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Original Article

Synthesis and evaluation of ^{68}Ga-labeled DOTA-2-deoxy-D-glucosamine as a potential radiotracer in μPET imaging

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Abstract: The purposes of this study were to develop an efficient method of labeling D-glucosamine hydrochloride with gallium 68 (^{68}Ga) and investigate the imaging properties of the resulting radiotracer in a human tumor xenograft model using micro-positron emission tomography (μPET). The precursor compound 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-2-deoxy-D-glucosamine (DOTA-DG) was synthesized from D-glucosamine hydrochloride and 2-(4-isothiocyanatobenzyl)-DOTA. Radiolabeling of DOTA-DG with ^{68}Ga was achieved in 10 minutes using microwave heating. The labeling efficiency and radiochemical purity after purification of ^{68}Ga-DOTA-DG were ~85% and greater than 98%, respectively. In A431 cells, the percentages of ^{68}Ga-DOTA-DG and ^{18}F-FDG uptakes after 60 min incubation were 15.7% and 16.2%, respectively. In vivo, the mean ± standard deviation of ^{68}Ga-DOTA-DG uptake values in A431 tumors were 2.38±0.30, 0.75±0.13, and 0.39±0.04 percent of the injected dose per gram of tissue at 10, 30, and 60 minutes after intravenous injection, respectively. μPET imaging of A431-bearing mice clearly delineated tumors at 60 minutes after injection of ^{68}Ga-DOTA-DG at a dose of 3.7 MBq. ^{68}Ga-DOTA-DG displayed significantly higher tumor-to-heart, tumor-to-brain, and tumor-to-muscle ratios than ^{18}F-FDG did. Further studies are needed to identify the mechanism of tumor uptake of this new glucosamine-based PET imaging tracer.

Keywords: Gallium 68, 2-deoxy-D-glucose, μPET imaging, microwave heating-assisted synthesis

Introduction

The positron-emitting radioisotope gallium 68 (^{68}Ga; $t_{1/2}$ = 68 minutes) is of great interest to practitioners of positron emission tomography (PET). This generator-produced nuclide does not require an on-site cyclotron for radioisotope production. ^{68}Ga has excellent physical properties relevant to PET, including decays by 89% through positron emission and 3.2% through gamma emission [1]. A number of ^{68}Ga-labeled peptides have been investigatror for in vivo PET imaging of tumors [2, 3]. ^{18}F-fluorodeoxyglucose (FDG) is routinely used in the clinic as a biomarker of metabolic activity for the detection and staging of cancer and monitoring of treatment response. ^{18}F-FDG is transported into tumor cells by the glucose transporters (GLUTs) and is a substrate of hexokinases. D-glucosamine (DG) is an attractive scaffold as a glucosyl ligand. Studies have shown that a derivative of DG with a bulky moiety attached to its amino group is the substrate of GLUTs and hexokinases [4, 5].

Previously, the synthesis of ^{68}Ga-labeled glucosamine through 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) radiometal chelator have been reported by us and others in abstract forms [6, 7]. Tworowska et al. [7] synthesized ^{68}Ga-DOTA-glucosamine by heating to 95 °C using conventional method for 10-20 min. To improve the radiolabeling efficiency, we used microwave to assist the labeling process. Microwave heating is used routinely in the synthesis of organic compounds [8]. This heating increases the rate of reaction without compromising the stability of reagents or

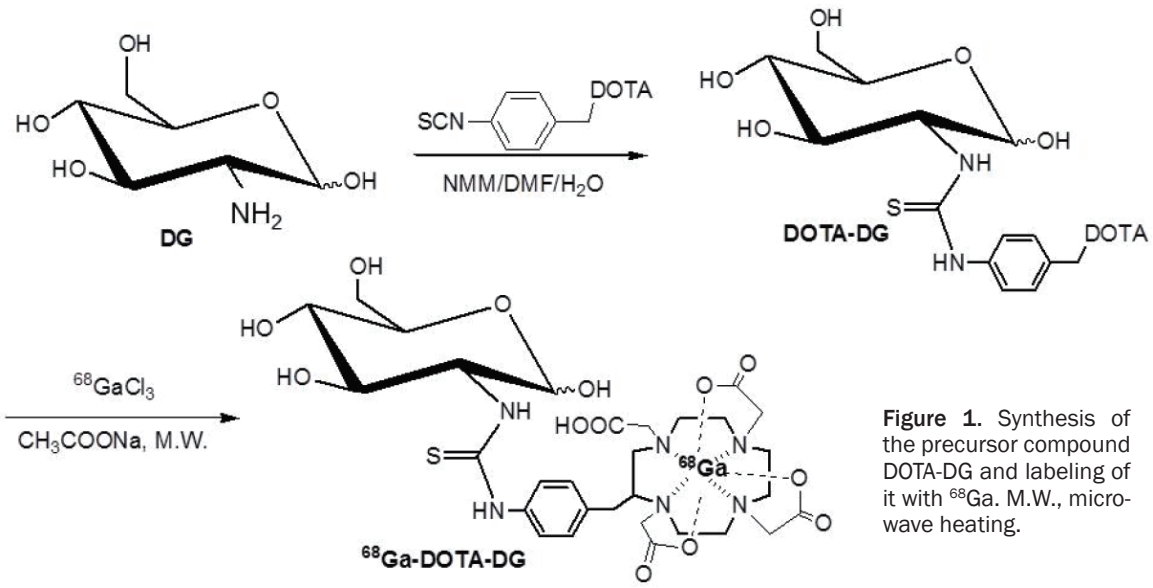

Figure 1. Synthesis of the precursor compound DOTA-DG and labeling of it with ^{68}Ga. M.W., microwave heating.

the safety of labeling procedures [9, 10]. Herein we report on our use of microwave heating-assisted labeling of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-DG with ^{68}Ga for tumor imaging. We found that the resulting radiotracer, ^{68}Ga-DOTA-DG, could clearly delineate tumor xenografts in nude mice with high tumor-to-nontumor ratios, making it a potentially useful radiotracer in PET imaging.

Materials and methods

Reagents

Dimethylformamide, sodium acetate, D-glucosamine hydrochloride, and *N*-methylmorpholine were purchased from Sigma-Aldrich (St. Louis, MO). 2-(p-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticacid (p-SCN-Bn-DOTA) was purchased from Macrocyclics (Dallas, TX). Other commercially available chemicals were purchased from VWR International (San Diego, CA). All reagents were used as received. ^{18}F-FDG was obtained from the Department of Nuclear Medicine at The University of Texas MD Anderson Cancer Center.

Synthesis of DOTA-DG

DG was reacted with p-SCN-Bn-DOTA in an aqueous solution at pH 7 for 12 hours at room temperature to produce DOTA-DG linked via a thiourea bond (**Figure 1**). The progress of the reaction was monitored using high-performance liquid chromatography (HPLC). DOTA-DG was analyzed via electrospray ionization mass spectrometry using an Agilent LC/MSD TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a Vydac C-18 column (4.6×250.0 mm, 7-μm particle size, 300-Å pore size (Grace, Deerfield, IL). The HPLC eluting conditions were as follows: solvent: A, 0.1% trifluoroacetic acid (TFA) in water; B, 0.1% TFA in acetonitrile; gradient: B, 0-10% over 0-10 minutes; B,10-50% over 10-12 minutes; B, 50-80% over 12-20 minutes; B, 80% to 10% over 20-21 minutes. The flow rate was 1 mL/minute.

Microwave heating-assisted ^{68}Ga labeling of DOTA-DG

$^{68}GaCl_3$ in 0.1 N hydrochloric acid (370 MBq/0.3 mL) was obtained from ^{68}Ge/^{68}Ga generator (Eckert & Ziegler, Berlin, Germany). Two methods were used for labeling of DOTA-DG with ^{68}Ga. In the conventional method, 370 MBq of $^{68}GaCl_3$ in 0.3 mL of a 1-M aqueous solution of sodium acetate (pH 4) was added to a solution of DOTA-DG in distilled water (100 μg in 0.1 mL). The mixture was allowed to react at 90 °C for 20 minutes in a water bath and then cooled to room temperature over 30 minutes. In the microwave heating-assisted method, the mixture of 370 MBq of $^{68}GaCl_3$ in 0.3 mL of a 1-M sodium acetate solution and 100 μg of DOTA-DG in 0.1 mL of distilled water was heated in a

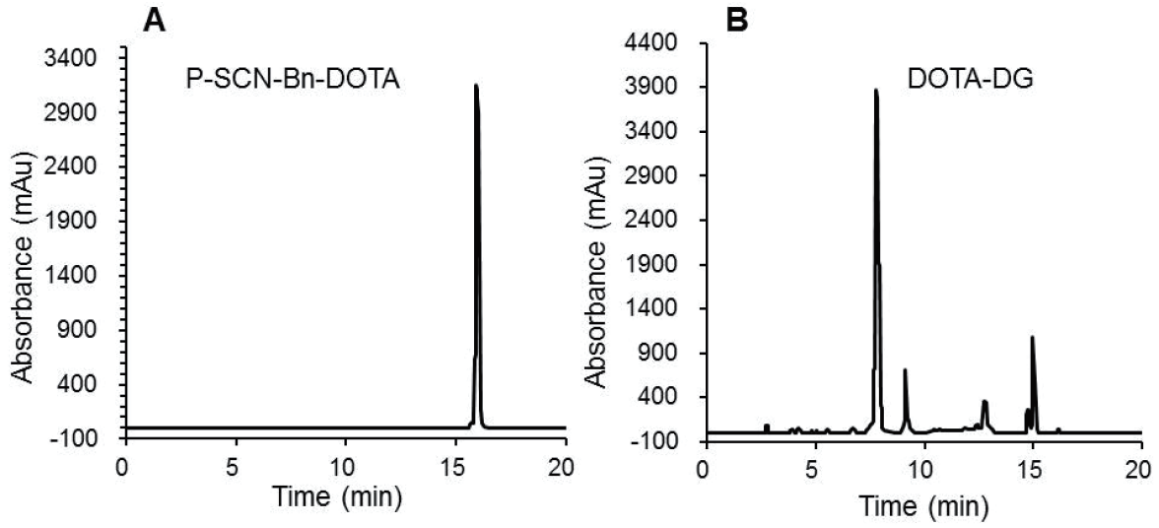

Figure 2. HPLC analysis of DOTA-DG conjugate. A. p-SCN-Bn-DOTA (Rt = 7.51 minutes). B. DOTA-DG (Rt = 7.51 minutes).

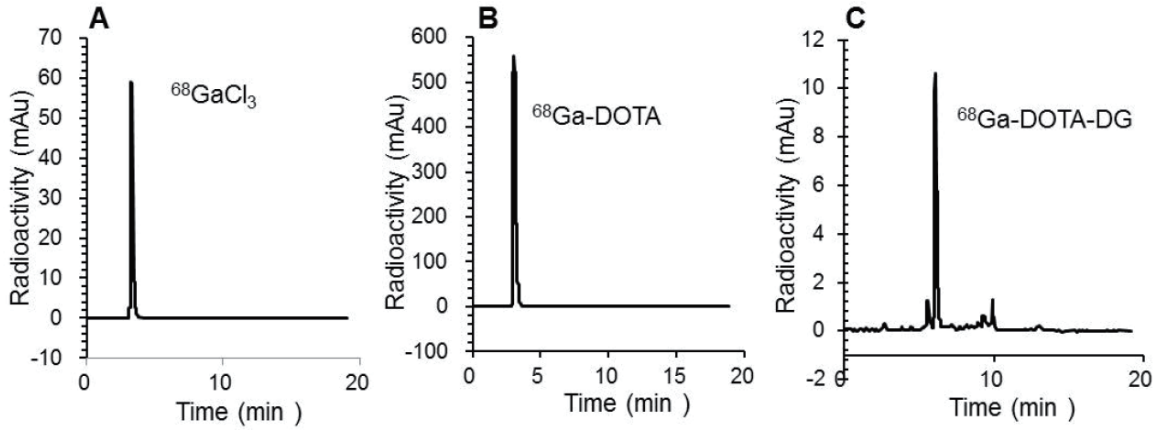

Figure 3. HPLC analysis of as-synthesized ^{68}Ga-DOTA-DG. A. $^{68}GaCl_3$ solution (Rt = 3.15 minutes). B. ^{68}Ga-DOTA (Rt = 2.92 minutes). C. ^{68}Ga-DOTA-DG (Rt = 7.03 minutes).

microwave reaction apparatus (Discover; CEM Corporation, Matthews, NC) at a power setting of 25 W for 5 minutes and 50 W for an additional 5 minutes. The reaction mixture was then kept at 40 °C for 5 minutes. DOTA was labeled with ^{68}Ga similarly to DOTA-DG, and the resulting radiotracer, ^{68}Ga-DOTA, was used as a control in biodistribution study.

Radiolabeled compounds were analyzed using HPLC. The specific activity of ^{68}Ga-DOTA-DG was determined with a radio-HPLC chromatogram by dividing the integrated peak radioactivity of the radiotracer by its physical quantity derived from the corresponding ultraviolet absorbance and a calibration curve of known quantities of the unlabeled compound. The labeling yields were determined using instant thin-layer chromatography (ITLC) (German Science, Ann Arbor, MI) developed with a solution of 0.1 M citric acid in saline. The strips were scanned using an ITLC scanner (AR-2000; Bioscan, Washington, DC).

In vitro cell uptake of radiotracers

A431 human epithelial carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37°C in a humidified atmosphere con-

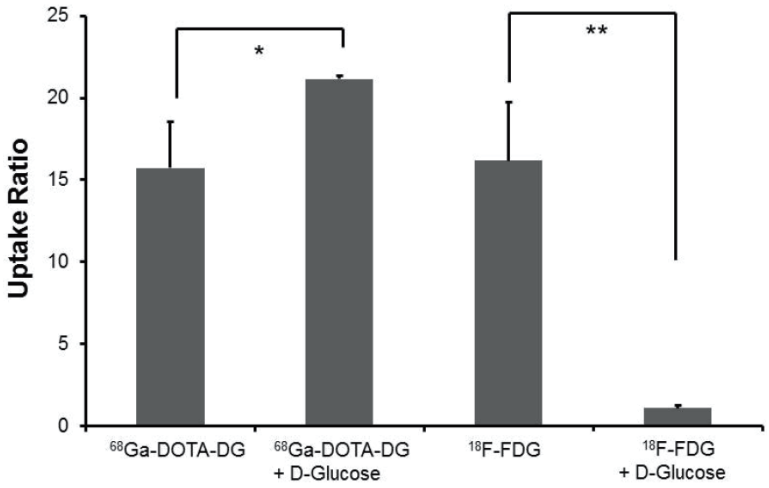

Figure 4. In vitro cellular uptake of ^{68}Ga-DOTA-DG and ^{18}F-FDG in A431 cells. In blocking experiments, both radiotracers were coincubated with an excessive amount of cold D-glucose. The graph shows a signficant difference in cell uptake between the radiotracers and their corresponding blocking groups. *p<0.05; **p<0.005.

taining 5% CO_2 in Dulbecco's modified Eagle's medium and Ham's F12 nutrient mixture containing 10% fetal bovine serum (Gibco, Grand Island, NY).

Cell uptake assays were performed after seeding 1.2×10^6 A431 cells/mL/well in 24-multiwell culture plates. When the cells were about 80% confluent, each well was injected with 74 KBq of ^{68}Ga-DOTA-DG or ^{18}F-FDG in 1 mL of culture media (2 μg/mL for both radiotracers). A blocking study was performed with the addition of both radiotracers with 1 mg of cold deoxy-D-glucose to the wells (100 μL/well). After 60 minutes of incubation at 37 ºC, the culture media were removed, and the cells were washed twice with ice-cold Hank's balanced salt solution (pH 7.3). The cells were then dissolved in 0.5% sodium dodecyl sulfate (0.5 mL/well). The radioactivity in the cells was measured using a gamma counter (Packard, Downers Grove, IL). The cell uptake of the radiotracers was calculated using the formula %uptake = (radioactivity of cells/total radioactivity) × 100%. This study was performed in triplicate.

Biodistribution

The mice were kept under specific pathogen-free conditions and were handled and maintained according to Institutional Animal Care and Use Committee guidelines. A431 cells were inoculated subcutaneously into the right thighs of nude mice (20-25 g; Harlan Sprague Dawley, Indianapolis, IN) by injecting 1×10^6 viable tumor cells in a suspension of phosphate-buffered saline. When the resulting tumors grew to a diameter of 6-8 mm, the mice were allocated to three groups of three mice each. They were then injected intravenously with ^{68}Ga-DOTA-DG or ^{18}F-FDG (3.7 MBq/mouse). The animals were killed at 10, 30, and 60 minutes after injection. Blood, heart, liver, spleen, kidney, lung, stomach, intestine, muscle, bone, brain, and tumor tissues were removed, weighed, and counted for radioactivity using a gamma counter. Uptake of each radiotracer in various tissues was calculated as the percentage of the injected dose per gram of tissue (%ID/g).

Micro-PET imaging

A431 tumor cells were inoculated into nude mice as described above. When the resulting tumors grew to 6-8 mm in diameter, the mice were injected intravenously with ^{68}Ga-DOTA-DG (20 μg, 14.8 MBq/mouse, 0.2 mL). The mice were then placed in the prone position for micro-PET (μPET) imaging. Prior to imaging, the mice were anesthetized with 2% isoflurane gas (Iso-Thesia, Rockville Centre, NY) in oxygen. During imaging, anesthesia in the mice was maintained with 0.5-1.5% isoflurane. μPET images were acquired 30-80 minutes after radiotracer injection using an R4 μPET scanner (Concorde Microsystems, Knoxville, TN).

Statistical analysis

Cell uptake and biodistribution data were analyzed using two-tailed, unpaired Student *t*-tests, with *p* values less than 0.05 considered to be statistically significant. The in vitro percentage of radiotracer uptake, in vivo percentage of injected radiotracer dose per gram of tissue, and tumor-to-nontumor ratios are presented as the mean ± standard deviation. All statistical

Table 1. Biodistributions of ^{68}Ga-DOTA-DG in mice bearing A431 tumors at different times at 10, 30, and 60 minutes after radiotracer injection

	Mean %ID/g (± standard deviation)		
Site	10 minutes	30 minutes	60 minutes
Blood	5.24±0.63	0.81±0.16	0.40±0.09
Heart	1.92±0.51	0.28±0.10	0.12±0.03
Liver	1.45±0.22	0.37±0.22	0.36±0.01
Spleen	1.09±0.14	0.24±0.07	0.21±0.02
Kidney	10.23±2.87	1.94±0.37	1.19±0.11
Lung	3.96±0.51	0.65±0.11	0.26±0.06
Stomach	1.93±0.08	0.30±0.05	0.12±0.01
Intestine	1.22±0.08	0.28±0.05	0.16±0.05
Muscle	0.99±0.14	0.16±0.10	0.12±0.13
Bone	0.86±0.16	0.16±0.04	0.07±0.03
Tumor	2.38±0.30	0.75±0.13	0.39±0.04
Brain	0.25±0.08	0.05±0.01	0.03±0.02

Table 2. Biodistributions of ^{68}Ga-DOTA-DG, ^{18}F-FDG, and ^{68}Ga-DOTA in mice bearing A431 tumors at 1 hour after radiotracer injection

	Mean %ID/g (± standard deviation)		
Site	^{68}Ga-DOTA	^{68}Ga-DOTA-DG	^{18}F-FDG
Blood	0.44±0.07	0.40±0.09	0.54±0.10
Heart	0.11±0.02	0.12±0.03	30.2±7.40
Liver	11.96±0.90	0.36±0.01	0.69±0.21
Spleen	2.77±1.13	0.21±0.02	1.50±0.60
Kidney	0.72±0.04	1.19±0.11	1.07±0.06
Lung	0.39±0.02	0.26±0.06	2.30±0.64
Stomach	0.11±0.05	0.12±0.01	2.93±1.50
Intestine	0.06±0.02	0.16±0.05	1.42±0.40
Muscle	0.03±0.00	0.12±0.13	2.37±0.16
Bone	0.07±0.01	0.07±0.03	2.06±0.44
Tumor	0.20±0.07	0.39±0.04	4.26±1.10
Brain	0.03±0.00	0.03±0.02	5.81±2.83
Tumor/muscle	7.82±2.40	6.75±5.55	1.80±0.47
Tumor/brain	7.83±2.56	14.03±2.00	0.79±0.18
Tumor/heart	1.75±0.33	3.30±0.71	0.15±0.04
Tumor/liver	0.02±0.00	1.08±0.11	6.40±1.62
Tumor/kidney	0.27±0.08	0.33±0.05	3.96±0.92

computations were performed using the Excel software program (Microsoft Corporation, Redmond, WA).

Results

DOTA-DG synthesis and characterization

DOTA-DG was synthesized via nucleophilic addition of DG to p-SCN-Bn-DOTA (**Figure 1**). The resulting product was purified via flash column chromatography. Electrospray ionization mass spectrometry revealed a mass/charge ratio (m/z) of 729.2082 for $[M-H]^-$, which agrees with the calculated value for $C_{30}H_{45}N_6O_{13}S^-$ of 729.2771. HPLC analysis demonstrated a retention time of 7.61 minutes with greater than 90% DOTA-DG purity (**Figure 2**).

Radiochemistry and stability of ^{68}Ga-DOTA-DG

^{68}Ga-DOTA-DG was synthesized using two methods: conventional and microwave-assist-

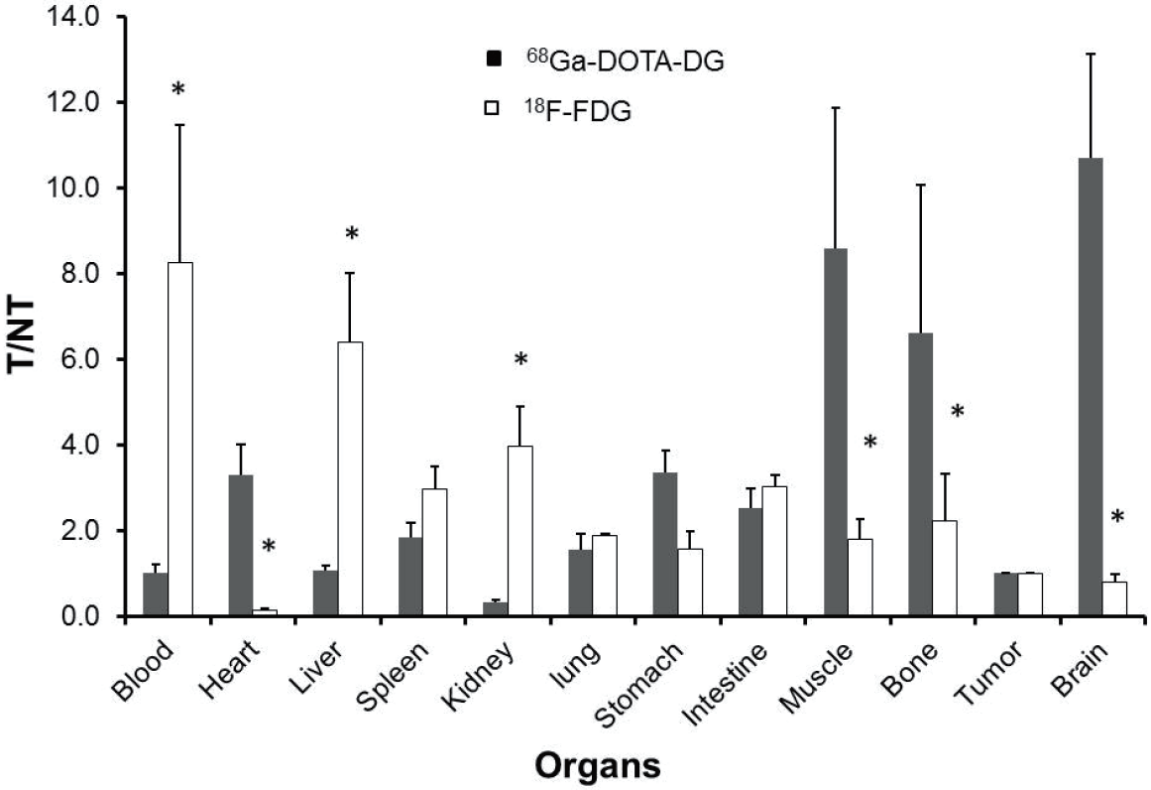

Figure 5. Comparison of the tumor: nontumor (T/NT) uptake ratios for ^{18}F-FDG and ^{68}Ga-DOTA-DG in mice bearing A431 tumors at 1 hour after radiotracer injection. *p<0.05.

ed heating. The radiochemical labeling efficiency of ^{68}Ga-DOTA-DG with the microwave heating technique was 85%. After purification of ^{68}Ga-DOTA-DG using a semipreparative HPLC column, the radiochemical purity was greater than 98% (Rt = 7.01 minutes). Radio-HPLC readily distinguished ^{68}Ga-DOTA-DG from $^{68}GaCl_3$ (Rt = 3.15 minutes) and ^{68}Ga-DOTA (Rt = 2.92 minutes) (**Figure 3**). In comparison, the radiochemical labeling efficiency for ^{68}Ga-DOTA-DG using the conventional method was 60%. The specific activity of ^{68}Ga-DOTA-DG using the microwave heating method was 790 Ci/mmol (2.9×10^{13} Bq/mmol).

Cell uptake of radiotracers in vitro

In vitro, ^{68}Ga-DOTA-DG and ^{18}F-FDG had similar uptake levels in A431 cells after 60 minutes of incubation (**Figure 4**). The percentages of ^{68}Ga-DOTA-DG and ^{18}F-FDG uptake were 15.7% and 16.2%, respectively. The presence of an excessive amount of cold deoxy-D-glucose (1 mg/mL water) significantly blocked the uptake of ^{18}F-FDG in these cells (p<0.001). However, deoxy-D-glucose could not block ^{68}Ga-DOTA-DG. To the contrary, the presence of cold deoxy-D-glucose increased the uptake of ^{68}Ga-DOTA-DG in A431 cells by 121% (p=0.03).

Biodistribution in tumor-bearing mice

The biodistributions of ^{68}Ga-DOTA-DG at 10, 30, and 60 minutes after radiotracer injection in mice bearing A431 tumors are presented in **Table 1**. A comparison of the biodistributions of ^{68}Ga-DOTA, ^{68}Ga-DOTA-DG, and ^{18}F-FDG at 60 minutes postinjeciton are presented in **Table 2**. The tumor-to-nontumor ratios for ^{68}Ga-DOTA-DG and ^{18}F-FDG are shown in **Figure 5**.

The ^{68}Ga-DOTA-DG blood activity at 10 min postinjection was 5.24 %ID/g, which is similar to that of the reported blood level of 5.4 %ID/g for ^{18}F-FDG [11]. The initial ^{68}Ga-DOTA-DG tumor uptake value at 10 minutes after injection was 2.38%ID/g. At 60 minutes after injection, the tumor uptake values for ^{68}Ga-DOTA, ^{68}Ga-DOTA-DG, and ^{18}F-FDG were 0.20%ID/g, 0.39%ID/g, and 4.26%ID/g, respectively. The

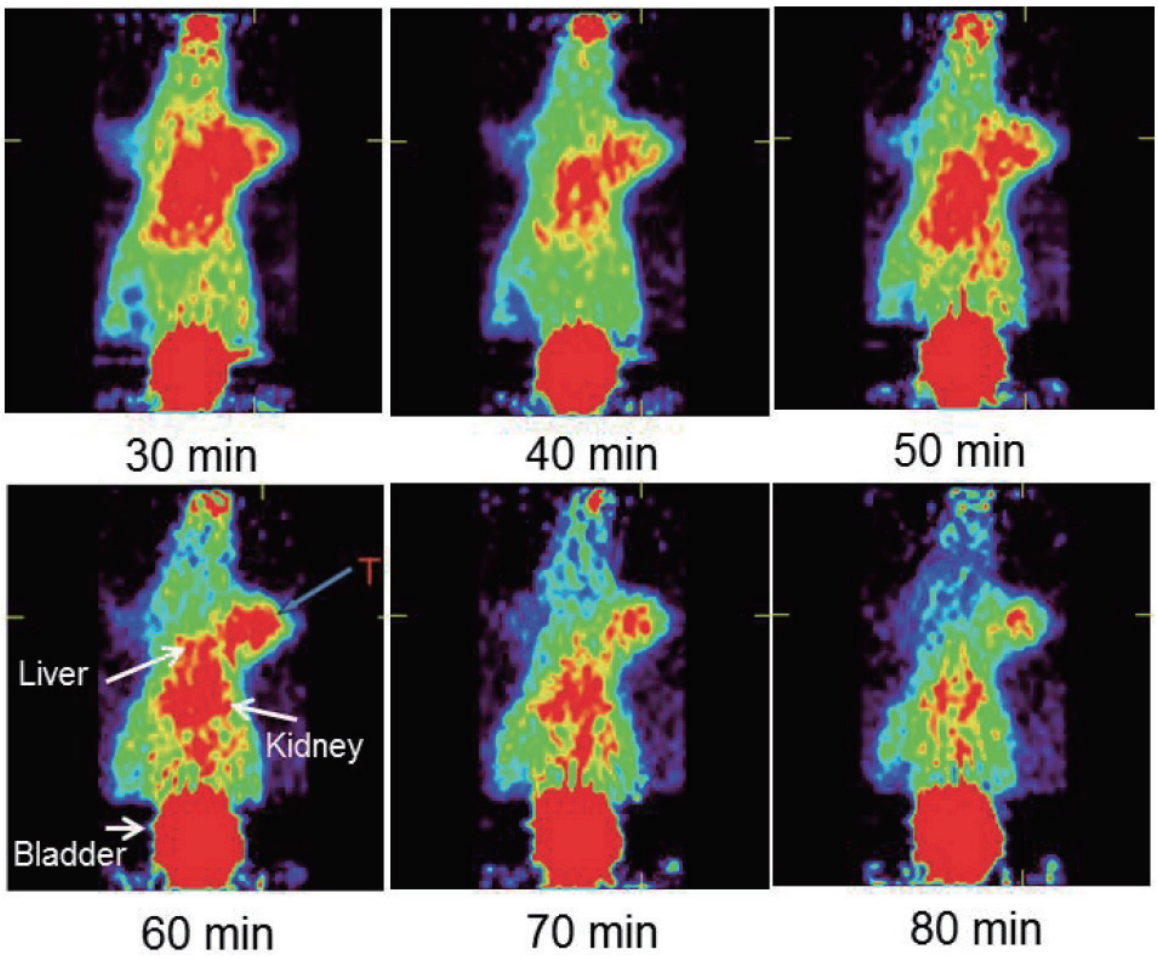

Figure 6. μPET images of a nude mouse bearing an A431 tumor acquired at 30-80 minutes after intravenous injection of ^{68}Ga-DOTA-DG. T, tumor.

blood activity levels at 60 min postinjection were similar for both ^{68}Ga-DOTA-DG (0.40 %ID/g) and ^{18}F-FDG (0.54 %ID/g) (**Table 2**). Thus, ^{18}F-FDG had significantly higher tumor-to-nontumor ratios than ^{68}Ga-DOTA-DG did at 60 minutes after injection in blood (p=0.05), liver (p=0.03), and kidney (p=0.02). On the other hand, ^{68}Ga-DOTA-DG had significantly higher tumor-to-nontumor ratios than ^{18}F-FDG did in muscle (p=0.05), brain (p=0.02), and heart (p=0.02) (**Figure 5**).

μPET images

μPET images of mice with A431 tumors obtained at different times after ^{68}Ga-DOTA-DG injection are shown in **Figure 6**. We observed relatively high activity of ^{68}Ga-DOTA-DG in the kidney and bladder 30-60 minutes after injection. The activity of ^{68}Ga-DOTA-DG in the kidney gradually cleared, and by 80 minutes after injection, ^{68}Ga-DOTA-DG was largely cleared from the body via the renal system. μPET imaging clearly showed uptake of ^{68}Ga-DOTA-DG in the tumors at all time points.

Discussion

In this study, we showed that DOTA-DG could be labeled with ^{68}Ga with high efficiency with the assistance of microwave. The observation that the cellular uptake of ^{68}Ga-DOTA-DG could not be blocked by a large excess of D-glucose suggests that ^{68}Ga-DOTA-DG is taken up by tumors cells via biological processes independent of GLUTs (**Figure 4**). At present, the structural features that govern the behavior of derivatives of DG are not clear. Although previous studies demonstrated that DG and its fluorescent analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)

amino]-2-deoxyglucose are substrates of GLUTs [4, 5, 12], the uptake of other derivatives of DG in tumor cells could not be blocked by D-glucose or DG. For example, several ^{99m}Tc- and near-infrared fluorescent dye-labeled DG compounds have similar characteristics as ^{68}Ga-DOTA-DG with respect to insensitivity to glucose concentration on their tumor uptake in vivo [13-15].

Several authors have reported the synthesis and in vivo imaging with gamma emitter-labeled DG. Bayly et al. [16] reported the successful synthesis of DG labeled with the tricarbonyls of ^{99m}Tc (I), but the resulting radiotracer was not very stable in the presence of cysteine and histidine. Yang et al. [15] demonstrated that ^{99m}Tc-labeled DG through an ethylenedicysteine chelator accumulated in murine tumors. Chen et al. [17] developed a one-step ^{99m}Tc-labeled diethylenetriaminepentaacetate-DG kit and showed accumulation of ^{99m}Tc-labeled diethylenetriaminepentaacetate-DG in MCF-7 human mammary tumors in nude rats. These studies did not attempt to determine whether these glucose analogs are actually involved in key steps in glucose metabolism. Further work is needed to delineate the mechanisms of cellular uptake of ^{68}Ga-DOTA-DG and other DG-based radiotracers.

μPET images acquired with ^{68}Ga-DOTA-DG clearly delineated A431 tumor grown in nude mice. With the current available data, it is not possible to ascertain whether the tumor uptake of ^{68}Ga-DOTA-DG is specific or nonspecific. Although DOTA is used widely for ^{68}Ga-labelling, DOTA is not an optimal chelator for ^{68}Ga. Other chelators such as 1,4,7-triazacyclononane-triacetic acid (NOTA) and triazacyclononane-phosphinate (TRAP) chelators have been shown to possess superior ^{68}Ga binding ability and yield higher specific activity [18]. Thus, future work should be also be directed at synthesizing and testing ^{68}Ga-NOTA-glucosamine and ^{68}Ga-TRAP-glucosamine conjugates.

In conclusion, we successfully synthesized ^{68}Ga-labeled DOTA-DG as a PET radiotracer. Microwave heating-assisted radiosynthesis of ^{68}Ga-DOTA-DG resulted in high labeling efficiency and short labeling times. μPET with ^{68}Ga-DOTA-DG demonstrated high tumor-to-nontumor ratios for muscle, brain, and heart in human tumor xenograft models. We also observed a significant difference between the biodistribution of ^{68}Ga-DOTA-DG and ^{18}F-FDG in the liver and kidney. Future work are needed to elucidate the biological processes responsible for tumor uptake of ^{68}Ga-DOTA-DG.

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