Bioorganic & Medicinal Chemistry 20 (2012) 4915–4920

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and characterization of a ^{68}Ga-labeled *N*-(2-diethylaminoethyl)benzamide derivative as potential PET Probe for malignant melanoma

Hee-Jung Kim [a,b], Dong-Yeon Kim [b,c], Jeong-Hoon Park [a], Seung-Dae Yang [a], Min-Goo Hur [a], Jung-Joon Min [c,d], Kook-Hyun Yu [b,*]

[a] Radiation Instrumentation Research Division, Korea Atomic Energy Research Institute, Jeongeup, Republic of Korea
[b] Department of Chemistry, Dongguk University-seoul, 30 Pildong-ro 1-Gil, Jung-gu, Seoul 100-715, Republic of Korea
[c] Department of Nuclear Medicine, Chonnam National University Hwasun Hospital, Hwasun, Republic of Korea
[d] Laboratory of In Vivo Molecular Imaging, Department of Nuclear Medicine, Chonnam National University Medical School, Gwangju, Republic of Korea

ARTICLE INFO

Article history:
Received 15 May 2012
Revised 27 June 2012
Accepted 27 June 2012
Available online 6 July 2012

Keywords:
Malignant melanoma
Benzamide derivatives
Melanoma imaging agent
Bifunctional chelating agent
^{68}Ge/^{68}Ga generator
Gallium-68
Positron emission tomography (PET)

ABSTRACT

Radiolabeled benzamides have been reported to be attractive agents for targeting malignant melanoma as they bind melanin and display high accumulation in melanoma cells. Herein, we report the synthesis and bioevaluation of a novel ^{68}Ga-labeled benzamide as a potential PET agent for malignant melanoma. The novel radiotracer was synthesized in good radiochemical yields (80% decay corrected yield) and high specific radioactivity (10 GBq/μmol). Cellular uptake of ^{68}Ga-SCN-NOTA-BZA was significantly higher in B16F10 cells (mouse melanoma) treated with L-tyrosine. Biodistribution and micro-PET studies of ^{68}Ga-SCN-NOTA-BZA in B16F10-bearing mice showed selective uptake into the tumor. The radiotracer was cleared via renal excretion without further metabolism. These results demonstrate that ^{68}Ga-SCN-NOTA-BZA is a potential PET probe for malignant melanoma.

1. Introduction

Malignant melanoma initiates in melanocytes, the pigment cells present in the skin, and develops from an existing mole or freckle. Malignant melanoma is the most acute form of skin cancer.[1] Because it may spread quickly to other parts of the body (i.e., metastasize), melanoma occurring anywhere on the body can cause death.[2] Moreover, incidence of malignant melanoma is increasing faster than that of any other cancer worldwide.[3–5] Early detection and accurate staging are crucial to achieving a positive outcome through therapy.[6,7]

Positron emission tomography (PET) is a non-invasive imaging method that can be used to determine the distribution of radioactive agents with high resolution (1–2 mm) and sensitivity (10^{-11}–10^{-12} M).[8,9] PET has become an important tool for the diagnosis and evaluation of cancer and cancer metastasis.

The most commonly used radionuclides for PET are cyclotron-produced ^{11}C and ^{18}F. Due to their short half-lives (^{11}C: 20.39 min, ^{18}F: 109.8 min), they must be used near the cyclotron. An alternative approach is the use of radiometals, such as ^{64}Cu, ^{68}Ga, and ^{86}Y. The isotope ^{68}Ga is easily produced from commercially available ^{68}Ge/^{68}Ga generators, allowing it to be prepared without a cyclotron.[10,11] The generator can be eluted several times per day, allowing for cost-effective and constant production of ^{68}Ga-labeled compounds.[12] ^{68}Ga is formed as the decay product of the long-lived parent radionuclide ^{68}Ge ($t_{1/2}$ = 270.8 days), allowing use of the generator for almost one year.[13] There is great interest in ^{68}Ga because it exhibits physical properties suitable for PET imaging, including a high positron yield of 89% and a half-life of 68 min.[14–16]

Bifunctional chelating agents (BCA), such as 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), are widely used to form complexes with ^{68}Ga. NOTA has been reported to be particularly excellent at chelating Ga^{3+} ions due to the formation of a highly stable complex.[17]

In this study, we conjugated a benzamide derivative with a NOTA-based bifunctional chelator and labeled the NOTA-benzamide conjugates with ^{68}Ga. Benzamide derivatives display specific binding to melanin. Several radiolabeled benzamide derivatives, such as *N*-(2-diethylaminoethyl)-4-[^{125}I]-iodo-benzamide ([^{125}I]IBZA),[18,19] *N*-(2-diethylaminoethyl)-4-[^{123}I]-iodo-benzamide [^{123}I]IBZA[20] *N*-(2-diethylaminoethyl)-2-[^{123}I]-iodobenzamide ([^{123}I]IBZA$_2$), [21,22] *N*-(2-diethylaminoethyl)-3-[$^{123/131}$I]-iodo-4-methoxy-benzamide,[23] 2-hydroxy-3-[^{123}I]-iodo-6-methoxy-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]benzamide,[24] [^{99m}Tc]-oxotechnetium-bis

* Corresponding author. Tel.: +82 2 2260 3709; fax: +82 2 2268 8204.
E-mail address: yukook@dongguk.edu (K.-H. Yu).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.06.047

(aminethiol)benzamide,[25] and [^{99m}Tc]-oxotechnetium-bis-aminethiol-*N*-(2-diethylaminoethyl)benzamide,[26] have been developed for single photon emission computed tomography (SPECT) and investigated for use in the diagnosis of melanoma. These radiolabeled derivatives for SPECT exhibit selective uptake by melanoma tumors and are useful in in vivo imaging. Moreover, the use of [^{123}I]IBZA[27] and [^{123}I]IBZA$_2$[28] for melanoma imaging in patients has been studied. Recently, the use of ^{18}F-labeled benzamide (*N*-[2-(diethylamino)-ethyl]-4-[^{18}F]-fluorobenzamide)[29,30] for PET was evaluated in a melanoma mouse model. This study showed its potential as a melanoma targeting agent. Complexes of ^{68}Ga are potential alternatives to ^{18}F-labeled compounds. In the present study, we report the synthesis, characterization, and preliminary biological evaluation of ^{68}Ga-SCN-NOTA-BZA.

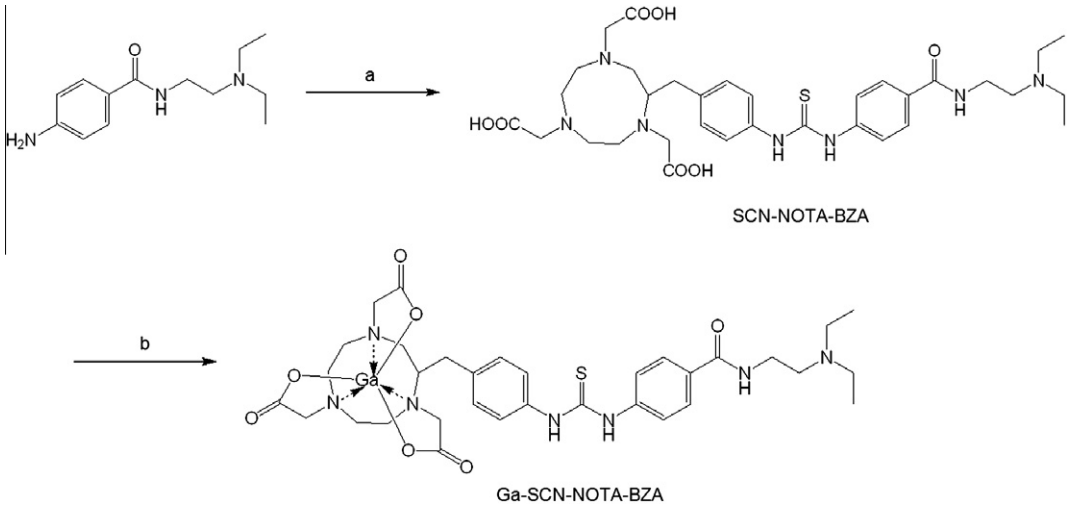
2. Results and discussion

2.1. Chemistry

Ga-SCN-NOTA-BZA was synthesized as shown in Scheme 1. The precursor, SCN-NOTA-BZA, was prepared by reacting 4-amino-*N*-(2-(diethylamino)ethyl)benzamide with *p*-SCN-Bn-NOTA in the presence of triethylamine (TEA) as a base at room temperature overnight. The resulting reaction mixture was purified by semi-preparative HPLC (gradient 10% of H_2O for 5 min and 10–70% of MeCN for 30 min; flow rate, 2 mL/min; 240 nm; t_R, 12 min) to obtain SCN-NOTA-BZA (75%) in high purity. Chelation of Ga^{3+} with SCN-NOTA-BZA was performed in aqueous solution by mixing SCN-NOTA-BZA and $GaCl_3$ in a 1:1 molar ratio. Purification of Ga-SCN-NOTA-BZA was performed by semi-preparative HPLC (gradient, 0–100% of MeCN for 30 min; flow rate, 3 mL/min; 240 nm; t_R, 14 min), with a 60% yield. Compound identity was confirmed by NMR and mass spectrometry (Supplementary data, Fig. S1–S6).

2.2. Radiochemistry

^{68}Ga was eluted from a ^{68}Ge/^{68}Ga generator by 0.1 N HCl and used directly for the reaction after adjusting the pH. Radiolabeling was conducted at a pH of 3 (1 M sodium acetate buffer, pH 5). A solution of 30 μg of SCN-NOTA-BZA in water (100 μL) was then added to a reaction vessel containing ^{68}Ga, and the mixture was heated at 100 °C for 10 min. ^{68}Ga-SCN-Bn-NOTA-benzamide (^{68}Ga-SCN-NOTA-BZA) was separated from the reaction mixture by semi-preparative HPLC. The identity of ^{68}Ga-SCN-NOTA-BZA was confirmed by co-injection with a non-radioactive reference compound (Ga-SCN-NOTA-BZA) using analytical HPLC. The entire labeling procedure was completed within 50 min, including the radioisotope incorporation, HPLC purification, rotary evaporation and formulation in saline, with a labeling efficiency in the 80–85% (decay-corrected yield) range. The radiochemical purity was greater than 97%



Scheme 1. Chemical synthesis of Ga-SCN-NOTA-BZA. Reagents and conditions: (a) *p*-SCN-Bn-NOTA, $CHCl_3$, triethylamine, room temperature, 24 h; (b) $GaCl_3$, 0.5 M sodium acetate buffer (pH 5), 100 °C, 30 min.

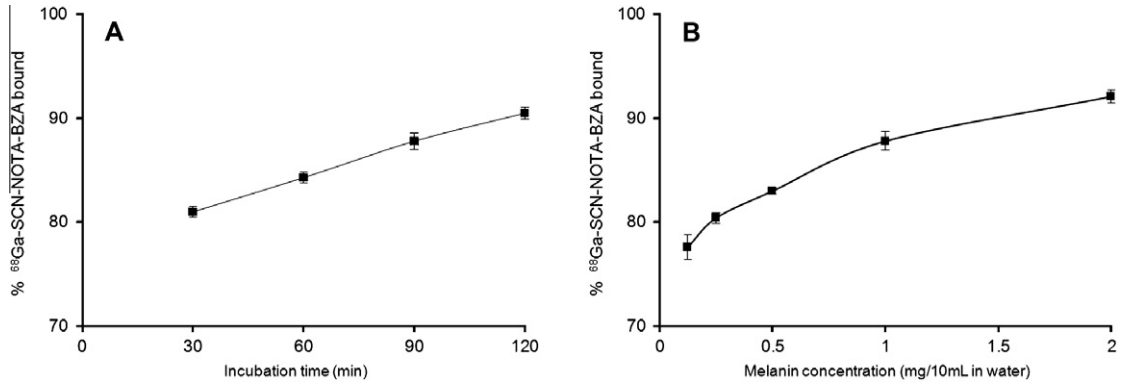


Figure 1. In vitro binding of ^{68}Ga-SCN-NOTA-BZA to melanin. (A) Effect of incubation time (30, 60, 90, and 120 min) at 37 °C. Data points are means ± SD of three measurements. (B) Effect of melanin concentration (0.125, 0.25, 0.5, 1.0, and 2.0 mg/10 mL) for 1 h incubation at 37 °C. Data points are the means ± SD of three measurements.

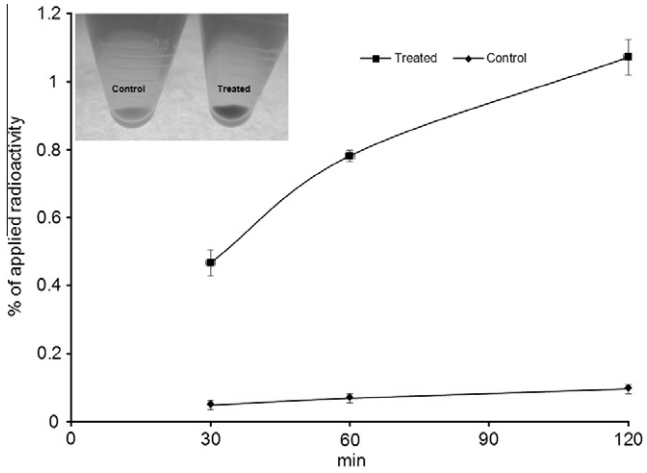


Figure 2. Cellular uptake of ^{68}Ga-SCN-NOTA-BZA in B16F10 cells. Photo shown is of a B16F10 cell pellet (non-treated (left) and treated (right) with 2 mM L-tyrosine for 24 h). All results are expressed as a percentage of applied radioactivity and are the means ± SD of three measurements.

and the specific activity at the end of synthesis was 10 GBq/μmol (Supplementary data, Fig. S7).

2.3. Partition coefficient (log *P*) and in vitro stability

The log *P* of the tracer was determined in a mixture of 1-octanol and phosphate buffered saline (PBS, pH 7.4). The measured log *P* value of ^{68}Ga-SCN-NOTA-BZA was −3.25 ± 0.05. The low log *P* value indicates that the compound is hydrophilic.

The in vitro stability of ^{68}Ga-SCN-NOTA-BZA in human plasma was evaluated at 37 °C. Stability was examined by ITLC-SG developed with 0.1 M Na_2CO_3 and 0.1 M HCl as eluents after 0.5, 1, 2, 3, and 4 h incubation time. The radiochemical purity of the tracer was >99% at 30 min and >97% after 4 h. ^{68}Ga-SCN-NOTA-BZA was found to be stable for up to 4 h at 37 °C (Supplementary data, Fig. S8).

2.4. In vitro studies

2.4.1. In vitro binding to melanin

To investigate the binding of ^{68}Ga-SCN-NOTA-BZA to melanin, its binding affinity was assessed for effects of incubation time and melanin concentration. Tyrosine-melanin (Sigma) was used for in vitro melanin binding studies. ^{68}Ga-SCN-NOTA-BZA uptake was dependent on the incubation time. Melanin uptake of ^{68}Ga-SCN-NOTA-BZA was rapid and linearly increased with time, reaching approximately 80% after 30 min and increasing to 90% after 120 min, for a melanin concentration of 0.5 mg per 10 mL (Fig. 1A). The uptake of ^{68}Ga-SCN-NOTA-BZA was also dependent on the concentration of melanin. The uptake increased gradually with increase in the concentration, showing almost a first order kinetics (Fig. 1B).

2.4.2. Cellular uptake studies

The melanin-dependent cellular uptake of ^{68}Ga-SCN-NOTA-BZA was evaluated using B16F10 (mouse melanoma) cells treated with L-tyrosine, which is the main substrate in the synthesis of melanin via the oxidative enzyme, tyrosinase. Treatment of B16F10 cells with L-tyrosine (2.0 mM) for 24 h noticeably darkened the cells compared with untreated cells (control cells) (Fig. 2). Cellular uptake values of ^{68}Ga-SCN-NOTA-BZA in B16F10 cells over incubation periods of 0.5, 1, and 2 h are shown in Figure 2. Cells treated with L-tyrosine showed a significantly increased uptake of ^{68}Ga-SCN-NOTA-BZA. Uptake by cells treated with L-tyrosine increased from 0.47% ± 0.04 at 0.5 h to 1.07% ± 0.05 at 2 h and was approximately 10-fold higher than that of control cells (0.048% ± 0.008 at 0.5 h to 0.096% ± 0.005 at 2 h).

The in vitro studies clearly demonstrate that ^{68}Ga-SCN-NOTA-BZA selectively binds to melanin.

2.5. In vivo evaluation

2.5.1. Biodistribution studies

The radioactivity distribution was measured in B16F10-bearing C57BL mice ($n = 6$) at 60 and 120 min after iv injection of ^{68}Ga-SCN-NOTA-BZA. As shown in Fig. 3, the highest uptake of

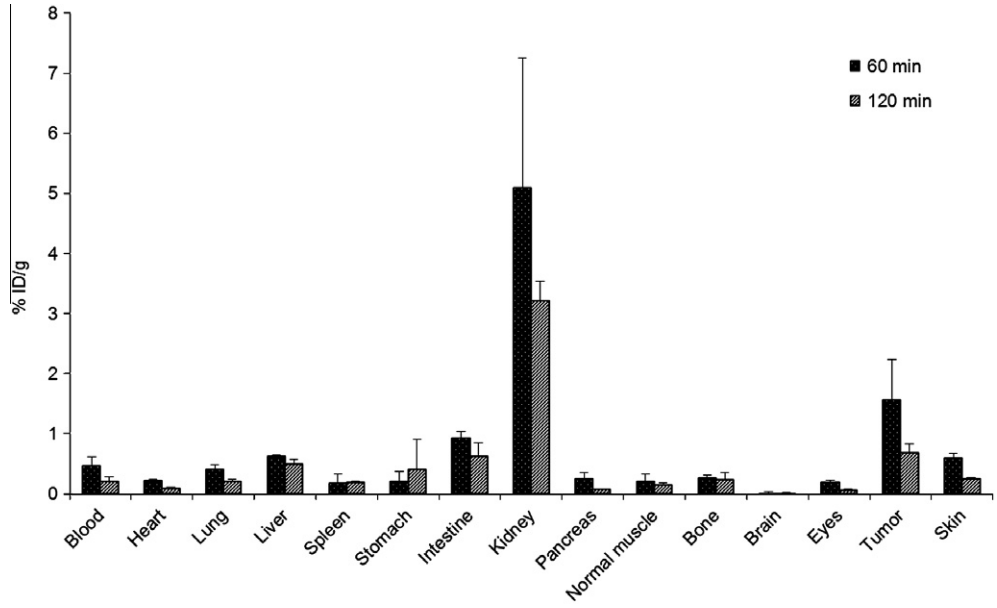


Figure 3. Biodistribution studies in B16F10-bearing mice at 60 and 120 min after an iv injection of ^{68}Ga-SCN-NOTA-BZA. Data are expressed as the percentage of administered activity (injected dose) per gram of tissue (% ID/g, $n = 6$). The tumor uptake of ^{68}Ga-SCN-NOTA-BZA was 1.57 ± 0.66% ID/g at 60 min after the radiotracer injection.

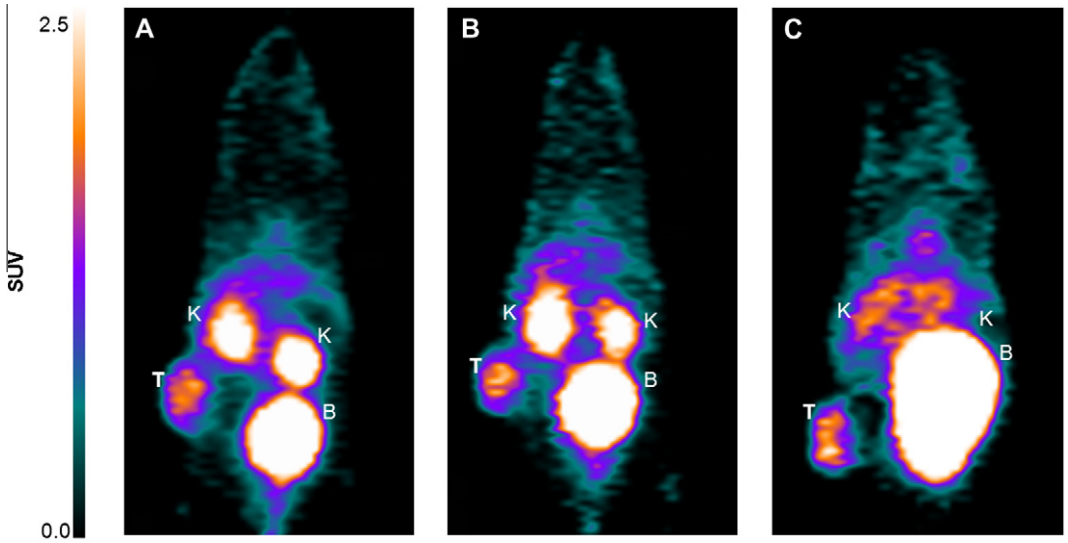


Figure 4. Coronal micro-PET imaging of B16F10-bearing mice of ^{68}Ga-SCN-NOTA-BZA. Shown are images at 0.5 (A), 1 (B), and 2 h (C) after an injection of ^{68}Ga-SCN-NOTA-BZA. B, bladder; K, kidney; T, tumor.

^{68}Ga-SCN-NOTA-BZA was found in the kidneys (5.09 ± 2.16% ID/g at 1 h) and in tumors (1.57 ± 0.66% ID/g at 1 h). In contrast, the lower values of uptake in the liver (0.62 ± 0.02% ID/g at 1 h) and intestine (0.93 ± 0.11% ID/g at 1 h) as compared to the kidneys indicated the predominance of the renal excretion route. Uptake by blood and other organs, including the heart, lung, spleen, pancreas, and muscle was very low. The tumor-to-blood (3.37 ± 0.3) and tumor-to-muscle (8.28 ± 1.41) ratios were very high. These results suggest that ^{68}Ga-SCN-NOTA-BZA is a promising melanoma imaging agent.

2.5.2. Small-animal PET studies

The melanoma mouse model biodistribution studies indicate that ^{68}Ga-SCN-NOTA-BZA can be a suitable tracer for melanoma imaging. To confirm the feasibility of using ^{68}Ga-SCN-NOTA-BZA to detect malignant melanoma in vivo, micro-PET scans were performed on a B16F10 tumor model at 0.5, 1, and 2 h after iv injection. The B16F10 tumors were clearly visualized with good contrast, with tumor to background contrast recorded at each time point (Fig. 4). ^{68}Ga-SCN-NOTA-BZA was excreted mainly through the kidneys, as predicted from the biodistribution study. The trend of increasing uptake in the tumor regions over time allowed ^{68}Ga-SCN-NOTA-BZA to accumulate in melanoma cells. Uptake of ^{68}Ga-SCN-NOTA-BZA into tumor cells in the B16F10-bearing model was significant, demonstrating the specific targeting of the tracer in the melanoma tumor model.

3. Conclusion

In this study, we described the synthesis and in vitro and in vivo characterization of ^{68}Ga-SCN-NOTA-BZA for malignant melanoma imaging. ^{68}Ga-SCN-NOTA-BZA was synthesized with 80% radiochemical yield with good radiochemical purities and high specific activity. ^{68}Ga-SCN-NOTA-BZA showed high uptake by melanin in a time-dependent fashion. The biodistribution of ^{68}Ga-SCN-NOTA-BZA demonstrated its targeting of melanoma through specific tumor uptake. Furthermore, micro-PET studies of ^{68}Ga-SCN-NOTA-BZA in a melanoma model showed that the tracer accumulates in tumor cells. These in vitro and in vivo results suggest that ^{68}Ga-SCN-NOTA-BZA could be a promising PET probe for malignant melanoma.

4. Materials and methods

4.1. General

2-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (*p*-SCN-Bn-NOTA) was purchased from Futurechem (Seoul, Korea). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA-500 FT-NMR spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C; Jeol, Tokyo, Japan). Spectra were recorded in D_2O and chemical shifts are reported in ppm relative to tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded on a JEOL JMS-AX505WA spectrometer (Tokyo, Japan), using fast atom bombardment (FAB) methods (FAB^+), at the National Center for Inter-University Research Facilities (Seoul, Korea). HPLC was performed using a SP930D pump, UV730D UV detector (Young-Lin Inc., Korea), and FC-3200 high energy gamma detector (Bioscan, USA) to measure radioactive flow. The UV detection wavelength was 240 nm for all experiments. Both semi-preparative (Phenomenex Luna, C18, 10 mm × 250 mm) and analytical (Phenomenex Gemini C18, 4.6 mm × 250 mm) reverse phase HPLC columns were used. The ^{68}Ge/^{68}Ga-generator was obtained from Eckert & Ziegler (Berlin, Germany). Instant thin layer chromatography-silica gel (ITLC-SG) plates were purchased from Merck (Darmstadt, Germany). A CRC-712MH radioisotope calibrator (Capintec Instruments, USA) was used for radioactivity measurements. ^{68}Ga analysis was performed with a 1480 WIZARD 3 gamma counter (Perkin Elmer, Waltham, MA, USA). PET studies were performed using micro-PET (Inveon, Siemens).

4.2. Chemistry

4.2.1. Synthesis of SCN-Bn-NOTA-benzamide (SCN-NOTA-BZA)

A mixture of *p*-SCN-Bn-NOTA (0.05 g, 0.09 mmol) and 4-amino-N-(2-(diethylamino)ethyl)benzamide (0.030 g, 0.11 mmol) in $CHCl_3$ (1 mL) containing TEA (45 μL, 0.27 mmol) was stirred 24 h at room temperature. The solvent was evaporated under reduced pressure. The resulting product was purified by semi-preparative HPLC (gradient 10% of H_2O for 5 min and 10–70% of MeCN for 30 min; flow rate, 2 mL/min; 240 nm; t_R, 12 min) to give SCN-NOTA-BZA as a pale

yellow solid (46 mg, 75%). MS (FAB) m/z 686, $(M+H)^+$; HRMS of $C_{33}H_{48}N_7O_7S$ 686.3336 (calculated) and 686.3340 (observed); ^1H NMR (500 MHz, D_2O): δ 7.68 (d, 1H), 7.34 (d, 2H), 7.20 (m, 4H), 3.67–3.65 (br, 2H), 3.54–3.50 (br, 2H), 3.39–3.29 (br, 4H), 3.20–3.12 (br, 6H), 3.09–2.99 (br, 8H), 2.92–2.52 (br, 3H), 1.20–1.14 (br, 8H); ^{13}C NMR (125 MHz, D_2O): δ 179.3, 178.5, 176.3, 174.4, 169.8, 142.0, 136.8, 136.1, 130.3, 130.0, 129.8, 128.8, 128.2, 126.0, 125.6, 124.5. 60.6, 57.8, 57.6, 57.2, 57.1, 52.4, 52.1, 51.6, 50.6, 47.6, 46.6, 46.0, 43.5, 34.9, 33.7, 33.6, 8.2, 8.1.

4.2.2. Synthesis of Ga-SCN-Bn-NOTA-benzamide (Ga-SCN-NOTA-BZA)

The non-radioactive reference compound Ga-SCN-NOTA-BZA was prepared by reacting SCN-NOTA-BZA with one molar equivalent of $GaCl_3$ in water. The pH was adjusted to 3, as measured by pH test paper, through addition of 0.5 M sodium acetate solution, and the mixture was stirred for 30 min at 100 °C. The reaction mixture was purified by RP-HPLC (gradient, 100% of H_2O for 5 min and 0–100% of MeCN for 30 min; flow rate, 3 mL/min; 240 nm; t_R, 14 min). MS (FAB) m/z 753, $(M+H)^+$; HRMS of $C_{33}H_{45}GaN_7O_7S$ 752.2357 (calculated) and 752.2350 (observed); ^1H NMR (500 MHz, D_2O): δ 7.62 (m, 2H), 7.32 (m, 2H), 7.18 (m, 4H), 3.70–3.55 (br, 6H), 3.43 (br, 1H), 3.34–3.13 (br, 8H), 3.12-2.99 (br, 6H), 2.96–2.71 (br, 4H), 1.12–1.08 (m, 8H); ^{13}C NMR (125 MHz, D_2O): δ 181.1, 147.8, 174.5, 174.2, 169.7, 130.0, 129.9, 129.8, 129.2, 128.4, 128.3, 124.6, 115.0, 65.3, 62.3, 61.9, 52.6, 51.1, 50.7, 47.9, 47.8, 47.7, 46.7, 34.9, 33.0, 23.1, 8.3, 8.2

4.3. Radiolabeling of ^{68}Ga-SCN-Bn-NOTA-benzamide (^{68}Ga-SCN-NOTA-BZA)

For the radiolabeling experiment, $^{68}GaCl_3$ (185–259 MBq) was eluted from a $^{68}Ge/^{68}Ga$-generator using 0.1 M HCl. SCN-NOTA-BZA (30 μg) was added to the HCl solution containing ^{68}Ga, followed by 1 M sodium acetate buffer (pH 5) to adjust the pH 3. The mixture was stirred for 10 min at 100 °C. The solution was cooled and injected into a semi-preparative HPLC column system equipped with a UV detector and a high energy gamma detector to purify the ^{68}Ga-SCN-NOTA-BZA (gradient, 0–100% of MeCN for 30 min; flow rate, 3 mL/min; 240 nm; t_R, 14 min). The identity of the radioproduct was confirmed by comparing its retention time with the non-radioactive Ga-SCN-NOTA-BZA using RP-HPLC. ^{68}Ga-SCN-NOTA-BZA was dried and dissolved in saline (0.9% NaCl aqueous solution) in a sterile multidose vial for in vitro and in vivo experiments. The total reaction time of ^{68}Ga-SCN-NOTA-BZA was less than 50 min, and the overall decay-corrected radiochemical yield was approximately >80%. Radiochemical purity was >97%, as determined by analytical HPLC (with the same gradient as used for semi-preparative HPLC). The specific activity of ^{68}Ga-SCN-NOTA-BZA was 10 GBq/μmol.

4.4. Lipophilicity

The log$P$ was measured by mixing 0.37 MBq of ^{68}Ga-SCN-NOTA-BZA with 1-octanol (3 mL) and sodium phosphate buffer (PBS, 3 mL; pH 7.40) in a test tube. The test tube was vigorously stirred for 20 min at room temperature, then centrifugated at 3000 rpm for 5 min. Aliquots of 100 μL PBS and 100 μL 1-octanol were removed and counted on a gamma counter. The log$P$ value was calculated by comparing the ratio (in cpm/ml) of 1-octanol to that of PBS and expressed as log$P$ = log [cpm/ml of 1-octanol /cpm/ml of PBS]. The experiment was performed in triplicate.

4.5. In vitro stability

For the labeling stability test, ^{68}Ga-SCN-NOTA-BZA (0.37 MBq/100 μL) was incubated with 1.0 mL of human serum in a 37 °C water bath 0.5, 1, 2, 3, and 4 h and then analyzed by ITLC-SG; 0.1 M Na_2CO_3 ($^{68}Ga^{3+}$ remained at the origin, and labeled compounds moved with the solvent front) and 0.1 M HCl ($^{68}Ga^{3+}$ moved with the solvent front, and labeled products remained at the origin) were used as eluents. All assays were performed in triplicate.

4.6. In vitro binding to melanin

The uptake of ^{68}Ga-SCN-NOTA-BZA was measured with different incubation times and melanin concentrations. For the determination of ^{68}Ga-SCN-NOTA-BZA uptake rates versus the incubation time, ^{68}Ga-SCN-NOTA-BZA (0.74 MBq) was incubated in 0.5 mg/10 ml melanin in water suspension during 0.5, 1, 1.5, and 2 h (using triplicate samples for each time) at 37 °C with stirring. To assess the effect of the incubation time on ^{68}Ga-SCN-NOTA-BZA uptake rate,^{68}Ga-SCN-NOTA-BZA (0.74 MBq) was incubated with five different melanin concentrations equal to 0.125, 0.25, 0.5, 1, and 2 mg/10 ml in water suspension (using triplicate samples for each concentration) for a 1 h at 37 °C with stirring. After incubation, the tubes were centrifuged at 30,000 g for 10 min, and aliquots of the supernatant were counted in a gamma counter. Control tubes contained the same radioactive preparation without melanin. The differences between the activities of aliquots from the supernatants of the test tubes (with melanin) and the control tubes (without melanin) allowed for the calculation of the percentage of unbound complexes. The experiment was carried out in triplicate.

4.7. Cellular uptake studies

Cellular uptake studies were performed on B16F10 cells. B16F10 cells were cultured in Dulbecco's modified Eagle high-glucose medium (Gibco Life Sciences) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were incubated at 37 °C in a 5% CO_2 in air atmosphere.

Cells were sub-cultured in 12-well plates (1×10^6 B16F10 cells) and pretreated with 2 mM L-tyrosine for 24 h. Non-treated cells were used as a control. The cells were incubated at 37 °C for 0.5, 1, or 2 h with 0.74 MBq of ^{68}Ga-SCN-NOTA-BZA. After incubation, the supernatant was removed. Subsequently, cells were washed 3 times with cold PBS to remove surface-bound radioactivity. The cells were suspended in 0.1% sodium dodecyl sulfate (SDS) in PBS. The radioactivity of the supernatant and cells was determined by a gamma counter. Data are expressed as the accumulation ratio (%) ± SD calculated by dividing the radioactivity in the pellet by the total radioactivity (supernatant + cell pellet).

4.8. Biodistribution studies

Animal care, all experiments, and euthanasia were performed in accordance with protocols approved by the Chonnam National University Animal Research Committee and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised 1985).

Biodistribution of radioactivity after injection of ^{68}Ga-SCN-NOTA-BZA were investigated. ^{68}Ga-SCN-NOTA-BZA (3.7 MBq) was intravenously injected in B16F10-bearing C57BL mice (18–20 g, Orient, Gyeonggi-do, Korea). Mice were sacrificed by cervical dislocation at 60 and 120 min after injection (n = 3 per time). Blood

samples were collected by heart puncture, and brain, blood, heart, lung, liver, spleen, stomach, intestine, kidney, pancreas, bone, muscle, eyes, skin, and tumor tissues were dissected and weighed. Radioactivity in these samples was measured using a gamma counter. The radioactivity determinations were normalized to the weight of the tissue and the amount of radioactivity injected to obtain the% ID/g.

4.9. Micro-PET studies

PET scans were obtained using an Inveon PET Scanner (Siemens Medical Solutions, USA). B16F10-bearing mouse model (18–20 g, Orient, Gyeonggi-do, Korea) were used in the PET study. Mice were anesthetized by ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (2.5 mg/kg). B16F10-allograft mice were imaged at 0.5, 1, and 2 h after iv injection of 3.7 MBq of ^{68}Ga-SCN-NOTA-BZA.

Acknowledgments

This research was supported by Nuclear R&D Program and the Converging Research Center Program (2011K000709) through the Korean Ministry of Education, Science and Technology.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.06.047.

References and notes

1. Balch, C. M.; Soong, S. J.; Gershenwald, J. E.; Thompson, J. F.; Reintgen, D. S.; Cascinelli, N.; Urist, M.; McMasters, K. M.; Ross, M. I.; Kirkwood, J. M.; Atkins, M. B.; Thompson, J. A.; Coit, D. G.; Byrd, D.; Desmond, R.; Zhang, Y.; Liu, P. Y.; Lyman, G. H.; Morabito, A. *J. Clin. Oncol.* **2001**, *19*, 3622.
2. Dancey, A. L.; Mahon, B. S.; Rayatt, S. S. *J. Plast. Reconstr. Aesthet. Surg.* **2008**, *61*, 1275.
3. Marks, R. *Cancer* **1995**, *75*, 607.
4. Jemal, A.; Siegel, R.; Xu, J.; Ward, E. *CA Cancer J Clin60*, 277.
5. Thompson, J. F.; Scolyer, R. A.; Kefford, R. F. *Lancet* **2005**, *365*, 687.
6. Hoefnagel, C. A. *Eur. J. Nucl. Med.* **1998**, *25*, 1567.
7. Ak, I.; Stokkel, M. P.; Bergman, W.; Pauwels, E. K. *Eur. J. Nucl. Med.* **2000**, *27*, 447.
8. Gambhir, S. S. *Nat. Rev. Cancer* **2002**, *2*, 683.
9. Sharma, V.; Luker, G. D.; Piwnica-Worms, D. *J. magn. reson. imaging* **2002**, *16*, 336.
10. Mukai, T.; Suwada, J.; Sano, K.; Okada, M.; Yamamoto, F.; Maeda, M. *Bioorg. Med. Chem.* **2009**, *17*, 4285.
11. Shetty, D.; Jeong, J. M.; Ju, C. H.; Kim, Y. J.; Lee, J. Y.; Lee, Y. S.; Lee, D. S.; Chung, J. K.; Lee, M. C. *Bioorg. Med. Chem.* **2010**, *18*, 7338.
12. Breeman, W. A.; Verbruggen, A. M. *Eur. J. Nucl. Med. Mol. Imaging* **2007**, *34*, 978.
13. Prinsen, K.; Li, J.; Vanbilloen, H.; Vermaelen, P.; Devos, E.; Mortelmans, L.; Bormans, G.; Ni, Y.; Verbruggen, A. *Bioorg. Med. Chem.* **2010**, *18*, 5274.
14. de Sa, A.; Matias, A. A.; Prata, M. I.; Geraldes, C. F.; Ferreira, P. M.; Andre, J. P. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 7345.
15. Maecke, H. R.; Hofmann, M.; Haberkorn, U. *J. Nucl. Med.* **2005**, *46*(Suppl 1), 172S.
16. Riss, P. J.; Kroll, C.; Nagel, V.; Rosch, F. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5364.
17. Forster, C.; Schubert, M.; Pietzsch, H. J.; Steinbach, J. *Molecules* **2011**, *16*, 5228.
18. Michelot, J. M.; Moreau, M. F.; Labarre, P. G.; Madelmont, J. C.; Veyre, A. J.; Papon, J. M.; Parry, D. F.; Bonafous, J. F.; Boire, J. Y.; Desplanches, G. G., et al *J. Nucl. Med.: Off. Publ. Soc. Nucl. Med.* **1991**, *32*, 1573.
19. Moreau, M. F.; Papon, J.; Labarre, P.; Moins, N.; Borel, M.; Bayle, M.; Bouchon, B.; Madelmont, J. C. *Nucl. Med. Biol.* **2005**, *32*, 377.
20. Moreau, M. F.; Michelot, J.; Papon, J.; Bayle, M.; Labarre, P.; Madelmont, J. C.; Parry, D.; Boire, J. Y.; Moins, N.; Seguin, H., et al *Nucl. Med. Biol.* **1995**, *22*, 737.
21. Mansard, S.; Papon, J.; Moreau, M. F.; Miot-Noirault, E.; Labarre, P.; Bayle, M.; Veyre, A.; Madelmont, J. C.; Moins, N. *Nucl. Med. Biol.* **2005**, *32*, 451.
22. Eisenhut, M.; Hull, W. E.; Mohammed, A.; Mier, W.; Lay, D.; Just, W.; Gorgas, K.; Lehmann, W. D.; Haberkorn, U. *J. Med. Chem.* **2000**, *43*, 3913.
23. Nicholl, C.; Mohammed, A.; Hull, W. E.; Bubeck, B.; Eisenhut, M. *J. Nucl. Med.* **1997**, *38*, 127.
24. Maffioli, L.; Mascheroni, L.; Mongioj, V.; Gasparini, M.; Baldini, M. T.; Seregni, E.; Castellani, M. R.; Cascinelli, N.; Buraggi, G. L. *J. Nucl. Med.* **1994**, *35*, 1741.
25. Friebe, M.; Mahmood, A.; Bolzati, C.; Drews, A.; Johannsen, B.; Eisenhut, M.; Kraemer, D.; Davison, A.; Jones, A. G. *J. Med. Chem.* **2001**, *44*, 3132.
26. Cheng, Z.; Mahmood, A.; Li, H.; Davison, A.; Jones, A. G. *Cancer Res.* **2005**, *65*, 4979.
27. Michelot, J. M.; Moreau, M. F.; Veyre, A. J.; Bonafous, J. F.; Bacin, F. J.; Madelmont, J. C.; Bussiere, F.; Souteyrand, P. A.; Mauclaire, L. P.; Chossat, F. M., et al *J. Nucl. Med.: Off. Publ. Soc. Nucl. Med.* **1993**, *34*, 1260.
28. Moins, N.; D'Incan, M.; Bonafous, J.; Bacin, F.; Labarre, P.; Moreau, M. F.; Mestas, D.; Noirault, E.; Chossat, F.; Berthommier, E.; Papon, J.; Bayle, M.; Souteyrand, P.; Madelmont, J. C.; Veyre, A. *Eur. J. nucl. med. mol. imaging* **2002**, *29*, 1478.
29. Garg, S.; Kothari, K.; Thopate, S. R.; Doke, A. K.; Garg, P. K. *Bioconjug. Chem.* **2009**, *20*, 583.
30. Ren, G.; Miao, Z.; Liu, H.; Jiang, L.; Limpa-Amara, N.; Mahmood, A.; Gambhir, S. S.; Cheng, Z. *J. Nucl. Med.* **2009**, *50*, 1692.