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^{99m}Tc-N4amG: Synthesis Biodistribution and Imaging in Breast Tumor-bearing Rodents

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Abstract

^{99m}Tc-N4-guanine (^{99m}Tc-N4amG) was synthesized and evaluated in this study. Cellular uptake and cellular fraction studies were performed to evaluate the cell penetrating ability. Biodistribution and planar imaging were conducted in breast tumor-bearing rats. Up to 17% ID uptake was observed in cellular uptake study with 40% of ^{99m}Tc-N4amG was accumulated in the nucleus. Biodistribution and scintigraphic imaging studies showed increased tumor/muscle count density ratios as a function of time. Our results demonstrate the feasibility of using ^{99m}Tc-N4amG in tumor specific imaging.

INTRODUCTION

Cancer cells are transformed from normal cells. During transformation, certain capacities are acquired by cancer cells, including self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasive & metastasis (Hanahan and Weinberg 2000). Of these cancer hallmarks, four out of six are cell survival and proliferation-related. In other words, un-controlled cell proliferation can be marked as the most important characteristic of cancer cell.

Modern imaging technologies provide non-invasive, high resolution functional imaging that assists in assessment of cancer cell proliferation. Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are two major molecular imaging modalities in nuclear medicine. With proper imaging probes, both modalities can be used to evaluate tumor growth potential, determine the degree of malignancy, and even provide physicians with an early prediction of treatment response. Much has been done to develop proper imaging probes for tumor proliferation assessment. It has been reported that the uptake of 2'-fluorodeoxyglucose (¹⁸F-FDG) correlates with cell proliferation *in vitro* and *in vivo* (Okada, Yoshikawa et al. 1992; Higashi, Clavo et al. 1993). Studies performed by various laboratories demonstrated that radiolabeled amino acid analogues may have similar potential to assess cell proliferation as well (Jager, Plaat et al. 2000; McConathy, Martarello et al. 2003). Indeed, both sugars (such as ¹⁸F-FDG) and amino acids play important roles in cell proliferation; however, nucleosides are the most essential elements in generation of nucleotides, which are required in cell proliferation. During cell proliferation, DNA must be duplicated. Therefore, uptake and consumption of nucleosides will be much greater in

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proliferating cells than in resting cells. Hence, radiolabeled nucleoside analogues are potentially the ideal tumor cell proliferative markers.

Development of radiolabeled nucleoside analogues which are involved in DNA/RNA synthesis or incorporated into DNA/RNA has been previously reported (Wright, Taylor et al. 1969; Uesugi, Kaneyasu et al. 1982; Marquez, Tseng et al. 1990; Buck, Halter et al. 2003; Francis, Visvikis et al. 2003). These probes made it possible to assess cell proliferation. For example, 3'-deoxy-3'- ^{18}F -fluorothymidine (^{18}F -FLT) is a clinically-tested tracer which images cellular proliferation by involving in DNA synthesis process. Many studies have been conducted to compare the sensitivity and specificity of ^{18}F -FDG and ^{18}F -FLT in different tumor types. For instance, comparison between ^{18}F -FDG and ^{18}F -FLT in glioma shows ^{18}F -FLT exhibits greater sensitivity than ^{18}F -FDG, with higher correlation to proliferation marker, Ki-67 (Chen, Cloughesy et al. 2005). Other studies were focused on the potential utility of ^{18}F -FLT to assess treatment response. Data from different studies have shown that ^{18}F -FLT can be used to predict survival rate of patient with glioma after bevacizumab and irinotecan treatment (Chen, Delaloye et al. 2007), define proliferation of tumor treated with cytostatic kinase inhibitor (Waldherr, Mellinghoff et al. 2005), and evaluate dose scheduling (Liu, Kolesar et al. 2008). However, DNA incorporation of ^{18}F -FLT is low, and the chemistry of making ^{18}F -FLT is complex (Buck, Halter et al. 2003; Francis, Visvikis et al. 2003). With such a complex chemistry, the radiochemical yield of ^{18}F -FLT is low, as expected. In addition, ^{18}F can only be obtained from cyclotron, which is inconvenient and expensive. All of these result in limited clinical applications of ^{18}F -FLT. Furthermore, although it has been accepted that the phosphorylation of ^{18}F -FLT by cellular thymidine kinase type 1 (TK1) is the key cellular factor for ^{18}F -FLT uptake, some clinical trials have shown a low or even no correlation between proliferation markers and ^{18}F -FLT tumor uptake (van Westreenen, Cobben et al. 2005; Westerterp, van Westreenen et al. 2005; Linecker, Kermer et al. 2008; Kimura, Yamamoto et al. 2009), indicating that other unknown cellular factors may mediate ^{18}F -FLT uptake. Some of these factors that mediate ^{18}F -FLT uptake include human nucleoside transporters (hNTs) (Paproski, Wuest et al. ; Paproski, Ng et al. 2008).

To overcome the low radiochemical yield and complex synthetic chemistry of ^{18}F -FLT, chelation chemistry plus in-house generator can be used to develop a novel tracer to measure changes in proliferative activity. Using chelation chemistry, radioisotopes can be labeled to a homing agent through a chelator. With this concept, single homing agent-chelator conjugate can be labeled with two or more different radioisotopes for various purposes. For example, $^{99\text{m}}\text{Tc}$ and ^{68}Ga -labeled compounds can be used for SPECT and PET imaging, respectively; ^{188}Re labeled compounds can be used for radiation therapy. Among all radioisotopes, $^{99\text{m}}\text{Tc}$ has been preferred to label imaging probes because of its favorable low energy (140 keV vs. 511 keV for ^{18}F), inexpensive isotope cost and capacity for kit product manufacturing. Unlike ^{18}F -labeled compounds that have to be delivered directly from manufacturing site shortly before administration, the cold kit product can be stored and labeled onsite with radioisotopes generated from an in-house generator when needed. Many $^{99\text{m}}\text{Tc}$ -labeling techniques have been developed. For instance, cyclam (N4), DTPA, DOTA, sulfur colloid and N_2S_2 have all been labeled with high radiochemical purity and yield (Van Nerom, Bormans et al. 1993; Laissy, Faraggi et al. 1994; Kao, ChangLai et al. 1998; Vriens, Blankenberg et al. 1998; Ohtsuki, Akashi et al. 1999; Canet, Casali et al. 2000; Wu, Chang et al. 2003). Among these chelators, N4 was chosen because it is a closed-ring structure, which is more rigid to stabilize isotopes. Also, the higher lipophilicity of N4 is preferred because it will help molecules to penetrate cell membrane, leading to greater uptake, decrease kidney accumulation and, thereby, reducing renal toxicity.

Nucleosides are constituted by three key elements: 1. The hydroxymethyl group, which is necessary for the phosphorylation of the molecule by cellular kinases to achieve biological activity. 2. The heterocyclic base moiety, which is crucial in the recognition process of the nucleoside through specific hydrogen bonds. 3. The ribose ring, which acts as a spacer between the hydroxymethyl group and the base and keeps them in the correct orientation. A variety of nucleoside analogues have been discovered; many of them have been successfully developed as therapeutic agents in both anticancer and antiviral medicine (Romeo, Chiacchio et al. 2010). There was few chelator-nucleoside conjugates used in tumor imaging, to explore novel nucleoside-based analogs using chelation radiochemistry, we synthesized a guanine analog using N4 as a chelator. In this report, the synthesis and assessment of tumor growth using ^{99m}Tc -N4-guanine (^{99m}Tc -N4amG) were evaluated.

MATERIALS AND METHODS

Chemicals and Analysis

Proton NMR and ^{13}C NMR analyses were performed using Bruker 300 MHz NMR spectroscope at the NMR core facility in UT MD Anderson Cancer Center (Houston, TX). Mass spectral analyses were conducted at the University of Texas M.D. Anderson Cancer Center (Houston, TX, USA). High resolution Mass spectra (multimode as ion source) were acquired on an Agilent 6200A accurate Mass time of flight (TOF) system (Santa Clara, CA, USA). HPLC analyses were performed using Waters C-18 column (Milford, MA). Two detectors on HPLC, UV and NaI detectors, were used to detect UV absorption and radioactivity, respectively. The UV absorption wave length was 254nm. Mobile system was acetonitrile to water: 8 to 2. Flow rate was 0.5mL per minute. Penciclovir was purchased from LKT Laboratories (St. Paul, MN). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Silica gel coated thin-layer chromatography (TLC) plates were purchased from Whatman (Clifton, NJ, USA).

Synthesis of 9-[4-amino-3-(hydroxymethyl)butyl]guanine (Guan, compound I)

—N²-(p-anisyl)diphenylmethyl)-9-[(4-tosyl)-3-p-anisyl)diphenylmethoxymethylbutyl]guanine (250 mg, 0.38 mmol) was placed in a dry round bottom flask. 10 mL N,N-dimethylformamide and sodium azide (80 mg, 1.2 mmol) was added. The reaction mixture was stirred at 100°C for 2 hrs. The reaction mixture was cooled to room temperature, and diluted with dichloromethane followed by water washing. The aqueous phase was extracted with dichloromethane. The combined organic phase was dried with anhydrous MgSO₄ and evaporated to yield the crude product. 225mg of the crude azido compound was obtained. ^1H NMR (CDCl₃): δ 8.04 (s, 1H), 7.22–7.45 (m, 20H), 6.86 (d, 4H, J = 9.0 Hz), 6.78 (d, 4H, J = 9.0 Hz), 3.79 (s, 3H), 3.76 (s, 3H), 3.55–3.65 (m, 2H), 3.25–3.35 (m, 2H), 1.63–1.73 (m, 2H), 1.32–1.42 (m, 1H), 1.16 (d, 2H, J = 3.6 Hz). Exact mass calculated for C₅₀H₄₆N₈O₄ [M + H]⁺ 823.3641 found 823.4025.

The crude azido compound (225mg) was dissolved in anhydrous tetrahydrofuran (30mL) and triphenylphosphine (325mg, 1.3 mmol) was added. The reaction mixture was stirred at room temperature for 16 hours. Hydrochloric acid (HCl 5N, 1ml) was added to the reaction mixture and the whole flask was heated under reflux for 5 hrs. The reaction mixture was cooled to room temperature and was filtered. The solution was evaporated to dryness. The residue was dissolved with water and washed with ethylene chloride. Sodium hydroxide (1N, 3.5ml) was added in drop-wise manner to adjust the pH to 7–8. Sephadex-G15 column chromatography was performed to separate the desired product from impurities. After freeze drying (Labconco, Kansas City, MO), 50mg of the desired product (compound I) was obtained in 76% yield. Ninhydrin (2% in methanol) spray test demonstrated the presence of amino group. Proton and ^{13}C NMR were performed. ^1H NMR (CDCl₃): δ 7.60 (s, 1H), 3.98

(t, 2H, $J = 7.2$ Hz), 3.46–3.61 (m, 2H), 2.90 (d, 2H, $J = 6.0$ Hz), 1.72 (m, 2H), 1.43 (m, 1H). FAB MS: 253 $[M + H]^+$.

Synthesis of cyclam mono acid-9-[4-amino-3-(hydroxymethyl)butyl]guanine conjugate (N4amG)—

1,4,8,11-tetraazacyclotetradecane (cyclam) (2g, 9.98 mmol) was dissolved in methylene chloride. Di-tert-butyl dicarbonate (5g, 22.91 mmol) was added to the cyclam solution. The resulting solution was stirred at room temperature for 2 hr. Then, the reaction was stopped and solvent was evaporated by Rotary Evaporator. After solvent evaporation, silica gel column chromatography was performed using methanol/methylene chloride system. 4.81 g of the desired product, Cyclam-(Boc)₃ was obtained in 86 % yield. Proton NMR and mass spectrometry analyses were conducted. ¹H NMR (CDCl₃): δ 3.08–3.22 (m, 12H), 2.81 (m, 2H), 2.55 (m, 2H), 1.81 (m, 2H), 1.67 (m, 2H), 1.38 (m, 27H). Exact mass calculated for C₂₅H₄₉N₄O₆ (M + H)⁺ 501.3574 found 501.3648.

Cyclam-(Boc)₃ (6.53 g, 13mmol) was dissolved in 20 mL of dimethyl formamide (DMF). Ethyl bromo acetate (1.34 mL) and potassium carbonate (2.70 g, 19.5 mmol) was added to the solution, and the reaction mixture was stirred under nitrogen gas and condenser at 80–85 °C for 16 hr. Reaction mixture was filtered; after filtration, filtrate was placed in Rotary Evaporator for solvent evaporation. After solvent evaporation, extraction was performed twice with chloroform as organic layer and water as aqueous layer. The organic layer was dried with magnesium sulfate and filtered. After solvent evaporation, silica gel column chromatography was performed using methanol/ methylene chloride system. 7.21 g of the desired product, Cyclam-(Boc)₃-EA was obtained in 83 % yield. Proton NMR and mass spectrometry analyses were conducted. ¹H NMR (CDCl₃): δ 4.31 (m, 2H), 3.32 (s, 2H), 3.08–3.22 (m, 12H), 2.81 (m, 2H), 2.55 (m, 2H), 1.81 (m, 2H), 1.67 (m, 2H), 1.38 (m, 27H), 1.29 (m, 3H). Exact mass calculated for C₂₉H₅₅N₄O₈ [M + H]⁺ 587.3942 found 587.5961.

Cyclam-(Boc)₃-EA (4.58 g, 7.8 mmol) was dissolved in 30 mL methanol. NaOH (1N, 15.5ml) was added for hydrolysis. Reaction mixture was stirred at room temperature for 2 hr. Methanol solvent was evaporated by Rotary Evaporator and the remaining aqueous layer was acidified with 1M HCl until pH dropped to 6. Extraction was performed twice with ethyl acetate. Ethyl acetate layer was dried with magnesium sulfate, filtered and after solvent evaporation, cyclam-(Boc)₃-AA was collected. Proton NMR and mass spectrometry analyses were conducted. ¹H NMR (CDCl₃): δ 3.32 (s, 2H), 3.08–3.22 (m, 12H), 2.81 (m, 2H), 2.55 (m, 2H), 1.81 (m, 2H), 1.67 (m, 2H), 1.38 (m, 27H). Exact mass calculated for C₂₇H₅₁N₄O₈ [M + H]⁺ 559.3629 found 559.5141.

Cyclam-(Boc)₃-AA (50mg, 0.19mmol) was dissolved in water (5ml). To this colorless solution, sulfo-NHS (95.5mg, 0.44 mmol), EDAC (84.5mg, 0.44mmol) and compound I (50mg, 0.20mmol) were added. The mixture was stirred at room temperature for 16 hr. The mixture was frozen dried. 10mL 50% trifluoroacetic acid (TFA) in methylene chloride was added to the dried residues. The reaction mixture was stirred at 50 °C for 30 min. Solvent was evaporated by Rotary Evaporator and the remaining was dissolved in water. The aqueous layer was dialyzed for 24 hours using Spectra/POR dialysis membrane with molecular weight cut-off at 500 Da. (Spectrum Medical Industries Inc., Houston TX). After freeze drying, 50mg of the desired product (N4amG) was obtained in 58% yield. Mass spectrometry and HPLC analysis were performed. The purity is >97%. ¹H NMR (D₂O): δ 7.60 (s, 1H), 3.98 (t, 2H, $J = 7.2$ Hz), 3.46–3.61 (m, 2H), 3.32 (s, 2H), 3.08–3.22 (m, 12H), 2.90 (d, 2H, $J = 6.0$ Hz), 2.81 (m, 2H), 2.55 (m, 2H), 1.81 (m, 2H), 1.72 (m, 2H), 1.67 (m, 2H), 1.43 (m, 1H). Exact mass calculated for C₂₂H₄₁N₁₀O₃ [M + H]⁺ 493.3285 found 493.3340.

Radiolabeling of N4amG with ^{99m}Tc

N4amG (5mg) was dissolved in 0.2 ml distilled water. Subsequently, 0.1 mL Tin(II) chloride solution (1 mg in 1 ml of water) and sodium pertechnetate ($\text{Na}^{99m}\text{TcO}_4$, 1.0 mCi, Mallinckrodt, Houston, TX) were added sequentially. The total volume was adjusted to 1.0 mL by adding adequate amount of distilled water. The reaction mixture was kept at room temperature for 10 minutes. The specific activity was 5mg/1mCi/mL. Radiochemical purity was determined by TLC (ITLC SG, Gelman Sciences, Ann Arbor, MI) eluted with saline. From radio-TLC analysis (Bioscan, Washington, DC) and HPLC analysis (Waters, Milford, MA), the radiochemical purity was more than 95%.

In vitro cell culture assay

To evaluate whether ^{99m}Tc -N4amG can penetrate cell membrane, *in vitro* cellular uptake study using ^{99m}Tc -N4amG and its chelator control (^{99m}Tc -N4) was conducted. Cells in 12-well plate (50,000 cells/ well) were incubated with ^{99m}Tc -N4amG or ^{99m}Tc -N4 at 37 °C for 30 min, 2 hr or 4 hr. Following the incubation period, cells were washed with PBS twice and detached with 0.5mL of trypsin solution. After centrifugation, radioactivity was measured by gamma counter. Data was processed and presented in mean $\pm$ S.D.

To determine the distribution pattern of ^{99m}Tc -N4amG within the cells, NE-PER extraction reagents were used stepwise to isolate cytoplasmic and nuclear extracts from breast cancer cells. ^{99m}Tc -N4amG (0.1mCi) was prepared in the concentration of 0.1 mg/mL. After 20 μL of ^{99m}Tc -N4amG (0.02 mg/well, 2 mCi) or ^{99m}Tc -N4 (control) was dispensed into each well of 6-well plate, cells (50,000 per well) were incubated at 37 °C for 2 h. Cells were transferred to a 2mL microcentrifuge tube and isolated by centrifugation at 500 g for 2–3 min. 200 μL of ice-cold kit reagent (CER I) was added to the cell pellet in other remaining tubes. Following 10 min incubation, 11 μL of ice cold CER II was added and incubated for an additional 1 min. The tubes were centrifuged for 5 min at maximum speed in a microcentrifuge and the supernatant (cytoplasmic extract) fractions were transferred to other tubes for counting. Insoluble (pellet) fractions, which contain nuclei, were resuspended in 100 μL of ice-cold NER. Following a total of 40 min incubation, the tubes were recentrifuged at a maximum speed for 10 min and the supernatant (nuclear extract) fractions were transferred to other tubes for counting. Insoluble (pellet) fraction, which contains nuclear material, was resuspended in 100 μL of 1_PBS and transferred to other tubes for counting. Samples were counted in a gamma counter.

To further investigate whether different cell cycle phases have different capacity in the uptake of ^{99m}Tc -N4-Guan, cell sorting assay was conducted. Cells were sorted into the various phases of cell cycle (G0/G1, S, and G2/M) using fluorescence activated cell sorter (FACS). Sorted cells were suspended in 15mL test tubes (50,000 cells/ tube). ^{99m}Tc -N4-Guan or ^{99m}Tc -N4 was added, and the cells were incubated at 37 °C for 60 min. Following the incubation period, cells were centrifuged and washed with PBS twice. After final centrifugation, radioactivity was measured by gamma counter. Data was processed and presented in mean $\pm$ S.D.

Biodistribution studies

The animals were housed in the University of Texas M.D. Anderson Cancer Center facility. The protocols involving rats and radioisotopes were approved by the M.D. Anderson Animal Use and Care Committee, and Radiation Safety Committee. Female Fischer 344 rats (150–175 g) (Harlan Sprague–Dawley, Inc., Indianapolis, IN) were inoculated subcutaneously in the right leg with breast cancer cells (10^6 cells/rat) from the breast cell line (known as DMBA-induced breast cancer cell line). Biodistribution studies were performed on day 14 after inoculation. 9 tumor-bearing rats were used and divided into three

groups, each group representing a time interval (0.5, 2, and 4 hr, n=3/time point). 20 μCi of $^{99\text{m}}\text{Tc}$ -N4amG was injected via a lateral tail vein. After administration of the radiotracers, the rats were sacrificed at certain time point and the selected tissues were excised, weighed and counted for radioactivity with a gamma counter. The distribution of radiotracer in each organ was expressed as percentage of the injected dose per gram of tissue (%ID/g). Tumor/normal tissue count ratios were determined from the corresponding %ID/g values. In statistical analysis, percent of injected dose per gram of tissue weight (%ID/g) and tumor-to-tissue ratios used in biodistribution studies will be presented as means $\pm$ SD.

Olinda dosimetry estimation

Human radiation dose estimation for $^{99\text{m}}\text{Tc}$ -N4amG were computed through Olinda/EXM software (Oakridge, TN)(Stabin, Sparks et al. 2005) from their respective biodistribution data. Olinda inputs were calculated using established method(Wei, Tsao et al. 2008; Tsao, Wang et al. 2011). Briefly, fitted residence time functions were plotted and multiplied by the exponential decay functions (i.e. half-life) for $^{99\text{m}}\text{Tc}$. These functions were then integrated analytically to determine the area under the curve (AUC) to yield the residence time of each organ. Mass correction factors were used to account for the different ratios of organ to total body weights in the rat and in humans, and allowed for the scaling of the rat residence times to human residence times. Residence times were then used to calculate target organ absorbed radiation doses with S-value tables for a standard 70 kg male model using the Olinda software package. Each organ dose was computed from the sum of self-dose plus the dose it received from each source organ in the body or from the remainder of the body. The estimated human radiation absorption dose was determined.

Scintigraphic imaging studies

To demonstrate the application of $^{99\text{m}}\text{Tc}$ -N4amG in breast tumor imaging, three breast tumor-bearing rats were administered with $^{99\text{m}}\text{Tc}$ -N4amG (300 μCi /rat, 1.0mg physical amount, i.v.) Scintigraphic images, using a gamma camera equipped with low-energy, parallelhole collimator, were obtained after 1, 2 and 4 hr. The standard source used for calibration was 30 μCi of $\text{Na}^{99\text{m}}\text{TcO}_4$. Computer outlined region of interest (counts per pixel) of the tumor and muscle (at the symmetric site) was used to determine tumor-to-muscle count density ratios.

RESULTS

Chemistry

Exact mass calculated for $\text{C}_{22}\text{H}_{41}\text{N}_{10}\text{O}_3$ (N4amG, $\text{M} + \text{H}^+$) was 493.3358 found 493.3340 (Fig. 2). HPLC spectra showed that the purity of N4amG was greater than 95% (Fig. 3). After NMR confirmation, the impurity (<5%, retention time: 14.915 min) was protected precursor. From radio-HPLC (Bioscan, Washington, DC) analysis (Fig. 4), the radiochemical purity was more than 95% (retention time: 5.288 min).

In vitro cell culture assays

High cellular uptake (up to 17%ID at 4 hr) of $^{99\text{m}}\text{Tc}$ -N4amG in breast tumor cells was observed in cellular uptake study (Fig. 5). The accumulation increased with increase in incubation time suggesting $^{99\text{m}}\text{Tc}$ -N4amG has tumor targeting potential. Nucleus fraction had 40% uptake of $^{99\text{m}}\text{Tc}$ -N4amG whereas cytosolic fraction had 60%. Both nucleus and cytosolic fractions in $^{99\text{m}}\text{Tc}$ -N4amG were higher than $^{99\text{m}}\text{Tc}$ -N4 (Fig. 6). The data indicated that $^{99\text{m}}\text{Tc}$ -N4amG, but not $^{99\text{m}}\text{Tc}$ -N4 can penetrate cell membrane, and about 40% of $^{99\text{m}}\text{Tc}$ -N4amG will accumulate in cell nucleus. In cell sorting assay, cells in S phase showed highest uptake of $^{99\text{m}}\text{Tc}$ -N4-Guan followed by G2/M phase (Fig. 7). Compare to

chelator-only control ($^{99m}\text{Tc-N4}$), $^{99m}\text{Tc-N4-Guan}$ showed greater uptake in each cell cycle phases. This data suggested $^{99m}\text{Tc-N4-Guan}$ uptake is S phase-related.

Biodistribution studies

In biodistribution studies, there was an increased uptake in tumor-to-blood and tumor-to-muscle ratios in breast tumor-bearing rats. The optimum tumor uptake and tumor-to-muscle ratios were 2–4 hr post-administrations. There was no increased uptake in thyroid and stomach suggesting in vivo stability of $^{99m}\text{Tc-N4amG}$ (Table 1). $^{99m}\text{Tc-N4amG}$ was excreted through liver and kidneys. Relatively high uptake in Bone and spleen was observed suggesting $^{99m}\text{Tc-N4amG}$ could target organs with high proliferation properties.

Olinda dosimetry estimation

The human residence time estimates were applied to the Olinda software to produce radiation dose estimates for $^{99m}\text{Tc-N4amG}$. The Olinda output was multiplied by a proposed human dose of 20mCi for $^{99m}\text{Tc-N4amG}$ to yield total absorbed dose from an imaging study (Table 2). Radiation exposure to the whole body, blood forming organs (red marrow, spleen), gonads (testes, ovaries), and effective dose equivalent for the proposed human single dose at 20 mCi fall below the limits of 3 rad annually and 5 rad total. The absorbed dose in all other organs (e.g. kidneys) was below the limits of single dose of 5 rad annually and 15 rad in total. $^{99m}\text{Tc-N4amG}$ showed total rad absorbed by each organ was below the proposed annual and total limits.

Scintigraphic imaging studies

In planar imaging studies, the tumors could be well visualized in breast tumor-bearing rats (Fig. 8). From computer outlined region of interest analysis, the average tumor-to-muscle count density ratios (n=3 rats) at 1, 2 and 4 hr were 3.69 ± 0.15 , 3.96 ± 0.52 and 4.93 ± 0.79 , respectively. The gradually linear increased tumor-to-muscle count density ratios indicated the tumor targeting potential of $^{99m}\text{Tc-N4amG}$.

DISCUSSION

Penciclovir was selected as the lead compound to synthesize N4amG because of four major concerns: 1. Penciclovir is a guanosine analogue which preserves the core guanine structure. Since guanine is one of the main nucleobases in human body, penciclovir will work as an agonist in human body with high affinity to human nucleoside transporters (hNTs) and can be effectively transported into cell (Colledge, Civitico et al. 2000). 2. The rest part of penciclovir is a carbon chain with two hydroxyl groups instead of a ribose (deoxyribose) in guanosine. The carbon chain makes penciclovir resistant to metabolizing enzymes, as a result, the circulation time and half-life of penciclovir in human body will be increased. 3. The two hydroxyl groups play a crucial role in pharmacodynamics. After being transported inside the cell, the hydroxyl groups of penciclovir can be phosphorylated by cellular deoxyguanosine kinase (Colledge, Civitico et al. 2000). Phosphorylated penciclovir will be trapped inside cell and may be further involved in DNA synthesis. Finally, since penciclovir is a clinical medicine with known pharmacokinetics, pharmacodynamics and toxicity, and it is also commercially available, it is favorable for us to use penciclovir as the lead compound.

In our biodistribution data, it showed relatively high uptake in bone and spleen (1.02 ± 0.21 and 1.18 ± 0.15 %ID per gram of tissue weight at 4 hr, respectively.) Since red bone marrow and spleen are blood forming organs, it suggests that $^{99m}\text{Tc-N4-Guan}$ can target highly proliferating cells. It is known that penciclovir can penetrate cell membrane through hNTs; furthermore, inside cells, phosphorylated penciclovir can be recognized as dGMP and

thereby involved in DNA synthesis (Colledge, Civitico et al. 2000). With the same nucleoside backbone, ^{99m}Tc -N4-Guan may work very similarly with penciclovir inside cells. In addition, from our cellular fraction studies, after two hours incubation, 40% of the ^{99m}Tc -N4-Guan accumulated in the nucleus fraction indicating ^{99m}Tc -N4-Guan is involved in cell nucleus activity. These data suggests that ^{99m}Tc -N4-Guan may be a potential cell proliferation marker for future malignant diseases assessment.

In medicine, correct diagnosis is essential to successful treatment. At the present time, ^{18}F -FDG PET is the most commonly-used diagnostic tool in clinical oncology. However, ^{18}F -FDG may not be specific enough due to high uptake in benign tissues affected with infection and/or inflammation, leading to false positive results (Barrington and O'Doherty 2003). Consequently, it is difficult to make the right diagnosis. An alternative method by targeting cell proliferation is currently being developed to avoid these false positives results. Although many radiotracers have been tested for assessment of tumor cell proliferation *in vitro* and *in vivo*, no agent has been approved for clinical use in patients yet. It is then desirable to have a novel radiotracer for imaging cell proliferation. Nucleoside-based radiotracers not only penetrate cell membrane through hNTs but also accumulate inside proliferating cells. By using homing agent-chelator conjugates labeled with a generator produced radioisotope, greater benefits could be achieved in terms of utility, chemistry and cost. Clinical data also supports that nucleoside-based imaging agents will have higher tumor specificity than ^{18}F -FDG in many cancers (Buck, Schirrmester et al. 2002; Yamamoto, Nishiyama et al. 2008). Therefore, ^{99m}Tc -N4-guan is anticipated to be more specific than FDG in detecting tumors. Furthermore, N4amG can be labeled with different isotopes for difference modalities (such as ^{68}Ga for PET) or even for therapeutic utility (^{188}Re for radiation therapy). Hence, a novel platform of radiolabeled N4-Guan could be developed for tumor-specific imaging and treatment.

In conclusion, we designed and synthesized a novel nucleoside-based imaging tracer that may target tumor cell proliferation, for which good imaging agents are currently not available. Our results demonstrated that N4-Guan can be synthesized in an appropriate yield and it can be labeled with ^{99m}Tc in high radiochemical yield and purity. *In vitro* and *in vivo* biological evaluations of ^{99m}Tc -N4-Guan showed it is feasible to ^{99m}Tc -N4amG in tumor specific imaging.

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Figure 1.

Schematic of N4amG synthesis. Briefly, one of the hydroxyl groups of penciclovir was selectively protected by a bulky protection group. The unprotected hydroxyl group was converted to a highly reactive leaving group (tosyl group) and then reacted with sodium azide to form the azido-penciclovir compound. Azido compound was reduced followed by deprotection to gain amine-penciclovir (NH₂-Guan). For the chelator part, cyclam was converted to cyclam mono-acid (cyclam-(Boc)₃-AA) in 3 steps. The carboxyl acid group of the cyclam mono-acid was reacted with NH₂-Guan to form amide linkage. The final product, N4amG, was obtained after deprotection and purification. The total synthetic yield of N4amG is 6%.

Sample Name	n4-am-g	Position	Vial 1	Instrument Name	Instrument 1	User Name	zhp
Inj Vol	0.5	InjPosition		SampleType	Sample	IRM Calibration Status	Success
Data Filename	n4-am-g0001.d	ACQ Method		Comment		Acquired Time	3/30/2010 10:28:13 AM

Figure 2. Mass spectrometry of N4amG. Exact mass calculated for $C_{22}H_{41}N_{10}O_3$ (M + H)⁺ was 493.3358 found 493.3340.

Figure 3.
HPLC analysis of N4amG. The spectra showed that the purity of N4amG was greater than 97%.

Figure 4. Radio HPLC analysis of $^{99m}\text{Tc-N4amG}$. The spectra showed that the radiochemical purity of $^{99m}\text{Tc-N4amG}$ was greater than 95%.

Figure 5. *in vitro* cellular uptake of ^{99m}Tc -N4amG. Breast tumor cells (50,000 per each well) were incubated with 2 μCi of ^{99m}Tc -N4amG at 37°C at 0.5–4 hr. There was a gradually increased uptake of ^{99m}Tc -N4amG in mammary tumor cells. Data are expressed in mean $\pm$ S.D. percent of incubation dose from three measurements.

***in vitro* Cellular Fractions of ^{99m}Tc -N4amG** **Figure 6.**

in vitro cellular fractions of ^{99m}Tc -N4amG. After 2ml of radioactive media containing 2 μCi (0.074MBq) of ^{99m}Tc -N4amG (0.02 mg/well) or ^{99m}Tc -N4 (control) was dispensed into each well of 6-well plate, cellular fractions were isolated by using NE-PER extraction kit. Nucleus fraction had 40% uptake of ^{99m}Tc -N4amG whereas cytosolic fraction had 60%. Both nucleus and cytosolic fractions in ^{99m}Tc -N4amG were higher than ^{99m}Tc -N4. Data are expressed in mean $\pm$ S.D. percent of incubation dose from three measurements.

***in vitro* Cell Sorting Assay of ^{99m}Tc -N4amG** **Figure 7.**

in vitro cell sorting assay of ^{99m}Tc -N4amG. Cells were sorted into the various phases of cell cycle (G0/G1, S, and G2/M) using fluorescence activated cell sorter. Sorted cells were suspended in 15mL test tubes (50,000 cells/ tube). ^{99m}Tc -N4-Guan or ^{99m}Tc -N4 was added, and the cells were incubated at 37 °C for 60 min. Following the incubation period, cells were centrifuged and washed with PBS twice. After final centrifugation, radioactivity was measured by gamma counter. Data was processed and presented in mean $\pm$ S.D.

Planar scintigraphy of ^{99m}Tc -N4amG in breast tumor-bearing rats **Figure 8.**

Planar scintigraphy of ^{99m}Tc -N4amG (300 μCi /rat, i.v., acquired 500,000 count) in breast tumor-bearing rats at 1–4 hr. The numbers indicate tumor-to-muscle ratios (counts/pixel). Data showed that ^{99m}Tc -N4amG could gradually accumulate inside tumor.

Table 1Biodistribution of ^{99}Tc -N4amG in breast tumor-bearing rats

% Of injected dose per gram of tissue weight (n=3/time interval iv)			
	0.5 h	2 h	4 h
Blood	1.47±0.03	0.91±0.12	0.61±0.09
Heart	0.41±0.06	0.25±0.03	0.17±0.03
Lung	0.88±0.13	0.61±0.14	0.42±0.08
Thyroid	0.67±0.02	0.51±0.06	0.38±0.09
Pancreas	0.29±0.03	0.19±0.04	0.13±0.01
Liver	8.39±2.26	8.07±0.28	8.25±1.30
Spleen	1.08±0.16	1.12±0.21	1.18±0.15
Kidney	4.30±0.57	6.71±0.53	7.06±1.04
Stomach	0.37±0.05	0.30±0.05	0.24±0.02
Intestine	0.35±0.04	0.24±0.04	0.21±0.07
Uterus	0.48±0.02	0.31±0.06	0.22±0.04
Tumor	0.43±0.04	0.37±0.06	0.32±0.06
Muscle	0.13±0.03	0.11±0.01	0.07±0.02
Bone	0.96±0.07	0.82±0.36	1.02±0.21
Brain	0.04±0.01	0.02±0.00	0.02±0.01
T/Blood	0.29±0.02	0.41±0.03	0.52±0.02
T/Muscle	3.24±0.38	3.49±0.41	4.30±0.59

Table 2Radiation dose estimates of the reference adult for $^{99}\text{Tc-N4amG}$

Organs		
Target organ	rad/mCi	Human dose (20 mCi)
Adrenals	1.01E-02	0.202
Brain	8.52E-04	0.017
Breasts	2.09E-03	0.042
Gall bladder wall	1.03E-02	0.206
LLI wall	9.35E-04	0.019
Small int	3.56E-03	0.071
Stomach	7.11E-03	0.142
ULI wall	3.53E-03	0.071
Heart wall	1.66E-02	0.332
Kidneys	7.60E-02	1.520
Liver	2.83E-02	0.566
Lungs	1.55E-02	0.310
Muscle	2.15E-03	0.043
Pancreas	1.23E-02	0.246
Bone surfaces	1.19E-02	0.238
Skin	9.45E-04	0.019
Testes	1.86E-04	0.004
Thymus	3.30E-03	0.066
Thyroid	7.62E-04	0.015
Urine bladder wall	4.18E-04	0.008
Uterus Uterus	1.06E-03	0.021
EFF dose	6.79E-03	0.136
Blood-forming organs		
Ovaries	1.24E-03	0.025
Red marrow	3.07E-03	0.061
Spleen	5.45E-02	1.090
EFF dose equivalent	1.45E-02	0.290
Total body	3.73E-03	0.075