Synthesis and Preliminary Evaluation of ^{68}Ga-NOTA-Biphenyl-c (RGDyK) for the Quantification of Integrin $\alpha_v\beta_3$

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Arginine-glycine-aspartate (RGD) peptide binds to the integrin $\alpha_v\beta_3$, which plays a crucial role in tumor angiogenesis and metastasis. Previously developed ^{68}Ga-labeled cyclic RGD peptides are rapidly excreted from the circulatory system. In the present study, we developed a ^{68}Ga-labeled cyclic RGD peptide with a biphenyl group between the chelator and RGD peptide, *i.e.*, ^{68}Ga-NOTA-biphenyl-c(RGDyK). Then, we performed the comparison with the reference compound, *i.e.*, ^{68}Ga-NOTA-c(RGDyK). ^{68}Ga-NOTA-biphenyl-c(RGDyK) was 37% less hydrophilic than ^{68}Ga-NOTA-c(RGDyK). For positron emission tomography imaging, ^{68}Ga-NOTA-biphenyl-c(RGDyK) had a longer retention time and showed a higher signal-to-noise ratio in tumors than ^{68}Ga-NOTA-c(RGDyK). However, the biphenyl-radiopeptide displayed the relatively high non-specific binding. From these perspectives, incorporation of the biphenyl group to the RGD generates pros and cons.

Keywords: Radiometals, ^{68}Ga-NOTA-c(RGDyK), ^{68}Ga-NOTA-biphenyl-c(RGDyK), Cyclic arginine-glycine-aspartate peptides, Positron emission tomography imaging

Introduction

Upregulation of the integrin $\alpha_v\beta_3$ is highly correlated with tumor progression and the integrin receptor-related angiogenesis has gained great importance for oncology.[1] Arginine-glycine-aspartate (RGD) peptides that bind specifically to integrin $\alpha_v\beta_3$ are potential biomarkers for angiogenesis.[2] Therefore, many radiolabeled RGD peptide analogues have been developed as radiopharmaceuticals for positron emission tomography (PET) and single photon emission computed tomography (SPECT).[3] Currently, eight RGD peptide PET radioligands have been utilized in human subjects, including ^{68}Ga-NOTA-PRGD2, ^{68}Ga-NOTA-RGD, ^{18}F-Galacto-RGD, ^{18}F-RGD-K5, ^{18}F-FPPRGD2, ^{18}F-Fluciclatide, ^{18}F-Alfatide, and ^{18}F-Alfatide II.[4]

Among the above peptides, all but one of them contains c(RGDyK) or c(RGDfK). To make stable complex with ^{68}Ga, many macrocyclic chelators such as have been developed. Representative chelators are DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), NOTA (1,4,7-triazacyclononane-N,N′,N″-triacetic acid), and NODAGA (1-(1,3-carboxypropyl)-4,7-carboxymethyl-1,4,7-triazacyclononane).[5,6] Multiple lines of evidence have demonstrated that the binding between ^{68}Ga and NOTA is more stable than that between ^{68}Ga and DOTA. Thus, NOTA is a frequently used chelator in clinical trials. In terms of radioisotopes, fluorine-18 (^{18}F) and gallium-68 (^{68}Ga) have been used for PET imaging. Unlike ^{18}F, the production of which requires an on-site cyclotron, ^{68}Ga is commercially feasible, allowing convenient batch production for small subjects from a generator elution system.[7]

Previously developed RGD radioligands have drawbacks such as rapid clearance from the blood pool and low tumor-to-background ratio because of their high hydrophilicity.[8,9] Consequently, efforts to improve the pharmacokinetic properties of these compounds are ongoing. Bogdanowich-Knipp *et al*. suggested that structural rigidity confers solution stability under neutral and acidic conditions.[10] Our group recently reported that incorporating a biphenyl moiety into a macrocyclic chelate backbone increased thermodynamic and kinetic stability.[11] Motivated by these observations, we introduced a biphenyl group between NOTA and c(RGDyK). Then, the resulting NOTA-biphenyl-c(RGDyK) was conjugated with ^{68}Ga. The aim of the study is to develop ^{68}Ga-NOTA-biphenyl-c (RGDyK) and to assess its biological characteristics.

Experimental

Preparation of RGD Peptides. NOTA-c(RGDyK) was purchased from Futurechem (Seoul, Korea), and NOTA-biphenyl-c(RGDyK) was synthesized as described in Appendix S1 (Supporting Information).

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Radiosynthesis. ^{68}Ga was produced from a ^{68}Ge/^{68}Ga generator ($T_{1/2}$ of ^{68}Ga = 68 min) made by ITG Company (Isotope Technologies Garching GmbH, Germany). $^{68}GaCl_3$ (~ 333 kBq in 1 mL) was completely dried by purging with nitrogen (99.9999%) gas in a V-vial (5 mL) and heated at 100°C. Then, each NOTA-c(RGDyK) and NOTA-biphenyl-c(RGDyK) solution (100 μg/100 μL of 1 M NaOAc, pH 5~6) was dispensed into separate V-vials. The mixtures were reacted at 80°C for 5 min. After being radiolabeled with ^{68}Ga, ^{68}Ga-NOTA-c(RGDyK), and ^{68}Ga-NOTA-biphenyl-c(RGDyK) were used without additional purification. The radiochemical purity was determined by radio-TLC (thin layer chromatography) using an eluent of 0.1 M citrate buffer of (pH = 5.0). In the present study, we used ^{68}Ga-NOTA-c(RGDyK) as a reference radiopeptide to comparative purpose.[12]

Animals. Female BALB/c mice were purchased from Nara-BioTec (3–5 weeks of age, Korea). The living chambers for the animals were automatically temperature and humidity controlled. A diet and water were provided *ad libitum.* The protocols were approved by the Institutional Animal Care and Use Committee at Korea Institute of Radiological & Medical Sciences.

Tumor Xenograft Model. U87MG cell was purchased from American Type Culture Collection (Manassas, VA, USA) and was maintained in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) and 1% pen/strep in 5% CO_2 at 37°C. The medium was renewed every 3 days. The 5×10^6 cells of U87MG was subcutaneously administrated into the left shoulder of mice (4–6 weeks of age). When the tumor volume reached around 0.5 cm in diameter (approximately 14 days after implantation), biodistribution and PET/CT experiments were separately conducted.

***In vitro* Cellular Uptake.** U87MG was plated in a six-well plate with optimized medium (cell density: 1×10^6 cells/well). After 4 h of incubation at 37°C, 111 kBq of ^{68}Ga-NOTA-c(RGDyK) and ^{68}Ga-NOTA-biphenyl-c(RGDyK) was distributed to wells and incubated for 30, 60, 90, and 120 min under an 5% CO_2 atmosphere at the ambient temperature (n = 4 each). The cells were washed with 2 mL × 2 with cold PBS (phosphate-buffer saline), and the radioactivity of the detached cells (down layer) and washed buffer (upper layer) were quantified by a γ-counter (1480 Wizard, USA). The percent uptake into cells of the compounds was calculated by % (counts in cells/total counts). All data points are displayed as the means ± SD.

Serum Stability. *In vitro* serum stability of ^{68}Ga-NOTA-c(RGDyK) and ^{68}Ga-NOTA-biphenyl-c(RGDyK) was evaluated by the radio-instant TLC method using an eluent of 0.1 M citrate buffer of (pH = 5.0). For each radiolabeled conjugate (14.1 MBq), a buffer solution was incubated at 37°C in 0.5 mL of mouse and human serum or PBS for the time intervals (30, 60, 90, and 120 min). Increment of free ^{68}Ga indicated the degradation of radiopeptides.

Determination of Lipophilicity. Radiolabeled conjugate of 3.7 MBq was added to the octanol-PBS system (1 mL). The vial was shaken for 5 min and it centrifuged at 12,500 rpm for 5 min. Determination of logP values were obtained from the measurement of the radioactivity in each phase by γ-counter (n = 3).

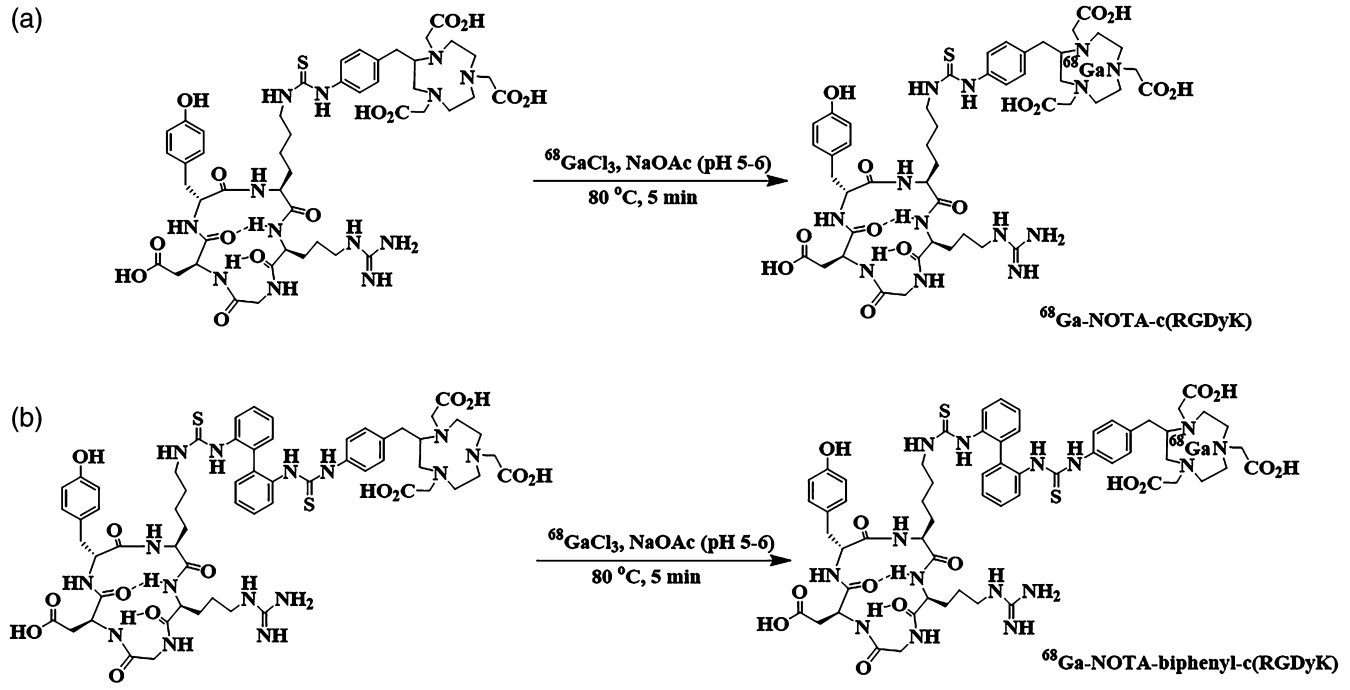

Figure 1. Reaction schemes for the RGD peptides. (a) ^{68}Ga-NOTA-c(RGDyK) and (b) ^{68}Ga-NOTA-biphenyl-c(RGDyK).

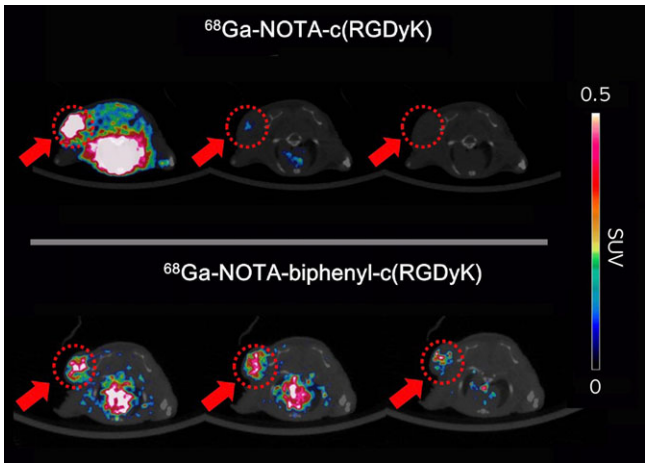

Figure 2. Representative PET/CT images of U87MG tumor-bearing mice injected with ^{68}Ga-NOTA-c(RGDyK) and ^{68}Ga-NOTA-biphenyl-c(RGDyK). Red arrows indicate the tumor.

***Ex vivo* Biodistribution.** After anesthetizing tumor xenograft mice with 1.5% isoflurane in oxygen, radiopeptides of 0.37 MBq were intravenously administrated. Mice (n = 5 each) were sacrificed, and the tissues of interest were extracted. Then weighed the tissue, and measured the radioactivity by the γ-counter. Each tissue uptake was presented as % ID/g.

Image Acquisition and Analysis. After anesthetizing mice with 1.5% isoflurane, the radiopeptides (7.4 MBq) were intravenously administrated via the tail vein for 1 min with a KDS 210 syringe pump (Holliston, MA, USA). Simultaneously PET/CT images were obtained from a Inveon PET scanner (Knoxville, TN, USA) for a certain time (120 or 150 min for ^{68}Ga-NOTA-c(RGDyK) and ^{68}Ga-NOTA-biphenyl-c(RGDyK), respectively. Raw PET data were reconstructed four static frames (acquisition every 30 min) using a 2D OSEM. The resultant PET images were converted to standard uptake value (SUV). The regions of interest are the tumor, liver, kidney, muscle, and heart.

Results and Discussion

The chemical structures of the NOTA-RGD peptides were illustrated in Figure 1. NOTA-biphenyl-c(RGDyK) was successfully prepared in five steps in a yield of 8% (Scheme S1 in Appendix S1). The identity of the peptide was checked by means of mass spectrometry. Based on HPLC (high performance liquid chromatography) quantities analysis, its chemical purity was over 95%.

The radiosynthesis was straightforward (labeling yield = 99%) and the radiochemical purities was over 99% (Figure S1). Both NOTA-RGD radioligands showed *in vitro* stability in the mouse and human serum. In case of ^{68}Ga-NOTA-biphenyl-c(RGDyK), the 95% initial authentic peak was remained after 2 h of incubation (Figure S2).

^{68}Ga-NOTA-biphenyl-c(RGDyK) (logP value of 2.96) showed higher lipophilicity than ^{68}Ga-NOTA-c(RGDyK) (logP value of 2.17). This difference may be explained by the conformational rigidity of the biphenyl group between the RGD peptide and NOTA chelate. The biphenyl group provides structural rigidity to the molecule.[13] Incorporating flexible molecules, such as polyethylene glycol, do not increase hydrophilicity but do decrease binding affinity.[14,15]

Its cell uptake rate was approximately fourfold higher than that of ^{68}Ga-NOTA-c(RGDyK) (Figure S3). We suspected that this result also reflected conformational rigidity because incorporation of cyclopentyl or cyclohexyl moiety increased binding affinity.

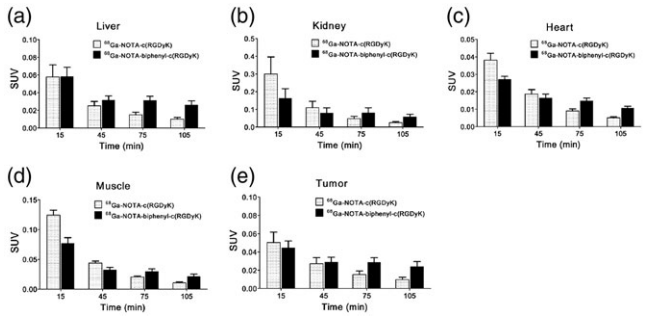

Figure 3. Radioactivity uptake patterns in the major organs for ^{68}Ga-NOTA-biphenyl-c(RGDyK) and ^{68}Ga-NOTA-c(RGDyK).

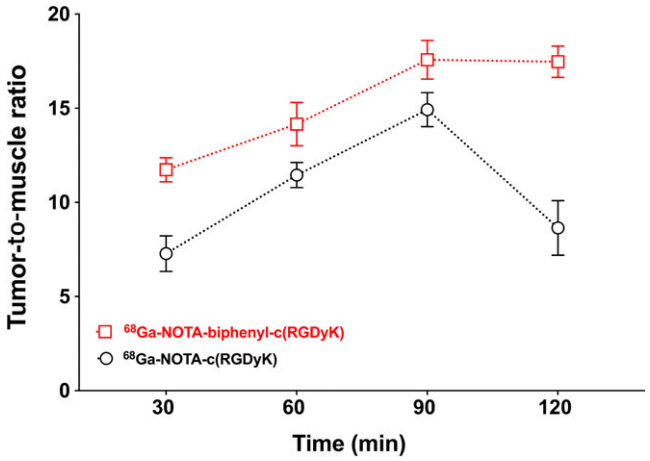

Figure 4. Comparison of the tumor-to-muscle ratios of ^{68}Ga-NOTA-biphenyl-c(RGDyK) and ^{68}Ga-NOTA-c(RGDyK).

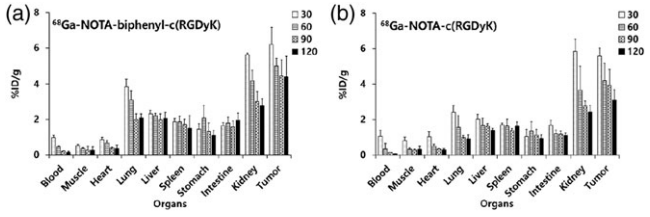

Figure 5. The *ex vivo* tissue biodistribution data of the U87MG tumor-bearing mouse model administered with an intravenous injection of (a) ^{68}Ga-NOTA-biphenyl-c(RGDyK) and (b) ^{68}Ga-NOTA-c(RGDyK).

According to the representative PET/CT images, ^{68}Ga-NOTA-biphenyl-c(RGDyK) showed the higher tumor uptake than the reference RGD radioligand (Figure 2).

In the previous study, NOTA-c(RGDyK) had 1.9 nM of IC50 value. We utilized this RGD peptide as a reference chelator and performed the cell uptake experiments in the identical condition. Figure 3 shows the comparative radioactivity uptake patterns. Both radioligands exhibited comparable liver uptake in the early phase (30 min), but ^{68}Ga-NOTA-biphenyl-c(RGDyK) showed a relatively high uptake that was 2–2.6 times (>90 min) greater than the reference radiopeptide. In the kidney, ^{68}Ga-NOTA-biphenyl-c(RGDyK) exhibited a relatively low uptake until 45 min, after which its uptake was 1.6–2.2 times (>90 min) higher than that of the reference. In addition, ^{68}Ga-NOTA-biphenyl-c(RGDyK) displayed 1.5–2 times higher muscle uptake than the reference compound (>75 min) indicating its enhanced non-specific binding.

Compared with ^{68}Ga-NOTA-c(RGDyK), the tumor regions are clearly visualized using ^{68}Ga-NOTA-biphenyl-c (RGDyK). In particular, after 90 min, the tumor uptake of ^{68}Ga-NOTA-c(RGDyK) was faint, whereas the signal for ^{68}Ga-NOTA-biphenyl-c(RGDyK) in the tumor remained for up to 150 min. These results indicated that incorporating the biphenyl group enhances the tumor uptake and prolongs the retention time. The radioactivities in the heart and muscle regions showed similar patterns over time to that in the kidney region.

To compare the specific-to-non-specific binding for both RGD radioligands, we obtained the tumor-to-muscle ratios (Figure 4). ^{68}Ga-NOTA-biphenyl-c(RGDyK) showed higher tumor-to-muscle ratios than those of ^{68}Ga-NOTA-c (RGDyK) and it reached to equilibrium from 90 min. This implies that ^{68}Ga-NOTA-biphenyl-c(RGDyK) has kinetic stability.

The *ex vivo* tissue biodistribution for both radiopeptides exhibited similar patterns to the results of the PET experiments (Figure 5). For ^{68}Ga-NOTA-biphenyl-c(RGDyK), the liver, lung, and intestine uptakes were higher than those of ^{68}Ga-NOTA-c(RGDyK). This non-specific binding may be due to the enhanced blood-pool effect caused by the lipophilicity of the compound. Henrotte and co-workers demonstrated that the biphenyl group non-covalently interacts with human serum albumin.[16] Tumor uptake of ^{68}Ga-NOTA-biphenyl-c(RGDyK) at 30, 90, and 120 min was 6.19 ± 0.96, 4.96 ± 0.44, and 4.44 ± 0.92%ID/g, respectively. These values were even higher values compared with those of ^{68}Ga-NOTA-c(RGDyK) (5.57 ± 0.44, 4.18 ± 0.99, and 3.94 ± 0.89%ID/g at the same time points).

Conclusion

^{68}Ga-NOTA-RGD peptides showed enhanced tumor uptake as well as increased non-specific binding. From these perspectives, we demonstrated that adapting biphenyl group on the RGD peptide has pros and cons.

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Supporting Information. Additional supporting information is available in the online version of this article.

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