchased from Fisher Scientific, Inc. (Waltham, MA). Transferrin was purchased from Sigma-Aldrich (St. Louis, MO). A 370MBq [68Ge-68Ga] generator was purchased from Eckert and Zeigler (Berlin, Germany). Sodium chloride solution (0.9%) was prepared in this laboratory using deionized water. Human serum was prepared from whole blood drawn from a volunteer. All chemicals were used without further purification.

Instruments

Peptide was purified using a preparative column on high-pressure liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) equipped with gradient pumps and UV/VIS detector, a NaI(Tl) radioactivity monitor, and a rate meter. The reverse phase C18 microbound column (4.6 mm × 250 mm) served as the stationary phase, and two solvents: 0.1% Trifluoroacetic acid (TFA) in H_2O and 0.1% TFA in acetonitrile as mobile phase. The gradient was such that 10% CH_3CN in aqueous 0.1% CF_3COOH to 100% CH_3CN in 0.1% CF_3COOH at a flow rate of 1 mL/min over 28 min at 22°C. For thin layer chromatography (TLC), pre-coated aluminum sheets with silica gel were purchased from Merck (Kenilworth, NJ) and radioactivity was measured on Perkin Elmer 2480 wizard^2 automatic gamma counter (Waltham, MA). The pH of the solutions was measured using either a pH meter (Sartorius, Gottingen, Germany) or pH strips (Fisher scientific Inc.)

Synthesis of peptide conjugate

Briefly, the PACAP analogue with C-terminal NODAGA/DOTA chelators was synthesized on a Wang resin using an ABI 341A peptide synthesizer (Applied Biosystems, Foster City, CA). Fmoc-Lys (ivDde) was first introduced at the C terminus of the peptide, followed by 4-aminobutyric acid (y-Aba). The 27-amino-acid-long PACAP sequence was then assembled by standard Fmoc coupling with the final histidyl residue, being a t-Boc–protected His(Trt) derivative. The capping t-Boc function was necessary to ensure that the N-terminal amino group remained protected during

subsequent deprotection and coupling cycles performed at the c amino group of the C-terminal lysine. The ivDde group at the C-terminal lysine was then selectively removed with 2% hydrazine, followed by coupling with DOTA or NODAGA. The peptide was cleaved from the resin using TFA/water/ phenol/thioanisole/ethanedithiol (82.5:5:5:5:2.5) and precipitated with diethyl ether. The crude peptide was purified to homogeneity by reversed phase HPLC using a preparative column. The peptide was characterized by matrix-assisted laser desorption/ionization- time of flight (MALDI-TOF) mass spectrometry.

Radiolabeling

For radiolabeling, 300 µL (14.5 – 18.5 MBq) of $^{68}GaCl_3$ was added in a clean glass test tube to which were added varying amounts of acetate buffer to study the influence of pH (1.0 – 6.0) and varying amount of a 4 mg/mL peptide-chelators water solution (1-20 µg). The mixture was heated at predetermined temperatures (25, 50, 70 and 90 °C) for pre chosen time periods (10, 15, 20 and 30 min) to study the kinetics of the radiolabeling reactions with respect to temperature and time. The radiolabeling was evaluated by Radio-HPLC.

Preparation of free $^{68}GaCl_3$, ^{68}Ga-hydrolyzed, ^{68}Ga-DTPA and ^{68}Ga-Tf as reference standard

To identify HPLC peaks of the ^{68}Ga species, HPLC peaks, that may have formed during the chelation experiments, free $^{68}GaCl_3$ (as eluted from generator with 0.1M HCl), ^{68}Ga-hydrolyzed products, ^{68}Ga-DTPA and ^{68}Ga-Transferrin (Tf) were prepared as described[25-26,13] and their retention times were recorded on radio-HPLC.

Stability (in vitro), transchelation and transmetallation studies

For the stability, transchelation and transmetallation studies, ^{68}Ga-peptide-chelators were prepared by mixing 300 µL (14.5 – 18.5 MBq) of $^{68}GaCl_3$ in 0.1 M HCl, 120 µL of 1.0 M sodium acetate and 5 µL (20 µg) of peptide-chelators. The preparation was incubated at 90°C for 30 min. Aliquots of ^{68}Ga-peptide-chelators solutions (0.3 mL, approx. 15 MBq) were alternatively mixed with (1) 0.3 mL of a 0.9% NaCl, (2) 0.3 mL of a 100 nM $Fe^{3+}/Zn^{2+}/Ca^{2+}$(for transmetallation study), (3) 0.3 mL of a 200 µM DTPA solution, (4) 0.3 mL of a 50 µM transferrin solution (for transchelation study) and (5) 0.3 mL of human serum solution. The mixtures were incubated at 37 °C and analyzed after 0, 10, 30, 60 and 120 min of incubation, respectively. A 10 µl (in 100µL of normal saline) aliquot preparations of (1), (2) and (3) were injected in the radio-HPLC while preparations (4, 5) were analyzed by ITLC-SG using 0.1M citric acid as a solvent.

Cell binding (in vitro) assay

Estrogen receptor-positive T47D human breast tumor cells from American Type Culture Collection were maintained in RPMI 1640 medium with 2 mM L-glutamine contained 10 mM HEPES, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and supplemented with 0.2 Units/ml 90 %bovine insulin, and 10% fetal bovine serum, at 37°C under 5% CO_2/95% air. The cells when confluent were detached using 0.25% trypsin-EDTA, washed and resuspended with RPMI-1640 medium at a concentration of ~ 5×10^6 cells/mL. Each test tube contained 1 mL of cell suspension into which 10 µL of ^{68}Ga-peptide-chelator (approx. 0.74 MBq) was added and incubated for 2 hr at 37°C in a water bath. The incubation was terminated by adding 0.5 ml of cold normal saline. The mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant from each wash was collected into marked test tubes. To the remaining pellet, 1.0 ml of normal saline was added and centrifuged again at 3000 rpm for 10 minutes. The supernatant was separated and collected in the marked test tube. The cells and supernatant were counted for radioactivity in a gamma counter and percentage of radioactivity bound on the cells was calculated.

Biodistribution studies

The biodistribution of ^{68}Ga labeled peptide was studied in normal (n = 10, athymic nude, female mice, 20-25 g). The resultant optimal preparation procedure was used which contained 300 µL of $^{68}GaCl_3$ in 0.1 N HCl, 120 µL of 1.0 M sodium acetate solution and 20 µg of peptide conjugate, incubated for 10 min at RT (for NODAGA-peptide) and 90ºC (for DOTA-peptide). Following determination of radiochemical purity (> 95%) by radio-HPLC analysis, ^{68}Ga-peptide (3.7 – 5.5 MBq) was administered intravenously through a lateral tail vein in 200 µL of the preparation (pH ≈ 5.5). Radioactivity in the syringe before and after administration was measured in an energy-calibrated dose calibrator (CRC-15; Capintec, Ramssey, NJ), and the exact quantity received by each animal was determined. A standard $^{68}GaCl_3$ solution of a known quantity was prepared at the time of injection. The animals (n = 5, each study) were sacrificed at 1 h post injection by CO_2 inhalation. Various organs were removed, washed free of blood, blotted dry, and weighed. The associated radioactivity was counted in the γ-counter and results were calculated as percentage injected dose per gram (% ID/g) of tissue.

Results

Purity of peptide-chelators conjugates

NODAGA- and DOTA-peptide purity as determined by HPLC was 99%. The MALDI-TOF demonstrated that, for NODAGA-peptide observed molecular mass was 3716 (calculated molecular mass-3718) whereas for DOTA-peptide observed molecular weight was 3787 (calculated molecular mass-3785). The MALDI peaks for the peptide conjugates are given in Figure 1A and B.

Radiolabeling kinetics

Optimal radiolabeling (> 95.0%) of the DOTA-conjugated peptide required pH 2-5 and higher (50-90ºC) temperature, while the NODAGA conjugated peptide needed incubation only at room temperature at same pH range. At higher temperature (90°C), the radiolabeling was not affected by pH for both peptide conjugates (Fig. 2A, B) and can be labeled at wide range of pH (1.0 – 6.0). NODAGA-peptide can bind ^{68}Ga at a minimal quantity of 1 µg but DOTA-peptide required minimum of 20 µg to yield optimal (≥ 95%) radiolabeling (Fig. 2C). The minimum incubation time needed for optimal (> 95%) radiolabeling was 10 minutes at 90°C (Fig. 2D). Radio-HPLC showed retention time for both radio-complexes was 9.9 ± 0.3 minutes as compared to 3.1 ± 0.2 minutes for free ^{68}Ga as shown in Fig. 3-b. The retention time was also determined for the reference standard like hydrolyzed $^{68}GaCl_3$, ^{68}Ga-DTPA and ^{68}Ga-transferrin and found to be 3.5 ± 0.2 minutes.

Stability studies

Both ^{68}Ga labeled peptide conjugates were stable in 0.9% normal saline (Fig. 4A) and showed minimal (1-2%) degradation against ($FeCl_3$, $CaCl_2$ and $ZnCl_2$) transmetallation (Fig. 4B-D) up to 2 hours. The transchelation study with DTPA (Fig. 5A) and transferrin (Fig. 5B) solution showed that both radio-complexes were remained intact over 2 hours. Radio-TLC showed ratio factor (Rf) value of 0.0-0.1 for ^{68}Ga labeled peptide and 0.9-1.0 for free $^{68}GaCl_{3..}$

Cell binding (*in vitro*) assay

Cell binding assay showed higher uptake for ^{68}Ga-NODAGA-peptide, 34.0% ± 0.8% as compared with 24.5% ± 0.9% for ^{68}Ga-DOTA-peptide at 15 minutes. However, at 2

hours of incubation at 37ºC, the uptake was nearly same, 48.9% ± 3.9% for ^{68}Ga-NODAGA-peptide and 45.2% ± 2.1% for ^{68}Ga-DOTA-peptide (Table – I).

Biodistribution studies

The biodistribution pattern of the both^{68}Ga labeled peptide conjugate is shown in Figure 6. In both cases, a higher uptake is seen in kidneys followed by liver, blood and lungs. It was evident that the ^{68}Ga-NODAGA-Peptide showed rapid blood clearance as compared to ^{68}Ga-DOTA-peptide. Liver uptake was 8.8% for ^{68}Ga-NODAGA-Peptide was lower ($p = 0.01$) as compared to 16.5% for ^{68}Ga-DOTA-peptide. Most of the activity was excreted through kidney.

Discussion

With its several advantages such as the availability in a generator form, cyclotron independency, multi elution ability in a single day, simplified radiolabeling chemistry with several biomolecules using bifunctional chelating agents, the use of $^{68}GaCl_3$ is rapidly growing for diversified clinical applications.[27] The presence of even µg levels of metallic impurities (with ageing of generator) in $^{68}GaCl_3$ or reagent used may hamper the radiolabeling complexation. The removal of radionuclide impurities however has helped expansion of its use for clinical application.[28]

The eluate of $^{68}GaCl_3$ from the generator may contain metal ion impurities such as Fe^{3+}, Cu^{2+}, Al^{3+}, Zn^{2+} and Sn^{4+} which could hamper the radiolabeling of biomolecules. It is important therefore to identify the suitable chelating agent for the radiolabeling which is least affected by these impurities as well the stability of the labeled compound.[29-30] DOTA is the most commonly used chelating agent and ^{68}Ga-DOTATOC is accepted in the European Pharmacopeia.[31] ^{68}Ga labeled DOTA biomolecules conjugates (like peptides, antibody, and small molecules) have been successfully developed and used for many clinical applications. The disadvantage of DOTA conjugate is its slow reaction kinetics for complex formation with ^{68}Ga.[32] These limitations prompted us to investigate the use of NODAGA for comparison with DOTA.

Chelator-peptide conjugates were synthesized by solid phase peptide synthesis method. Both peptide conjugates were synthesized with high purity (~ 99.0%). NODAGA-peptide conjugate showed faster kinetics as compare to DOTA- peptide conjugate and less influenced by the pH variation. NODAGA peptide conjugate yielded higher radiolabeling efficiency at minimal quantity of peptide (1 µg), at room temperature and within short incubation (5 min) time. For the preparations with short lived radionuclides like ^{68}Ga, these characteristics of NODAGA are advantageous. The faster radiolabeling

kinetics may be attributable to its ionic size compatibility with cavity size of the nine membered triazomacrocycle of NODAGA and therefore, yielding a high radiolabeling in 5 min at room temperature.[33] A low radiolabeling was observed in acidic (pH-1.0) conditions for both peptide conjugates when incubated at temperature (25-70°C). It may attribute to strongly acidic environment which might have protonated the donor atoms of the ligand and destabilized the ^{68}Ga complex.[34] At high temperature (~ 90°C), the radiolabeling was found to be independent of pH variation (Fig. 2 a-b). However at pH 7.0 and above, labeling decreases (data not included). This may be due to a fact that at acidic pH, ^{68}Ga remains predominant in cation Ga^{3+} form (> 90%), though soluble complex ions $Ga(OH)^{2+}$, $Ga(OH)^{+}_{2}$ may be present in the solution. As the pH of the solution shifts towards basic (> 7.0), the hydrolyzed form of Gallium, $Ga(OH)^{-}_{4}$ increases and decreases the radiolabeling yield.[35] Once the peptide conjugate is radiolabeled, then adjusting the pH to physiological pH 7.0 does not adversely influence the chelated Ga-68.

The use of sodium acetate buffer maintained the pH and allowed strong formation BFC-^{68}Ga complex. The other advantage of using sodium acetate buffer is its biocompatibility for human use.[36] DOTA peptide conjugate showed labeling at high temperature. Therefore, DOTA may not be useful for those peptides which can denature at higher temperature and become unsuitable for molecular imaging. On the other hand, radiolabeling of NODAGA-peptide at room temperature ruled out major thermal denaturation. Radio-HPLC showed that the formation of the ^{68}Ga-NODAGA and DOTA peptide conjugate has retention time of around 9.9 min whereas free ^{68}Ga eluted at 3.6 min (Fig. 3-b) and complex formation between ^{68}Ga and NODAGA-peptide conjugate is shown in Fig. 3-a . HPLC UV detector also detected the UV peak for NODAGA and DOTA peptide conjugate at around 9.9 min. Therefore, it confirmed the complexation of the ^{68}Ga with peptide conjugate which leads to the retention of the ^{68}Ga labeled peptide approximately at the same time.

Both radio-complexes were found to be stable in normal saline (Fig. 4a) and human serum over 120 minute. The stability in human serum (at 37°C) indicated its suitability for *in vivo* applications and showed their inertness towards the metal ion/species that may be present in the serum. The transmetallation study was important not because these ions are present as impurities in the eluate but these ions are also present in serum in appreciable quantity.[37] ^{68}Ga-NODAGA and DOTA peptide conjugates showed minimal (1-2%) degradation in radiolabeling after 120 minute incubation with respective ion solution at 37°C (Fig. 4-b-d). This could also provide an indication about the stability of these radio-complexes in *in vivo* environment. Transferrin is the major protein in the plasma which is known to form a strong complex with ^{67}Ga-citrate.[38] The minimal transchelation (~1-2%) of ^{68}Ga-peptide conjugates with transferrin showed the stability of radio-complexation (Fig. 5-a-b).

The cell binding assay was performed on T47D breast cancer lines which express VPAC-1 receptors on their surface.[19] ^{68}Ga-NODAGA-peptide showed higher uptake in 15 minute as compared to ^{68}Ga-DOTA-peptide but the uptake was almost same at 2hr (Table-1) which may be due to saturation of the receptor site. The biodistribution data (Fig. 6) showed that both radio-complexes were cleared through kidneys. Hence, renal system was the major route of clearance from the body. ^{68}Ga-NODAGA-peptide was cleared rapidly form the blood and had significantly less liver uptake ($p < 0.05$) as compare to ^{68}Ga-DOTA-peptide at 1 hr. The fast clearance from the blood may be advantageous in reducing the background radioactivity for PET imaging but it could also limit the accumulation of radioactivity at target (tumor) area. Hence, a detailed *in vivo* investigation will be required further for any conclusion.

In the present study, we have showed that ^{68}Ga-NODAGA-peptide exhibited most favorable features from radiochemistry point of view. Chelator NODAGA has flexibility over the influence of temperature and acidic environment.[13] Labeling at room temperature provides an advantage and could prevent any deleterious effect on peptide conjugate due to heating. Both peptide conjugates were found to be stable in serum, against transchelation and transmetallation experimentations. A detailed *in vivo* study is in progress to investigate the influence of chelators on the biodistribution and tumor targeting.

Conclusions

A comparison between NODAGA and DOTA revealed that NODAGA-peptide conjugate could be efficiently radiolabeled with ^{68}Ga at room temperature with high yield. ^{68}Ga-NODAGA-peptide conjugate remained stable in serum, in presence of major metal ions impurities, against transchelation with DTPA or transferrin. The biodistribution data showed its clearance from kidneys, which minimize the radiation burden on the organs. The clearance of ^{68}Ga-NODAGA-peptide from blood was faster as compare to ^{68}Ga-DOTA-peptide and would be advantageous for PET imaging.

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Disclosure Statement

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