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Research paper

PET imaging and biodistribution analysis of the effects of succinylated gelatin combined with L-lysine on renal uptake and retention of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ *in vivo*



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ABSTRACT

^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$, an $\alpha_v\beta_3$ integrin-targeting tetrameric cyclic RGD peptide probe, is a potential theranostic compound for positron emission tomography (PET) of tumor angiogenesis and for internal radiotherapy owing to the multiple decay modes of ^{64}Cu . Since kidneys are dose-limiting organs in internal radiotherapy, we aimed to reduce the renal accumulation of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ by co-injection with Gelofusine (GF), a succinylated gelatin solution, and/or L-lysine (Lys), and to explore, for the first time, the related mechanisms using the noninvasive and quantitative PET imaging technology. Biodistribution assays, dynamic and static PET scans, and metabolism studies with radio-thin-layer chromatography (radio-TLC) were performed in healthy or $\alpha_v\beta_3$ -positive tumor-bearing mice. In the results, co-injection with GF markedly reduced the renal uptake and slightly increased the tumor uptake of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$. L-Lysine alone had no effect on the probe biodistribution, but the combined use of Lys and GF tended to enhance the effect of GF. Dynamic PET and metabolite analysis by radio-TLC highly revealed that GF blocks the renal reabsorption of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$, but does not interfere with its metabolism and excretion. In conclusion, administration of GF and Lys is a useful strategy for kidney protection in ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ -based internal radiotherapy.

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1. Introduction

$\alpha_v\beta_3$ Integrin, a transmembrane glycoprotein receptor highly expressed on the surface of activated endothelial cells during angiogenesis as well as on some types of tumor cells, is one of the key biomarkers for tumor angiogenesis and plays important roles in tumor growth, invasion, metastasis, and angiogenesis [1–3]. By using a Regioselectively Addressable Functionalized Template (RAFT) cyclodecapeptide scaffold (Fig. 1), we have previously developed a cRGD (cyclic pentapeptide containing the tripeptide sequence Arg-Gly-Asp) probe encompassing (1) the $\alpha_v\beta_3$ -targeting domain, a cluster of 4 copies of a cyclo(-RGDfK-) monomer and (2) a bifunctional chelator 1,4,8,11-tetraazacyclotetradecane (cyclam) for ^{64}Cu radiolabeling. This compound was referred to as ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ [4–6]. ^{64}Cu ($t_{1/2}$ 12.7 h) is a promising radionuclide with multiple decay modes— β^+ (17.8%) used for positron emission tomography (PET) [7] and β^- (38.4%) and Auger electron (43%) used for internal radiation therapy [8]. Our recent study demonstrated that ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ PET enables clear visualization of tumor angiogenesis and aids in

Abbreviations: PET, positron emission tomography; GF, Gelofusine; Lys, L-lysine; Radio-TLC, radio-thin-layer chromatography; RAFT, Regioselectively Addressable Functionalized Template; cRGD, cyclic pentapeptide containing the tripeptide sequence Arg-Gly-Asp; cyclam, 1,4,8,11-tetraazacyclotetradecane; PRRT, peptide receptor radionuclide therapy; GF \pm Lys, GF in the presence or absence of Lys; RP, reversed phase; NS, normal saline; s.c., subcutaneous; i.v., injected via tail vein; p.i., post-injection; %ID/g, percentage of the injected dose per gram of tissue; ROIs, regions of interest; VOI, volume of interest; %ID, percentage of the total injected dose; AUC, area under the time–activity curve; AARs, amino acid residues; CAARs, charged AARs.

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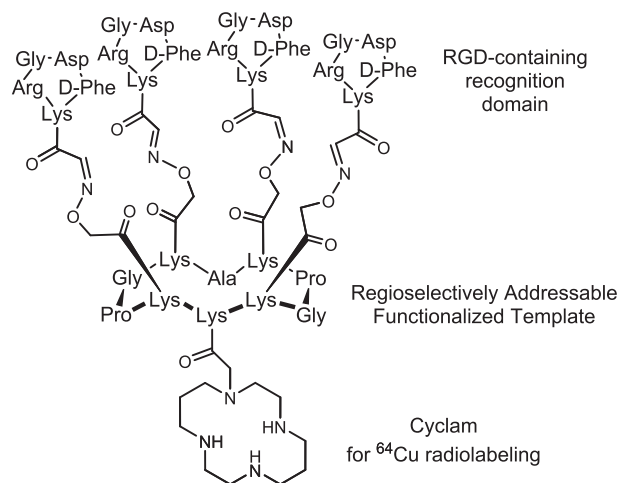


Fig. 1. Molecular structure of cyclam-RAFT-c(-RGDfK-)₄.

monitoring the effectiveness of antiangiogenic therapy in a mouse model [9]. Subsequently, we plan to investigate the therapeutic potential of this compound for internal radiation cancer therapy, also known as peptide receptor radionuclide therapy (PRRT) [10]. It is important to note that ⁶⁴Cu-cyclam-RAFT-c(-RGDfK-)₄-based PRRT would be used in diverse solid tumor types because it targets not only $\alpha_v\beta_3$ -positive tumor cells but also $\alpha_v\beta_3$ -overexpressed neoendothelial cells during angiogenesis, a key event required for tumor growth. However, ⁶⁴Cu-cyclam-RAFT-c(-RGDfK-)₄ was predominantly excreted through the kidneys, with more than half of the injected radioactivity eliminated within 1 h after injection and a significant amount of radioactivity being retained in the kidneys in an $\alpha_v\beta_3$ -nonspecific manner even 24 h after injection [6]. Because the kidney is the principal dose-limiting organ in internal radiotherapy with radiolabeled peptides [11], reducing the renal accumulation of ⁶⁴Cu-cyclam-RAFT-c(-RGDfK-)₄ is an essential step before examining its treatment potential.

In general, peptides are filtered through the glomerulus and are subsequently reabsorbed by the proximal tubular cells [11]. Infusion of the amino acids lysine and arginine has been reported to reduce renal tubular reabsorption of the radiolabeled somatostatin analogs pentetreotide or octreotide in animals and humans [12–14]. It was hypothesized that the positively charged lysine or arginine may competitively block the binding between a peptide containing positively charged groups and a negatively charged site on the tubular cell surface. Infusion of 25 g each of L-lysine (Lys) and L-arginine, which was found to be both effective and safe, is used as a standard procedure for kidney protection during PRRT with radiolabeled somatostatin analogs [15].

Gelofusine (GF), a succinylated gelatin solution, is a widely used plasma expander for patients suffering from massive hemorrhage, severe trauma, or dehydration. ten Dam et al. reported that infusions of low doses of GF in healthy male subjects resulted in urinary excretion of low-molecular-weight protein β_2 -microglobulin, suggesting that such an effect was most likely due to competitive inhibition of tubular protein reabsorption [16]. van Eerd et al. and Vegt et al. hypothesized that specific components in GF may attenuate the tubular reabsorption process. Subsequent studies on rats and mice showed that GF significantly reduced the renal uptake of ¹¹¹In-octreotide as effectively as lysine did [17], and studies on healthy volunteers showed that relatively small amounts of GF (<420 mL) could effectively reduce the renal uptake of ¹¹¹In-octreotide [18].

Regarding RAFT-c(-RGDfK-)₄, Briat et al. recently reported that GF induced >50% reduction in the renal accumulation of ¹¹¹In-labeled DOTA-RAFT-c(-RGDfK-)₄ in mice [19]. Renal uptake

and retention of radiopharmaceuticals are dependent not only on the characteristics of the targeting molecule, but also on the type of radionuclide and chelating agent used. We observed that the renal uptake levels of ¹¹¹In-DOTA-RAFT-c(-RGDfK-)₄ and ⁶⁴Cu-cyclam-RAFT-c(-RGDfK-)₄ were substantially different, with biodistributions at 24 h after injection of $\sim 40\%$ ID/g and $\sim 10\%$ ID/g, respectively [6,19]. Therefore, in this study, we determined the effect of GF on the renal uptake and retention of ⁶⁴Cu-cyclam-RAFT-c(-RGDfK-)₄ in normal and tumor-bearing mice. In comparison with the published work on the ¹¹¹In-labeled analog, the present study particularly evaluated (1) the dose–effect relationship of GF, (2) the combined effect of GF and Lys, (3) the spatiotemporal changes in renal radioactivity caused by GF in the presence or absence of Lys (GF \pm Lys), and (4) the influence of GF \pm Lys on the metabolism of ⁶⁴Cu-cyclam-RAFT-c(-RGDfK-)₄. Another novelty is that the present study explored the mechanisms underlying the action of GF and Lys using the noninvasive and quantitative PET imaging technology.

2. Materials and methods

2.1. Cyclam-RAFT-c(-RGDfK-)₄ and ⁶⁴Cu radiolabeling

Cyclam-RAFT-c(-RGDfK-)₄ (MW 4119.6) was synthesized as reported previously [5], and radiolabeled with ⁶⁴Cu in accordance with our previous report [6] with minor modifications. In brief, 0.08 mM cyclam-RAFT-c(-RGDfK-)₄ in dimethyl sulfoxide and 1.48 MBq/ μ L ⁶⁴CuCl₂ in ammonium citrate buffer (100 mM, pH 5.5) were mixed in a ratio of 1:1 (v/v) and incubated at 37 °C for 1 h. The radiolabeling efficiency, as determined by reversed phase (RP) high-performance liquid chromatography, was >98%, and the specific radioactivity was ~ 18.5 MBq/nmol.

2.2. GF and Lys solutions

Gelofusine (Braun Medical, Oss, Netherlands), kindly provided by Dr. Lucie Sancey (University of Lyon 1, France), consists of a 40 g/L solution of succinylated gelatin for intravenous infusion, and was diluted in normal saline (NS) for use in the present study. L-Lysine (Sigma-Aldrich, Buchs, Switzerland) was dissolved in NS and added to the injectate prior to administration.

2.3. Cell culture, animal, and tumor model

Human glioblastoma U87MG cells naturally expressing $\alpha_v\beta_3$ were cultured as previously described [6]. Animal procedures were approved by Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS; Chiba, Japan). Normal or tumor-bearing mice (female BALB/cA₁Jcl-*nu/nu*; CLEA Japan, Inc., Tokyo, Japan) at 7–8 weeks of age were examined. The tumors, 7–10 mm in diameter, were developed by subcutaneous (s.c.) injection of 1×10^7 cells into the left shoulder region of the mice.

2.4. Biodistribution studies

Mice were injected via tail vein (i.v.) with 0.74 MBq ⁶⁴Cu-cyclam-RAFT-c(-RGDfK-)₄ with or without co-injection of GF, Lys, or both (GF + Lys). The biodistribution study consists of the following 3 sequential experiments. *Experiment 1* ($n = 3$ –4/group) and *Experiment 2* ($n = 5$ /group) were designed to investigate the dose–effect relation of GF (at 80, 120, and 200 mg/kg) and the combinatorial effect of GF (80 mg/kg) and Lys (400 mg/kg) using normal mice. *Experiment 3* ($n = 5$ –7/group) was performed to determine whether GF or GF + Lys could affect the specific tumor uptake of

^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ in addition to their effects on the kidneys, using tumor-bearing mice. It should be noted that throughout this study, each injectate was adjusted to a 0.2 mL volume with NS to avoid any possible effect due to the injected volume. At 3 and/or 24 h post-injection (p.i.), the mice were sacrificed and their blood was drawn. The kidney, tumor, and other major organs of interest were dissected and weighed, and the radioactivity was measured using a gamma counter with decay correction. Radioactivity concentration was expressed as a percentage of the injected dose per gram of tissue (%ID/g) normalized to a body weight of 20 g.

2.5. PET imaging and quantification

Tumor-bearing mice ($n = 4/\text{group}$) received an i.v. injection of ~ 18.5 MBq ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ with or without co-injection of 80 mg/kg GF \pm 400 mg/kg Lys. Using a small-animal PET system (Inveon; Siemens Medical Solutions USA, Inc., Malvern, PA), dynamic PET imaging for a duration of 60 min (12 scans of 5 min each) was performed immediately p.i., followed by 30-min static imaging at 3.5 and 24 h p.i. During scanning, the mice in prone position were anaesthetized with 1–1.5% isoflurane, while maintaining normal body temperature. Images were reconstructed using a 3D maximum a posteriori (MAP) method (18 iterations with 16 subsets; $\beta = 0.2$) without attenuation correction. Image analysis was performed using the ASIPro VMTM Micro PET Analysis software (Siemens Medical Solutions, USA, Inc.). The total injected dose was calculated by decay correction of total activity present at the time of injection ($t = 0$). For radioactivity quantification in the tumor, both kidneys, and urinary bladder, regions of interest (ROIs) encompassing the whole tissue area on each of coronal slices were drawn manually, and all ROIs were linked to form a 3D volume of interest (VOI) using the 3D (VOI) dimensionality tool. For each VOI, the percentage of the total injected dose (%ID) was calculated to represent the total activity accumulation in the urinary bladder and both kidneys and the mean %ID/g to represent tumor uptake, assuming a tissue density of 1 g/mL. To quantify the radioactivity in the renal cortex, ROIs encompassing the cortex were drawn from 3 coronal slices, the mean %ID/g of each slice was recorded, and the average value of mean %ID/g from the 3 slices was calculated. To estimate the radioactivity in the blood pool, a ROI with a fixed size of 0.1 cm² was placed over the heart, and the blood radioactivity was quantified as the mean %ID/g.

2.6. Metabolite analysis by radio-TLC

Normal mice ($n = 3/\text{group}$) were treated with the same injection schedule as in the aforementioned PET study. At 1 and 24 h p.i., the mice were sacrificed and urine, blood, kidney, and liver were sampled. Blood samples were immediately subjected to centrifugation at 2300g for 15 min at 4 °C, and the plasma supernatants were collected. Liver and kidney samples were homogenized in ice-cold phosphate-buffered saline supplemented with protease inhibitor cocktail (1:30 dilution; Sigma–Aldrich, St. Louis, MO, USA). After centrifugation at 9100g for 30 min at 4 °C, the supernatants were collected. The extraction efficiency was approximately 80% for kidney and liver samples, and >90% for blood samples.

Partisil[®] RP TLC plate (KC-18 Silica Gel 60 Å; Whatman Inc., Clifton, NJ, USA) as the stationary phase was loaded with 2–2.5 μL of plasma, urine, tissue supernatant, injectate, and undiluted ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ or ^{64}Cu solution, and developed in the mobile phase of methanol/10% ammonium acetate (70/30 v/v). The radioactive components separated on the plate—corresponding to ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$, its

radioactive metabolites, and free ^{64}Cu —were exposed to an imaging plate, and scanned using a bioimaging analyzer as previously described [6]. The proteins were then visualized by exposure to iodine vapor. Samples from the same mouse and the injectate as the internal standard were analyzed on one TLC plate, with several samples, including urine and the injectate, being appropriately diluted in NS.

2.7. Statistical analysis

Quantitative data were presented as mean \pm SD and compared using one-way ANOVA followed by Bonferroni test for multiple comparisons. P values < 0.05 were considered statistically significant.

3. Results

3.1. Biodistribution studies

Table 1 shows the effect of various doses of GF on the biodistribution of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ in normal mice at 3 h p.i. Renal radioactivity was significantly reduced by 35.3% in the presence of 80 mg/kg GF; however, increased doses of 120 and 200 mg/kg did not lead to further reductions. Blood radioactivity (as low as $0.03 \pm 0.01\%$ ID/g) was not significantly influenced by GF at any of the doses tested. In other organs, co-injection with GF tended to result in a slight increase in radioactivity, independent of the doses used. Based on these results, the dose of 80 mg/kg was selected for all subsequent studies.

Fig. 2 shows the effect of Lys and the combined effect of GF and Lys on the biodistribution of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ in normal mice at 3 and 24 h p.i. L-lysine alone did not affect the biodistribution of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ at either 3 or 24 h p.i. in any of the organs examined, except in the stomach. When Lys was added to GF, the 31.5% (3 h p.i.) and 26.6% reductions (24 h p.i.) in renal radioactivity caused by GF alone were increased to 36.1% ($P > 0.05$) and 37.9% reductions ($P = 0.03$), respectively. Interestingly, unlike GF alone, GF + Lys did not significantly affect accumulation of radioactivity in other organs.

The effect of GF \pm Lys was examined in mice bearing $\alpha\text{v}\beta_3$ -positive U87MG tumors (Table 2). The tumor uptake of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ was slightly increased in the presence of GF \pm Lys. After co-injection with GF alone, the tumor-to-kidney

Table 1
Biodistribution of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ in normal mice with or without co-injection of GF at various doses.

Organ	Control	GF		
		80 mg/kg	120 mg/kg	200 mg/kg
Blood	0.03 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Heart	0.63 ± 0.17	0.90 ± 0.05^a	0.84 ± 0.08	0.82 ± 0.07
Lung	1.28 ± 0.33	1.85 ± 0.11^a	1.88 ± 0.06^a	1.78 ± 0.26
Liver	4.13 ± 1.08	5.30 ± 0.15	4.75 ± 0.39	5.78 ± 0.77^a
Pancreas	0.44 ± 0.10	0.57 ± 0.05	0.53 ± 0.02	0.57 ± 0.06
Spleen	1.69 ± 0.33	2.48 ± 0.23	2.23 ± 0.19	2.55 ± 0.58
Stomach	1.30 ± 0.41	1.71 ± 0.39	1.42 ± 0.35	1.77 ± 0.55
Intestine	1.44 ± 0.30	1.87 ± 0.07	1.58 ± 0.18	1.88 ± 0.30
Kidney	17.3 ± 3.71	11.2 ± 0.93^b	10.6 ± 1.18^b	10.7 ± 0.87^b
Skin	1.68 ± 0.49	2.25 ± 0.25	1.79 ± 0.26	1.97 ± 0.37
Muscle	0.39 ± 0.11	0.59 ± 0.07	0.53 ± 0.02	0.66 ± 0.19
Bone	1.00 ± 0.23	1.30 ± 0.12	1.38 ± 0.52	1.33 ± 0.19

Tissue radioactivity was assessed at 3 h p.i. and is expressed as %ID/g (mean \pm SD). $n = 3$ –4.

^a $P < 0.05$ vs. Control (mice that received probe alone).

^b $P < 0.01$ vs. Control (mice that received probe alone).

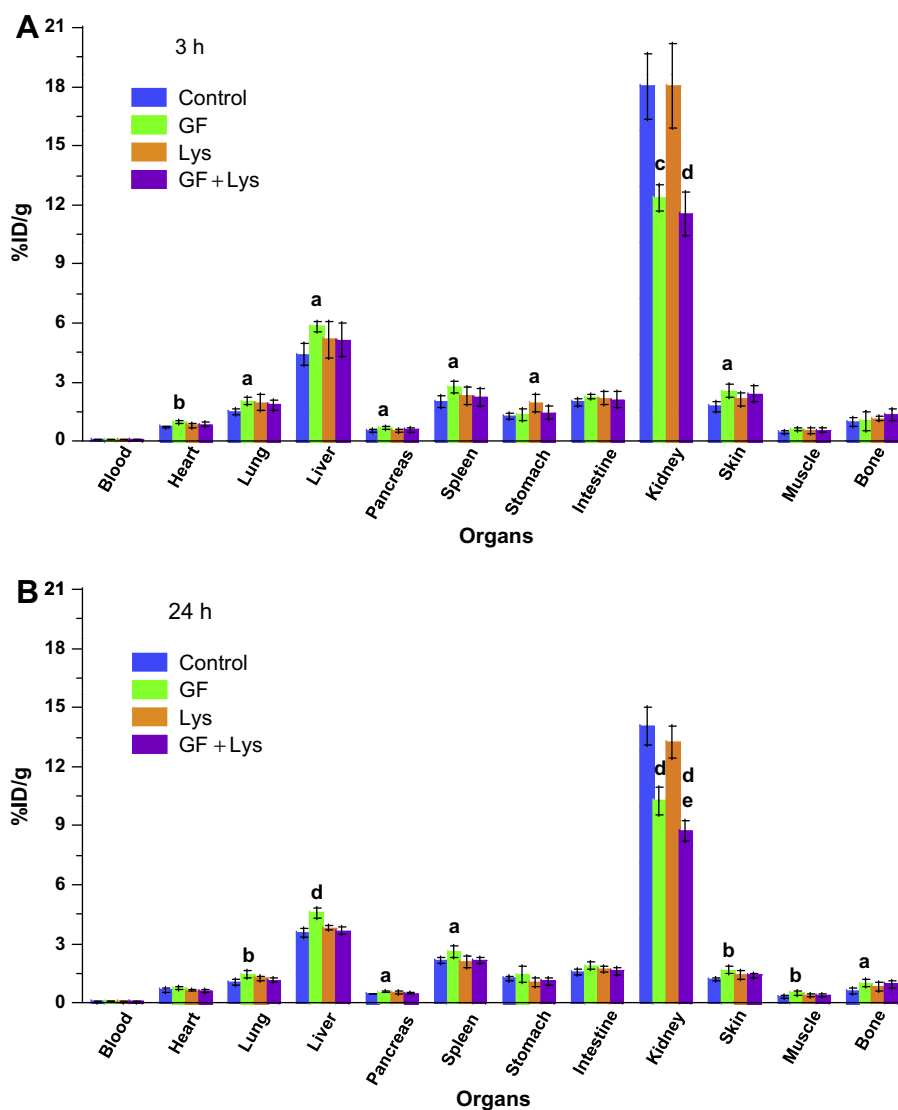


Fig. 2. Biodistribution of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ in normal mice at 3 h (A) and 24 h (B) p.i. of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ alone (control) or with co-injection of GF, Lys, or GF + Lys. $n = 5$; ^{a,b,c,d} $P < 0.05, 0.01, 0.001, \text{ and } 0.0001$, respectively vs. control (i.e. the group of mice receiving probe alone); ^e $P < 0.05$ vs. GF group.

Table 2

Biodistribution of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ in U87MG tumor-bearing mice with or without co-injection of GF ± Lys.

Organ	3 h p.i.			24 h p.i.		
	Control	GF	GF + Lys	Control	GF	GF + Lys
Blood	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01
Liver	4.50 ± 0.77	5.84 ± 0.34 ^a	5.94 ± 0.25 ^a	3.30 ± 0.27	3.87 ± 0.54	3.48 ± 0.34
Kidney	19.9 ± 3.52	13.1 ± 0.76 ^b	13.6 ± 0.40 ^b	14.7 ± 1.06	10.31 ± 1.00 ^c	9.47 ± 0.57 ^c
Muscle	0.36 ± 0.05	0.43 ± 0.09	0.42 ± 0.04	0.27 ± 0.03	0.32 ± 0.07	0.29 ± 0.08
Tumor	6.95 ± 1.59	8.19 ± 1.28	9.26 ± 1.36	4.40 ± 0.70	5.39 ± 0.94	5.22 ± 1.08
T/K	0.35 ± 0.06	0.63 ± 0.10 ^b	0.68 ± 0.09 ^b	0.30 ± 0.05	0.53 ± 0.094 ^a	0.56 ± 0.13 ^a
T/M	19.4 ± 3.71	19.7 ± 4.14	22.5 ± 4.21	16.0 ± 1.71	17.2 ± 3.51	19.4 ± 6.93

Tissue radioactivity was assessed at 3 and 24 h p.i. and is expressed as %ID/g (mean ± SD). T/K: tumor-to-kidney ratio; T/M: tumor-to-muscle ratio. $n = 5-7$.

^a $P < 0.01$ vs. Control (mice that received probe alone).

^b $P < 0.001$ vs. Control (mice that received probe alone).

^c $P < 0.0001$ vs. Control (mice that received probe alone).

uptake ratios were significantly increased by 80% ($P = 0.0008$) and 76.7% ($P = 0.005$) at 3 and 24 h p.i., respectively. Similarly, with GF + Lys, the tumor-to-kidney uptake ratios were significantly increased by 94.3% ($P = 0.0002$) and 86.7% ($P = 0.0018$) at 3 and

24 h p.i., respectively. However, no statistical significance was observed between the GF alone and GF + Lys groups. Further, the tumor-to-muscle uptake ratios were not significantly affected by co-injection with GF ± Lys.

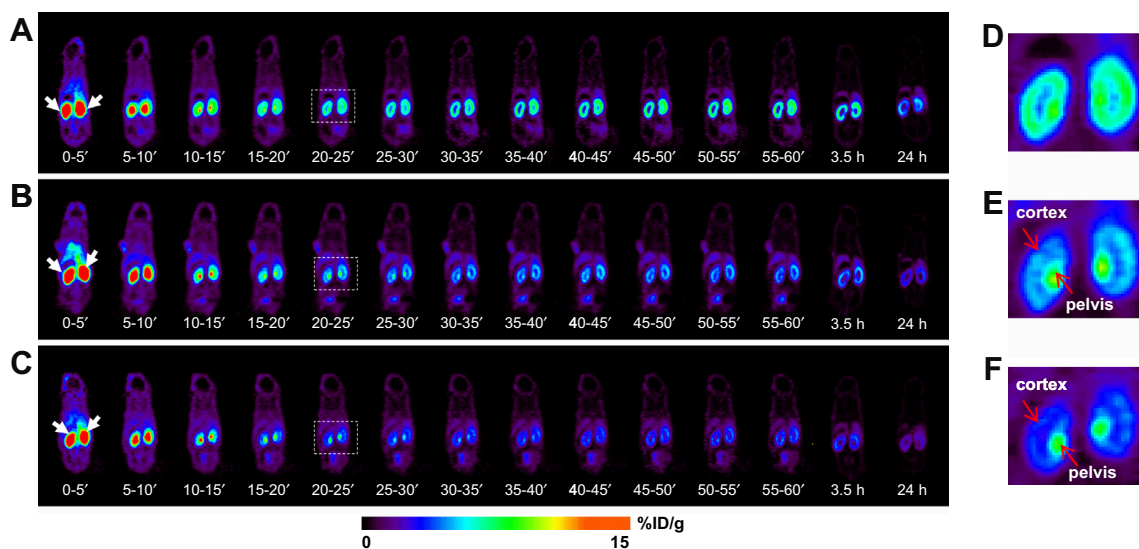


Fig. 3. Representative coronal PET images of U87MG tumor-bearing mice administered with ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ alone (A) or with co-injection of GF (B) or GF + Lys (C). Dynamic imaging was performed 5 min (') for each scan from 0 up to 60' p.i., and static images were acquired at 3.5 and 24 h p.i. Arrows in A, B, and C depict both kidneys. D, E, and F are zoomed (4.5 \times) images of kidneys delineated in dotted square in A, B, and C, respectively.

3.2. PET imaging and quantification

Fig. 3 shows the optimum coronal sections for both kidneys from the representative PET images of U87MG tumor-bearing mice. These images were derived from a 1-h dynamic scan and static scans at 3.5 and 24 h p.i. of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ alone (control) or with co-injection of GF \pm Lys, allowing improved visualization of the spatiotemporal distribution of renal radioactivity. In the control mouse, the radioactivity levels in both kidneys indicated rapid uptake within 0–5 min p.i., fast washout until 15–20 min p.i., and significant retention in the renal cortex at later time points (Fig. 3A and D). When compared to the control mouse, mice co-injected with either GF (Fig. 3B and E) or GF + Lys (Fig. 3C and F) displayed differences in both the intensity and distribution of renal radioactivity from 15–20 min p.i., with retention in the renal cortex being lower than that in the renal pelvis (up to 35–40 min p.i.). Fig. 4 shows another set of coronal sections for optimum visualization of the tumors. The kinetics of uptake, washout, and retention of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ were observed to be comparable among all of the tumors from the control and GF \pm Lys-administered mice, with this $\alpha_v\beta_3$ -positive tumor clearly detected with high contrast against collateral tissue from as early as 30 min up to 24 h p.i.

Quantitative analysis of dynamic PET images revealed a steady increase in the amount of radioactivity accumulated in the urinary bladders during the 60-min scanning period for all groups of mice, reflecting cumulative urinary excretion of the injected radioactivity (Fig. 5A). Co-injection with GF \pm Lys significantly increased percentage urinary excretion, a quantity roughly corresponding to the decreased percentage in total renal radioactivity. The value of area under the time–activity curve (AUC) from 12.5 to 57.5 min p.i. was 2293 ± 39 for the control group, which was slightly increased to 2382 ± 111 and 2416 ± 78 for the GF and GF + Lys group, respectively. Fig. 5B displays the kinetics of total renal radioactivity. Co-injection with GF \pm Lys tended to decrease renal radioactivity after the initial uptake and washout of the probe within 12.5 min p.i., resulting in significantly lower radioactivity levels in retention. AUC from 12.5 to 57.5 min p.i. was 210 ± 41.1 for the control group, which was significantly reduced to 152.4 ± 11.5 ($P = 0.048$) and 143.1 ± 21.3 ($P = 0.022$) for the GF and GF + Lys group, respectively. Fig. 5C shows the blood time–activity curves. Co-injection with

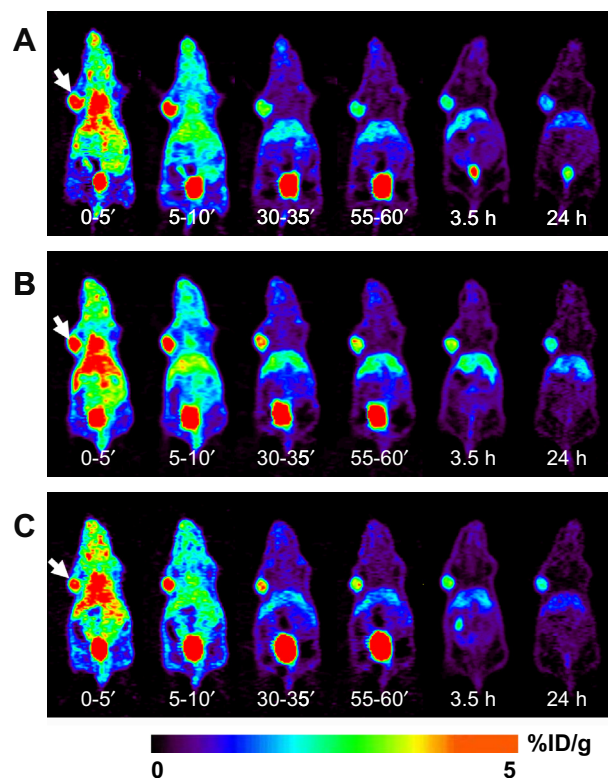


Fig. 4. A set of coronal sections of PET images derived from the same set of mice described in Fig. 2. Tumor (arrow) uptake of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ is clearly visible.

GF \pm Lys did not affect the blood clearance pattern of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$.

Fig. 6A shows the time–activity curves for the renal cortex, the main localization site of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ in the kidneys, exhibiting similar kinetics pattern to the corresponding time–activity curves for the whole kidney. Co-injection with

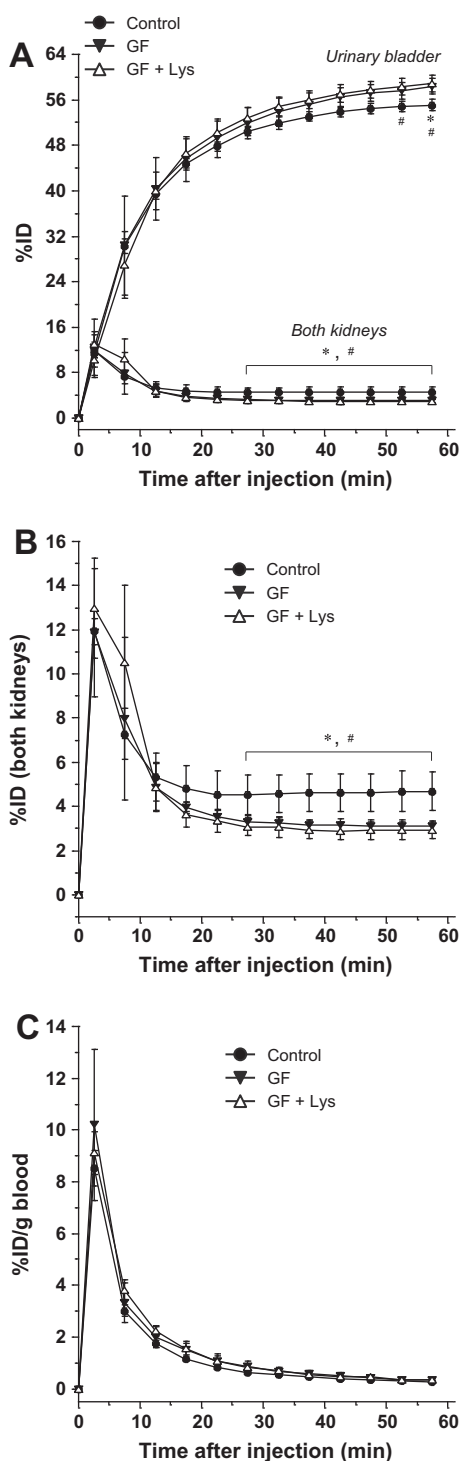


Fig. 5. (A and B) Time course of accumulation of radioactivity in both kidneys and the urinary bladder. (C) Clearance of radioactivity from blood pool over time. The data were obtained by quantitative analysis of dynamic PET images of U87MG tumor-bearing mice at 0–60 min p.i. of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ alone (control) (black circle) or with co-injection of GF (black inverted triangle) or GF + Lys (empty triangle). The graph in A is enlarged in B with a smaller maximum value for the Y-axis. $n = 4$; GF vs. Control: $P < 0.05$; GF + Lys vs. Control: $^{\#}P < 0.05$.

GF \pm Lys significantly reduced the radioactivity concentration in the renal cortex for a longer duration, i.e. from 27.5 min to 24 h p.i., compared to the control injection. A 41.9%, 38.4%, and 31.9% reduction was achieved by co-injection with GF alone at 57.5 min, 3.5 h, and 24 h p.i., respectively. Addition of Lys enhanced the effect of GF, as shown by the slightly increased

reduction ratios of 45.2%, 43.1%, and 36.5% observed at 57.5 min, 3.5 h, and 24 h p.i., respectively. Tumor uptake increased in GF \pm Lys-administered mice compared to that in the control mice, with statistical significance observed for the GF alone group at indicated time points (Fig. 6B).

3.3. Radio-TLC metabolite analysis

Fig. 7 shows representative results of radio-TLC analysis of plasma, urine, liver, and kidney samples from normal mice at 1 and 24 h p.i. of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ alone (control) or with co-injection of GF \pm Lys. Three independent experiments yielded similar results. Iodine vapor staining revealed that the protein components of plasma and tissue extracts remained at the origin (data not shown). Except in the urine and plasma at 24 h p.i., one or two closely overlapping spots were observed in all samples from control mice at similar or nearby positions from the intact probe. The urine sample at 1 h p.i. showed a spot matching with the intact probe, whereas, at 24 h p.i., it showed an irregularly shaped spot that migrated faster than the time required for detection of the intact probe, indicating excretion of the mixture of radiolabeled metabolites. At 24 h p.i., the plasma was barely detected because of very low radioactivity. Co-injection with GF \pm Lys was observed to have no significant effect on the metabolism of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$.

4. Discussion

In recent years, there has been increasing interest in developing radiolabeled peptides for cancer theranostics [20,21] because peptides, in general, have many key advantages over proteins, such as faster clearance from the blood and non-target tissues, more rapid tissue penetration, lower immunogenicity, and easier and less expensive production [10]. Further, reduction in renal retention of radioactive metabolites is important for PRRT in order to avoid potential nephrotoxic effects and widen the therapeutic windows [11,20]. Therefore, based on the therapeutic potential of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$, an efficient strategy to reduce renal uptake levels of this probe is required.

In the current study, we demonstrated that co-injection with GF efficiently reduced the uptake of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ in mouse kidneys by 30–40% (i.e. from 30 min to 24 h p.i.). Briat et al. recently reported that either pre- (2–5 min before) or co-injection with GF (200 mg/kg; 4 mg/mouse for a 20-g mouse) could reduce the renal uptake of ^{111}In - or fluorescent dye-labeled RAFT-c(-RGDfK-) $_4$ in mice by >50% at 1 and 24 h p.i. [19]. The 2 studies demonstrated that GF could primarily affect the behavior of the peptide, with somewhat varied efficiency depending on the type of conjugate used. Comparison of the biodistribution data obtained for ^{111}In - and ^{64}Cu -labeled RAFT-c(-RGDfK-) $_4$ at 24 h p.i. showed that renal uptake for the former probe is far greater than that for the latter ($42.3 \pm 9.3\text{ID/g}$ vs. $14.4 \pm 1.0\text{ID/g}$). This difference in renal uptake may be caused by at least partially distinct mechanisms involved in the renal uptake of the 2 probes. Here, we examined a range of GF doses and demonstrated that 80 mg/kg of GF was sufficient to reduce the uptake of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ in mouse kidney, and no further enhancement could be achieved at higher doses. Melis et al. reported similar findings with the ^{111}In -labeled somatostatin analog octreotate in rats [22]. In humans, one study showed that infusion of relatively small amounts of GF (average of 12.9 g in less than 420 mL NS) can effectively reduce the renal uptake of ^{111}In -octreotide by 45% without side effects [18].

The influence of GF on the uptake of ^{64}Cu -cyclam-RAFT-c(-RGDfK-) $_4$ in other major organs and $\alpha_v\beta_3$ -positive tumors was

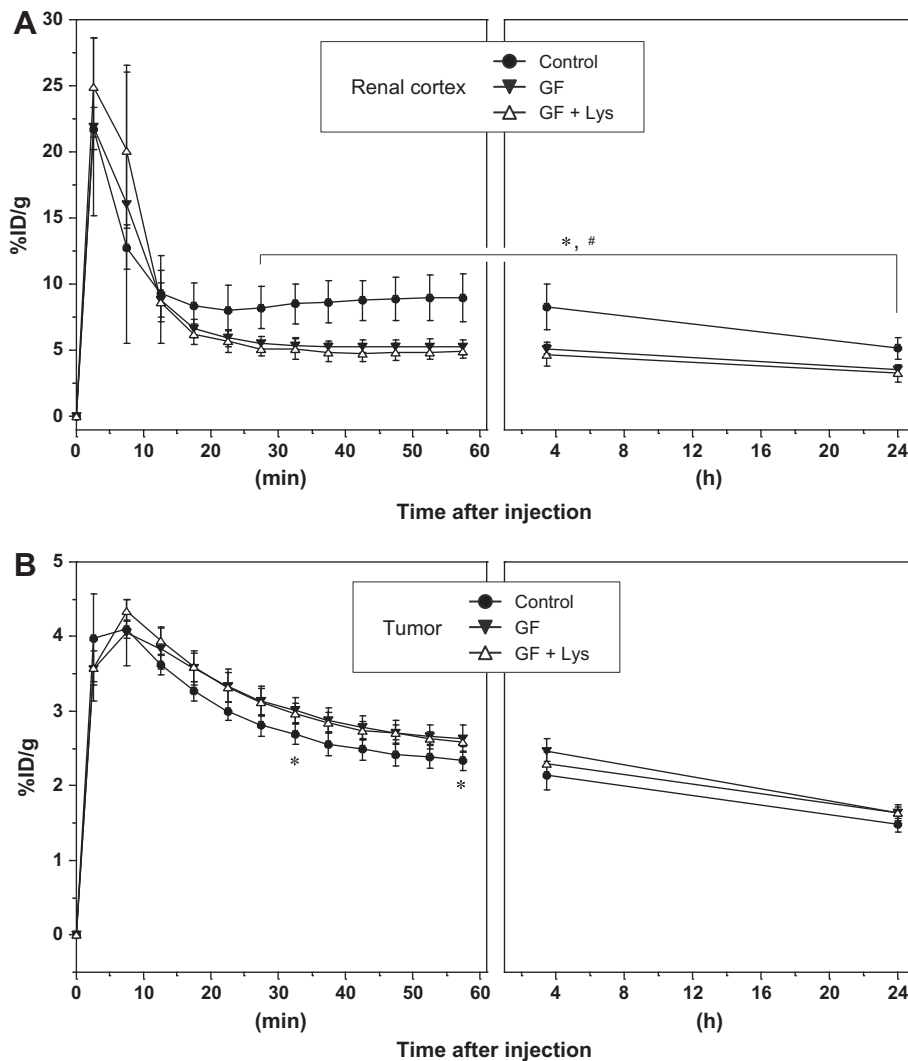


Fig. 6. Time course of radioactivity concentration in renal cortex (A) and tumor (B). The data were obtained by quantitative analysis of dynamic (0–60 min p.i.) and static (3.5 and 24 h p.i.) PET images of the same set of experimental groups presented in Fig. 5. GF vs. Control: $^*P < 0.05$; GF + Lys vs. Control: $^{\#}P < 0.05$.

carefully examined. It was observed that GF co-injection did not alter the blood clearance rate of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$. For several other healthy organs, slight but significant increases in accumulation of radioactivity were observed in biodistribution studies. Similarly, tumor uptake was also found to be slightly yet significantly enhanced in quantitative analysis of PET imaging within 1 h p.i. In addition, the tumor-to-kidney uptake ratios were found to be significantly increased by 80% and 76.7% at 3 and 24 h p.i., respectively, indicating that co-injection with GF could broaden the therapeutic window considerably. Our observation concerning slightly increased tumor uptake is in accordance with the results of Briat et al., who reported a 16.4% increase in tumor uptake with GF co-injection [19]. The effect of GF on other organs aside from the kidneys may relate to its volumetric effect as a blood volume expander. Regarding the combined use of GF and Lys, GF and Lys were reported to additively reduce the renal uptake of ^{177}Lu -octreotate and ^{111}In -octreotide in rats [22,23]. However, in the present study, the effects of Lys alone on the renal uptake of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ were not observed, and the combined use of Lys and GF tended to enhance the efficiency of GF only to a limited extent. In consideration of the liver uptake that was found significantly increased by GF alone but not GF + Lys (Fig. 2), it might be even safer to use both of GF and Lys for

co-injection with ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$ in internal radiotherapy.

One important characteristic of the present study is that dynamic PET imaging was successfully used to directly analyze the mechanism of how and when GF affected the renal accumulation and elimination of ^{64}Cu -cyclam-RAFT-c(-RGDFK) $_4$. During a 1-h scan, we observed that GF primarily affected the phase between the initial rapid washout of the peptide after renal uptake and the final retention of peptide. This process was presented as slow decline in renal radioactivity (an indication of strong tubular reabsorption) in the absence of GF, which was replaced by relatively faster decline of the radioactivity in the presence of GF, suggesting impairment of tubular reabsorption. Dynamic PET images clearly showed that radioactivity was predominantly found in the cortex of the kidneys in control mice as early as 20–25 min p.i. and was retained for long periods thereafter. In addition to reduced radioactivity in the renal cortex, radioactivity in mice co-injected with GF could be clearly visualized in the renal pelvic area even up to 35–40 min p.i., which is indicative of the active transit of the radioactivity into the urinary bladder. Co-injection of GF resulted in increase in urinary bladder radioactivity, which corresponded to a decrease in total renal radioactivity, indicating that decreased renal uptake was due to the blockade of renal reabsorption of

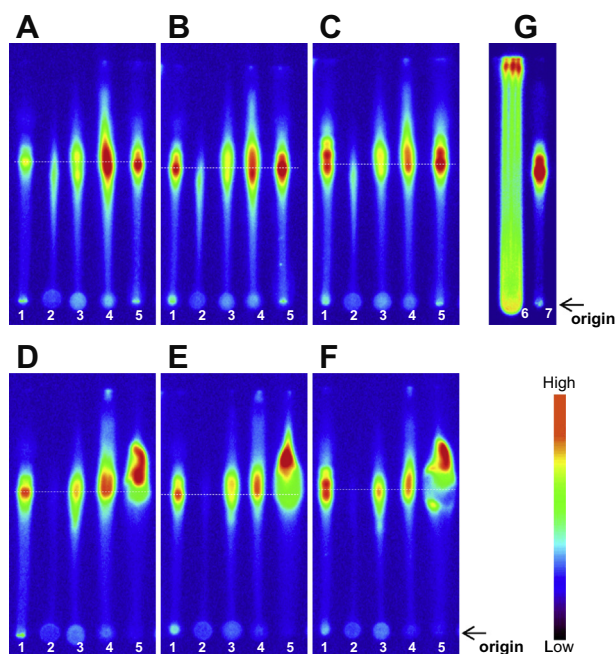


Fig. 7. Radio-TLC analysis of the metabolites in blood, urine, liver, and kidney of normal mice receiving ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$ alone (A and D) or with co-injection of GF (B and E) or GF + Lys (C and F). A–C: at 1 h p.i. D–F: at 24 h p.i. G: Standards. Lane 1 injectate, Lane 2 plasma, Lane 3 liver, Lane 4 kidney, Lane 5 urine, Lane 6 $^{64}\text{CuCl}_2$, Lane 7 ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$. Dotted lines the position of standard probe; Arrows indicate the origin.

^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$, the predominant radioactive component detected in the urine samples of mice with or without co-injection of GF \pm Lys at 1 h p.i. In addition, neither PET nor biodistribution studies showed the effect of GF on the blood clearance of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$, and *in vivo* metabolite analysis did not reveal the effect of GF on the metabolism of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$. Taken together, these data strongly suggest that co-injection with GF can result in reduced renal accumulation of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$, which is possibly achieved through suppression of tubular reabsorption.

Megalin, a multiligand receptor expressed exclusively on the apical membrane of proximal tubular cells, can bind to a variety of structurally distinct proteins, peptides, drugs, and other molecules [24–27]. Megalin-mediated endocytosis has been reported to play a significant role in the renal reabsorption of several radiolabeled peptides irrespective of their molecular targets, molecular weights, numbers of amino acid residues (AARs), or numbers of charged AARs (CAARs) [24,26]. Based on these studies, we consider that megalin may also be involved in the renal reabsorption of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$. The number of CAARs in a radiolabeled peptide has been shown to be related to its renal uptake levels [26,28]. Gotthardt et al. reported a positive relationship between the renal uptake levels of radiolabeled peptides and the numbers of CAARs (Glu, Lys, Asp, or Arg) contained in the peptides in the following order: exendin (10 CAARs) > minigastrin (7 CAARs) > octreotide (1 CAARs) > bombesin (0 CAARs) [28]. ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$ contains 8 CAARs including 4 Arg (positive charge) and 4 Asp (negative charge). The Lys residues contained in this probe are capped and therefore have no charge. Owing to the presence of 8 CAARs, the renal uptake of the probe would increase substantially. The positively charged Lys was found to reduce the renal uptake of the radiolabeled somatostatin analogs pentetate, octreotide, and octreotide (containing a single Lys residue each) through a putative competitive mechanism [12–14,28]. In the present study, co-injection with Lys did

not reduce the renal uptake of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$, possibly because of the lack of charged Lys residues. In addition to the number and type of CAARs, factors such as their structure and distribution inside a molecule may also contribute to renal reabsorption mechanisms. Unlike Lys, GF reduced the renal uptake of all the radiolabeled peptides examined [19,26,28], including ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$ investigated in this study. This could be because GF is a polypeptide-based succinylated gelatin composed of several molecules of varying sizes and structures, with both negative and positive CAARs; it may therefore possess the ability to interact with several binding domains of megalin simultaneously, thereby efficiently blocking the renal reabsorption of various molecules.

Aside from co-injection with Lys and GF, other strategies have been reported to reduce the renal uptake and retention of radiolabeled peptides, especially somatostatin analogs [13,29–32]. In addition to these, modification of the peptide by coupling it with another molecule (such as polyethylene glycol) can alter the pharmacokinetics by increasing the size and hydrophilicity of the molecule and masking its charges [11], which may also be considered in future studies for reducing the renal accumulation of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$. In addition, our subsequent studies on the development of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$ internal radiotherapy will also focus on estimating and determining the therapeutic but non-nephrotoxic doses of this radioactive compound.

5. Conclusion

Co-injection with GF effectively reduced uptake of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$ in mouse kidney. L-lysine alone had no effect on the probe biodistribution, but the combined use of Lys and GF tended to enhance the effect of GF. Dynamic PET imaging enabled visualization and quantification of the spatiotemporal change in renal radioactivity caused by GF and strongly suggested that the mechanism of action of GF at least partially occurs via inhibition of renal tubular reabsorption of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$. The use of GF should be included in future studies exploring the therapeutic potential of ^{64}Cu -cyclam-RAFT-c(-RGDfK) $_4$.

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