

# Iodine 125-labeled mesenchymal–epithelial transition factor binding peptide-click-cRGDyk heterodimer for glioma imaging

Eun-Mi Kim,<sup>1</sup> Min-Hee Jeong,<sup>1</sup> Dong Wook Kim,<sup>1</sup> Hwan-Jeong Jeong,<sup>1,2</sup> Seok Tae Lim<sup>1</sup> and Myung-Hee Sohn<sup>1</sup>

<sup>1</sup>Department of Nuclear Medicine, Research Institute of Clinical Medicine, Cyclotron Research Center, Chonbuk National University Medical School and Hospital, Jeonbuk, South Korea

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In our previous study, mesenchymal–epithelial transition factor (c-Met)-binding peptides (cMBP) had been readily radiolabeled with radioactive iodide for glioma imaging because of five histidine amino acids. However, iodinated cMBP showed relatively unfavorable *in vivo* kinetics. For this reason, we tried to design dual peptide ligands that would be advantageous in recognizing both c-Met receptor and integrin  $\alpha_v\beta_3$ . A cMBP-click-cRGDyk (cyclic Arg-Gly-Asp-Tyr-Lys) heterodimer was synthesized from mini polyethylene glycol-conjugated cMBP-3 glycine (GGG)-a single name of amino acids (SC) (Ser-Cys) and cRGDyk through a click (1 + 3 cycloaddition), and then labeled with iodine 125 (I-125) via histidine in the cMBP and tyrosine in the cRGDyk. The receptor-binding characteristics and tumor-targeting efficacy of cMBP-click-cRGDyk were tested *in vitro* and *in vivo*. A cMBP-click-cRGDyk had comparable integrin  $\alpha_v\beta_3$ -binding affinity with cRGDyk. The results of the biodistribution of <sup>125</sup>I-cMBP-click-cRGDyk at 4 h showed higher tumor-to-blood, tumor-to-liver, and tumor-to-muscle ratios: 10.07, 6.76, and 11.12, compared to 2.34, 1.99, and 5.18 of <sup>125</sup>I-cMBP-GGG-SC, respectively. U87MG tumor xenografts could be visualized by single photon emission computed tomography (SPECT)/CT using <sup>125</sup>I-cMBP-click-cRGDyk and also image contrast and overall quality were improved compared to <sup>125</sup>I-cMBP-GGG-SC. As the results of *in vivo* inhibition using free cRGDyk or cMBP-GGG-SC indicated, the tumoral uptake of <sup>125</sup>I-cMBP-click-cRGDyk decreased. This finding means that <sup>125</sup>I-cMBP-click-cRGDyk was specifically uptaken by integrin  $\alpha_v\beta_3$  and the c-Met receptor. Although imaging quality was improved, additional experiments are needed to acquire significant image-quality improvement. (*Cancer Sci* 2011; 102: 1516–1521)

Many types of receptors are uniquely expressed or markedly overexpressed in tumors, and have been used as potential targets for cancer diagnosis and therapy. The ability to measure and identify specific receptor expression is crucial for the accurate diagnosis, staging, restaging, and classification of tumors, and for monitoring patient response to therapy.<sup>(1,2)</sup> Many research groups are therefore interested in peptide radiopharmaceuticals. Compared to high molecular weight polymers, small peptides are structurally well defined and are generally cleared from circulation much faster, which might lead to a higher target-to-background ratio.

Multivalent ligands can be homomultivalent, with multiple copies of the same ligand, or heteromultivalent, with different types of ligands targeting different types of receptors. Multivalent ligands consist of multiple binding moieties (pharmacophores) that are bound together via chemical linkers. Multivalent binding can lead to increased functional affinity and binding specificity.<sup>(3–5)</sup> A wide spectrum of binding moieties has been studied, including small peptide fragments, truncated versions of antibodies, and carbohydrate analogs.<sup>(5–7)</sup>

Integrin  $\alpha_v\beta_3$  is highly expressed in invasive tumors, such as late-stage glioblastomas, breast and prostate tumors, malignant melanomas, and ovarian carcinomas, as well as in newborn blood vessels.<sup>(2,8)</sup> The expression level of integrin  $\alpha_v\beta_3$  is an important factor in determining malignant invasiveness and metastatic potential in both preclinical animal models and cancer patients.<sup>(9)</sup> Over the past several years, many researchers have successfully developed series RGD peptide radiotracers with favorable *in vivo* kinetics for tumor integrin  $\alpha_v\beta_3$  imaging.<sup>(10,11)</sup> Mesenchymal–epithelial transition factor (c-Met) is a receptor tyrosine kinase known to stimulate invasive cancer cell growth and increase metastatic potential; it is also expressed and mutates in a variety of solid tumors.<sup>(12–14)</sup> Mesenchymal–epithelial transition factor is overly expressed in human glioblastomas, with expression levels correlating to glioma malignancy grade and vascularity. The activated endothelial cells around c-Met-positive tumor tissues express high levels of integrin during tumor angiogenesis, invasion, and metastasis. We previously reported that iodine 125 (I-125)-radiolabeled, c-Met-binding peptides (cMBP) bound specifically to U87MG cells and *in vivo* tumors.<sup>(14)</sup> A glioma tumor (U87MG) expresses both c-Met and integrin  $\alpha_v\beta_3$ , and we hypothesize that a peptide ligand recognizing both receptors would be advantageous over a single receptor-binding probe. Chen *et al.* previously studied this concept.<sup>(7)</sup>

Currently, the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and alkynes, commonly called “click chemistry”, plays a crucial role in a wide range of biomedical applications.

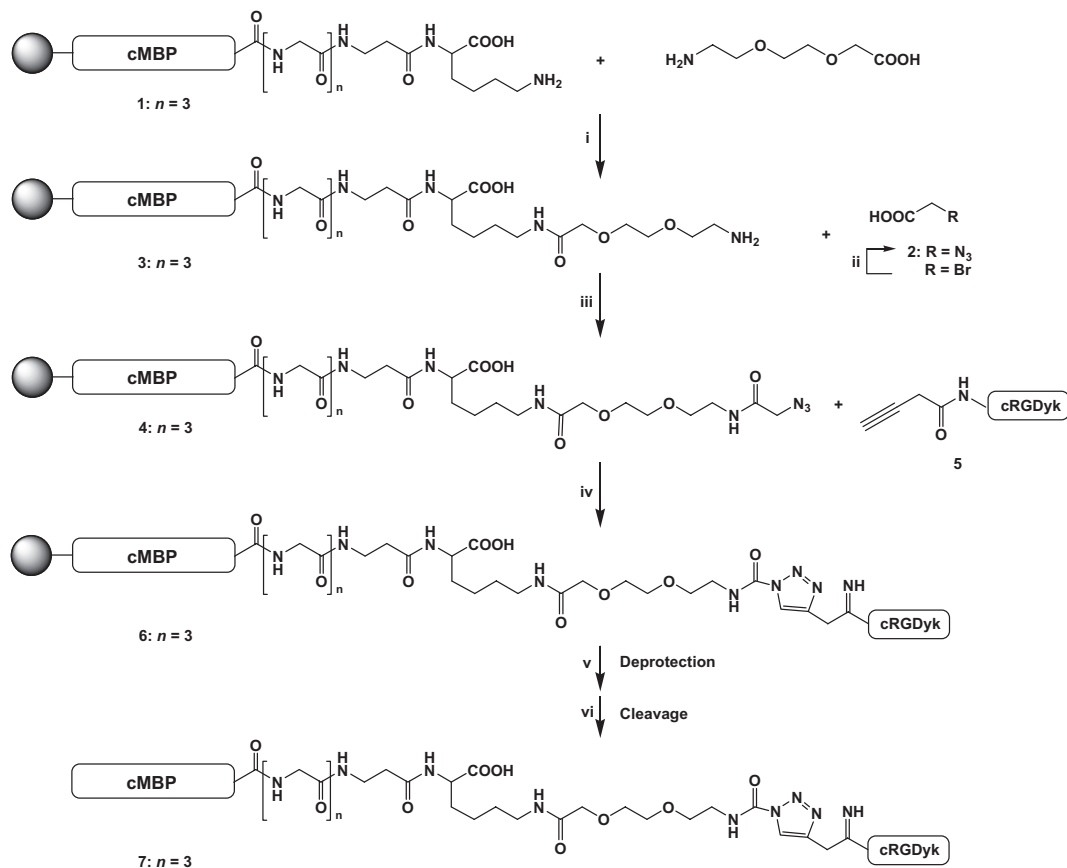
This reaction can be carried out in high yields under mild conditions, and the 1,2,3-triazole formed has a similar polarity and size with an amide bond. Due to these favorable aspects, the use of click chemistry to conjugate two (bio)molecular components has recently been reported.<sup>(12,15,16)</sup>

In this study, we hypothesized that the introduction of cRGDyk using click reaction methodology would help reduce radioactive accumulation in major organs by enhancing elimination, while increasing tumoral uptake. We developed a 1,2,3-triazole-associated, cMBP-click-cRGDyk peptide heterodimer that recognizes c-Met receptors through the cMBP motif and integrin  $\alpha_v\beta_3$  through the RGD motif. After radiolabeling this synthesized heterodimer, we conducted studies targeting integrin and c-Met receptors both *in vitro* and *in vivo*.

## Materials and Methods

**Reagents and chemicals.** Na iodine 125 (Na<sup>125</sup>I) and <sup>125</sup>I-e-chistatin were obtained from Perkin Elmer Life Science (Boston, MA, USA). The KSLSRHDHIIHHH-3 glycine (GGG)- $\beta$ -A-K

<sup>2</sup>To whom correspondence should be addressed. E-mail: jayjeong@chonbuk.ac.kr



**Fig. 1.** Synthesis of mesenchymal-epithelial transition factor-binding peptide-click-cRGDyk (cMBP-click-cRGDyk) heterodimer. Reagents and conditions: (i) 2-(2-[2-Fmoc aminoethoxy]ethoxy)acetic acid, DIC, HOBT, DMF, rt, 2 h; (ii) NaN<sub>3</sub>, water, 4°C, 16 h; (iii) DIC, HOBT, DMF, rt, 2 h; (iv) Cu(I)Br, DIPEA, DMF, propiolic cRGDyk, rt, 4 h; (v) 20% piperidine in DMF, rt, 10 min (Fmoc deprotection); (vi) triisopropylsilane, thioanisole, water, ethanedithiol, trifluoroacetic acid (2.5/2.5/2.5/2.5/90), rt, 3 h (cleavage). DIC, *N,N'*-diisopropylcarbodiimide; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; HOBT, hydroxybenzotriazole.

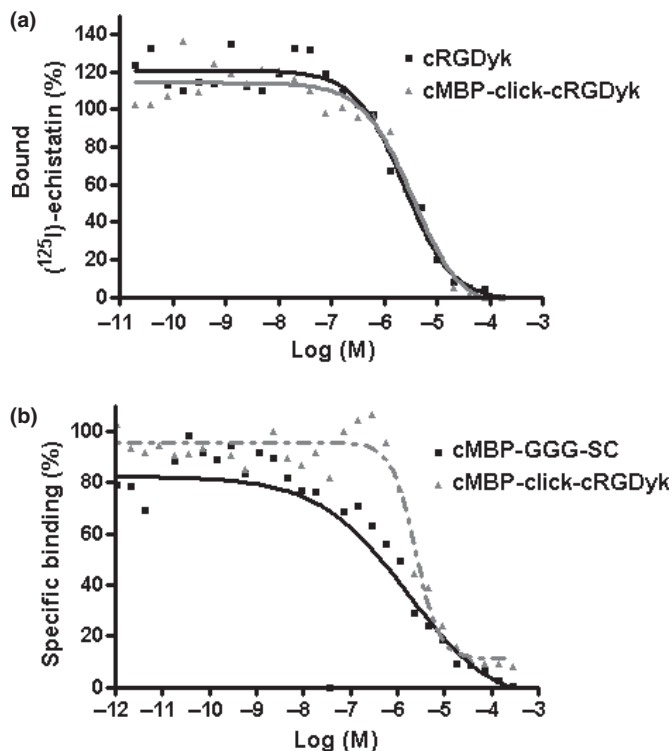
mini polyethylene glycol azide (cMBP-GGG-N<sub>3</sub>) was synthesized by Pepton (Seoul, Korea) using standard Fmoc chemistry. Propiolic-cyclic RGDyk was purchased from Future Chem (Seoul, Korea). The purity of all samples was >90%.

**Preparation of cMBP-click-cRGDyk.** The synthesis of azidoacetic acid and peptides was described previously.<sup>(12)</sup> Compound 7 was synthesized on an automated, solid-phase peptide synthesizer (ASP48S; Pepton, Korea) using traditional Fmoc chemistry and Rink amide 4-methylbenzhydrylamine hydrochloride salt (MBHA) resin (25 μmol). Each coupling step was performed using *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBT) and appropriate Fmoc-protected amino acids (100 μmol), including the following side-chain-protecting groups: Gln(Trt), Trp(Boc), His(Trt), and Ser(tBu). After washing the resin (with DMF, iPrOH), 2 eq propiolic-cyclic RGDyk, 0.2 eq Cu(I)Br, and 2 eq *N,N*-diisopropylethylamine (DIPEA) were added to the resin. The click reaction was shaken for 4 h at room temperature. The final product was cleaved by a standard procedure using a cocktail containing triisopropylsilane, thioanisole, water, ethanedithiol, and trifluoroacetic acid (mixing ratio of 2.5/2.5/2.5/2.5/90%, respectively). Typical yields of the crude peptide were 75–85%. The crude material was purified by reverse phase (RP)-HPLC, and electrospray ionization mass spectrometry (ESI-MS) was used to determine the molecular mass of the prepared peptide (HPLC: Retention time = 6.78 min for 7; ESI-MS (*m/z*) found (calculation): 2772 (2771.57) for 7).

**Radioiodinated peptide.** For *in vivo* imaging, cMBP-click-cRGDyk was radiolabeled with Na<sup>125</sup>I using chloramine-T.

Briefly, 10 μg (≅3.6 nmol) cMBP-click-cRGDyk was labeled with approximately 0.4 mCi Na<sup>125</sup>I (23 ng <sup>125</sup>I) using 30 μg chloramine-T in 10 μL phosphate-buffered saline (PBS; 0.5 M, pH 7.5). The reaction was terminated with sodium bisulfite (30 μg in 10 μL PBS). Radiochemical purity was assessed by instant thin layer chromatography-silica gel (0.9% normal saline). Radioactivity was determined with a radio-TLC scanner (Bioscan, Washington, DC, USA). The partition coefficient (*logP*) value of the <sup>125</sup>I-cMBP-click-cRGDyk and <sup>125</sup>I-cMBP-GGG-a single name of amino acids (SC) was previously described.<sup>(14)</sup>

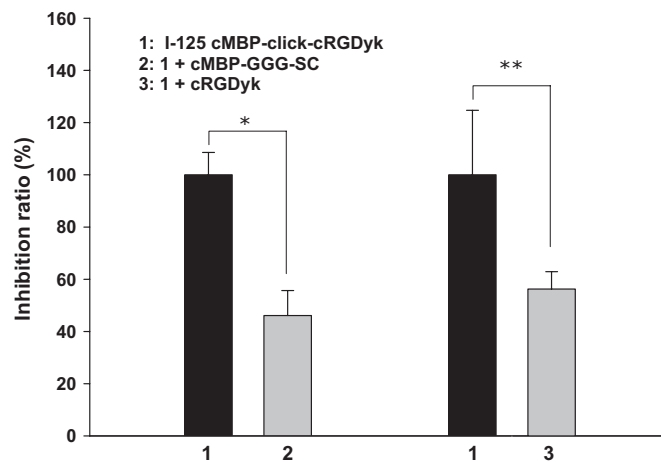
***In vitro* binding assays.** *In vitro* c-Met-binding affinity and the specificity of cMBP-click-cRGDyk were assessed via a displacement cell-binding assay using <sup>125</sup>I-cMBP (100 ng/well) as the radioligand. Experiments were performed on U87MG cells by a previously-published method.<sup>(14)</sup> For the *in vitro* integrin-binding affinity analysis, U87MG cells were seeded onto 96-well plates at 1 × 10<sup>4</sup> cells per well, and incubated overnight at 37 °C. Serial dilutions of cMBP-click-cRGDyk and <sup>125</sup>I-echistatin (corresponding to concentrations of 0.04 nM–150 μM) were added to the 96-well plates. The plates were then incubated for 1 h at 37 °C, washed, and dried; 0.1 mL of 2 N NaOH was then added to each well to facilitate cell lysis. The lysates were collected and counted in a gamma counter (Packard, Meriden, CT, USA). Binding affinities (IC<sub>50</sub>) for both receptors were calculated by non-linear regression analysis (sigmoidal dose-response equation) using the GraphPad Prism 4.0 computer-fitting program (Graph-Pad Software, San Diego, CA, USA).



**Fig. 2.** Inhibition of iodine 125 ( $^{125}\text{I}$ )-echistatin binding to integrin  $\alpha_v\beta_3$  on U87MG cells by cRGDyk and mesenchymal-epithelial transition factor (c-Met)-binding peptide (cMBP)-click cRGDyk (a). Mesenchymal-epithelial transition factor receptor tyrosine kinase binding of cMBP 3 glycine (GGG)-a single name of amino acids (SC) and cMBP-click cRGDyk on U87MG cells (b).

**Cell inhibition study.** For the blocking study, U87MG cells were seeded onto 24-well plates at  $1 \times 10^5$  cells per well. The next day, the cells were co-treated with  $^{125}\text{I}$ -cMBP-click-cRGDyk (0.18 MBq/2 pmol/well) and cMBP-GGG-SC (60 nmol) or cRGDyk (60 nmol) as blockers. The cells were incubated for 40 min at 37 °C. After incubation, the radioactive medium was aspirated, and the cells were washed with acid buffer (PBS, 0.1 M, pH 7.5). After washing, the plate was dried, and 0.1 mL of 2 N NaOH was added to the wells to facilitate cell lysis. The lysates were collected and counted in a gamma counter. The experiment was repeated five times.

**Tumor model and *in vivo* gamma imaging.** All animal experiments were performed in compliance with the policies and procedures of the Institutional Animal Care and Use Committee for animal treatment of Chonbuk National University (Jeonbuk, South Korea). Female athymic, nude mice (4 weeks, *nu/nu*) were obtained from Orient-Bio (Seoul, Korea). Glioblastoma tumors were established by subcutaneous injection of  $5 \times 10^6$  cells mixed with Matrigel into the right flank. The mice underwent single photon emission computed tomography (SPECT)/computed tomography (CT) studies when the tumor volume reached 250–350 mm<sup>3</sup> (tumor volume eq. = length  $\times$  wide<sup>2</sup>  $\times$  0.5, 4–5 weeks after inoculation). Iodine-125 gamma camera imaging and imaging processing were performed using a small animal imaging system with pin-hole collimation (aperture diameter = 1 mm, focal length = 9 cm) and a 15–45 keV photopeak energy window (X-SPECT/CT; GE Healthcare, Uppsala, Sweden). For the U87MG tumor model, mice were injected via the tail vein with approximately 14.8 MBq (400  $\mu\text{Ci}$ ) of  $^{125}\text{I}$ -cMBP-click-RGDyk under isoflurane anesthesia. For the *in vivo* inhibition studies,  $^{125}\text{I}$ -cMBP-click-cRGDyk, free cRGDyk (0.16  $\mu\text{mol}$ ), and cMBP-GGG-SC (0.16  $\mu\text{mol}$ )



**Fig. 3.** Inhibition study of iodine 125 ( $^{125}\text{I}$ )-mesenchymal-epithelial transition factor-binding peptide (cMBP)-click cRGDyk (0.18 MBq) with cMBP-3 glycine (GGG)-a single name of amino acids (SC) and cRGDyk in U87MG cells. All of the points were performed five times. Data are presented as the mean  $\pm$  SD. \* $P = 0.000$ , \*\* $P = 0.012$ .

**Table 1.** Biodistribution of iodine 125-mesenchymal-epithelial transition factor-binding peptide-click cRGDyk ( $^{125}\text{I}$ -cMBP-click cRGDyk)

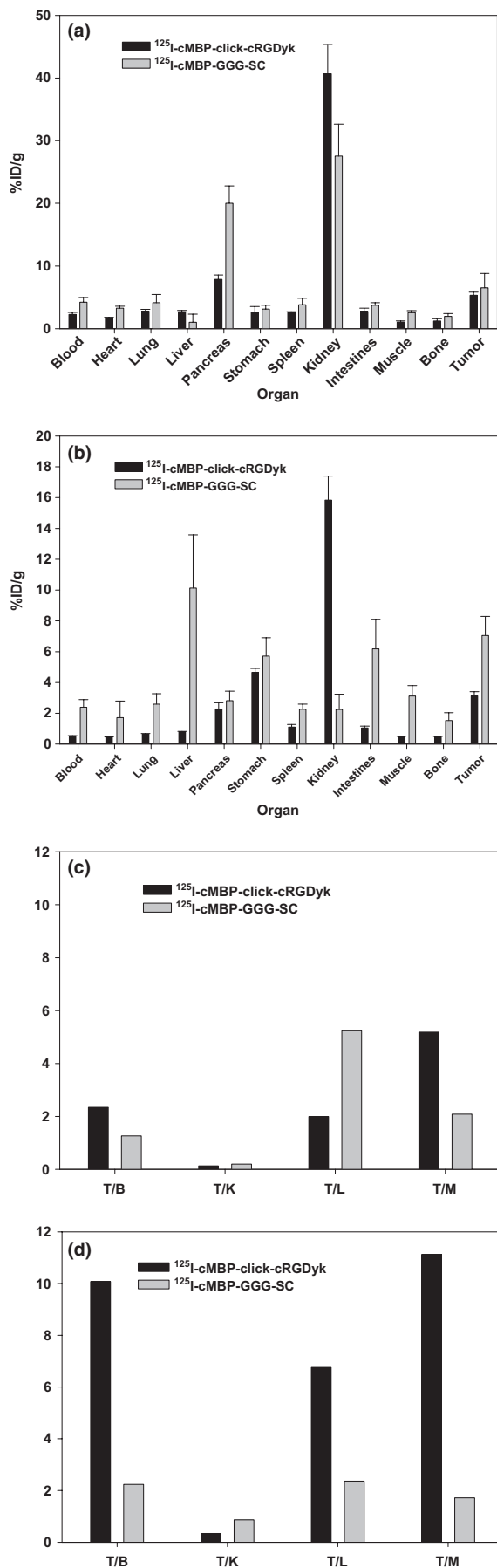
Tissue	$^{125}\text{I}$ -cMBP-click-cRGDyk (%ID/g)		
	30 min	1 h	4 h
Blood	2.28 $\pm$ 0.32	2.35 $\pm$ 0.63	0.53 $\pm$ 0.02
Heart	1.64 $\pm$ 0.16	1.75 $\pm$ 0.59	0.44 $\pm$ 0.02
Lung	2.80 $\pm$ 0.28	1.81 $\pm$ 0.28	0.64 $\pm$ 0.04
Pancreas	7.86 $\pm$ 0.70	16.07 $\pm$ 5.07	2.27 $\pm$ 0.40
Intestine	2.76 $\pm$ 0.50	2.97 $\pm$ 0.84	1.04 $\pm$ 0.12
Stomach	2.63 $\pm$ 0.89	9.77 $\pm$ 3.73	4.65 $\pm$ 0.56
Liver	2.68 $\pm$ 0.22	2.54 $\pm$ 0.71	0.79 $\pm$ 0.02
Spleen	2.65 $\pm$ 0.05	2.64 $\pm$ 0.76	1.09 $\pm$ 0.17
Kidney	40.67 $\pm$ 4.67	20.72 $\pm$ 7.71	15.83 $\pm$ 1.56
Muscle	1.03 $\pm$ 0.20	2.20 $\pm$ 0.55	0.48 $\pm$ 0.01
Bone	1.21 $\pm$ 0.37	1.63 $\pm$ 0.50	0.46 $\pm$ 0.03
Tumor	5.34 $\pm$ 0.49	4.60 $\pm$ 2.08	3.13 $\pm$ 0.27

Activity concentrations (mean  $\pm$  SD,  $n = 3$ ) at various times, post-injection. %ID/g, percentage of the injected dose per organ weight.

were co-injected into a U87MG tumor model via a lateral tail vein ( $n = 2$ ). Potassium iodide was not administered. Five-minute pin-hole planar images were acquired at 30 min, 1, 2, and 4 h after injection. At 1 h, SPECT scans were performed at 32 projections over 360 degrees (radius of rotation = 7.6 cm, 60 s/projection). Reconstructed data from SPECT and CT were visualized and co-registered using AMIRA 3.1 (GE Healthcare).

**Biodistribution study.** Biodistribution studies were carried out on U87MG xenografted female athymic, nude mice. Tumors were allowed to grow for 4–5 weeks after inoculation, at which time the animals received 0.01 mCi of  $^{125}\text{I}$ -cMBP-click-cRGDyk ( $\approx 0.5$  pmol) in 100  $\mu\text{L}$  PBS via lateral tail vein injection. The animals were then euthanized at specified time points (30 min, 1 h, 4 h,  $n = 3$ ). After the mice were killed, selected tissues and organs of interest were removed and weighed, and their activity measured in a gamma counter. The percentage of the injected dose per organ weight (%ID/g) was then calculated.

**Statistical analysis.** Data are expressed as mean  $\pm$  SD. Paired *t*-test (SPSS12.0; SPSS, Chicago, IL, USA) was used to determine statistical significance at the 95% confidence level, with  $P < 0.05$  considered statistically significant.



**Fig. 4.** Comparison of tissue distribution at 30 min (a) and 4 h (b) after post-injection between iodine 125 ( $^{125}\text{I}$ )-mesenchymal-epithelial transition factor-binding peptide (cMBP)-click cRGDy and  $^{125}\text{I}$ -cMBP 3 glycine (GGG)-a single name of amino acids (SC) in athymic nude mice bearing U87MG tumors (mean  $\pm$  SD). Comparison of tumor (T) with muscle, kidney, and liver ratios of  $^{125}\text{I}$ -cMBP-click-cRGDy and  $^{125}\text{I}$ -cMBP-GGG-SC at 30 min (c) and 4 h (d) after injection (i.v.) for athymic nude mice bearing U87MG tumors. T/B, tumor to blood; T/K, tumor to kidney; T/L, tumor to liver; T/M, tumor to muscle. %ID/g, percentage of the injected dose per organ weight.

## Results

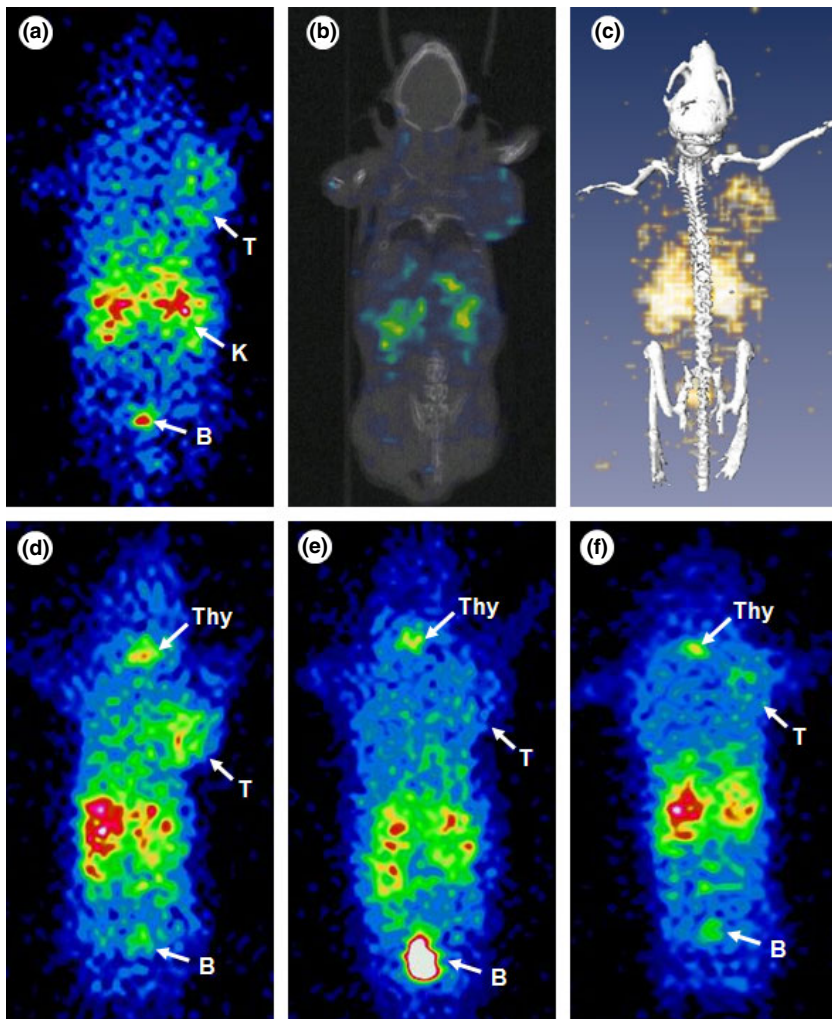
**Radiolabeling.** The structure of the cMBP-click-cRGDy target peptide is shown in Figure 1. The radiochemical purity of the  $^{125}\text{I}$ -labeled peptide conjugates were 90–95% at 24 h post-labeling. Radiochemical purity following incubation in human serum was over 90% at 4 h. The  $\log P$  value of the  $^{125}\text{I}$ -cMBP-click-cRGDy was  $-2.40 \pm 0.05$ , and  $^{125}\text{I}$ -cMBP-GGG-SC was  $-2.75 \pm 0.05$ .

**Competitive binding assay and cell inhibition study.** The binding affinities of cMBP-click-cRGDy and cMBP-GGG-SC for c-Met and integrin were evaluated for U87MG cells (Fig. 2). The results of the cell-binding assays were plotted in sigmoid curves for the displacement of  $^{125}\text{I}$ -cMBP and  $^{125}\text{I}$ -echistatin from U87MG cells as a function of increasing the concentration of the cMBP-click-cRGDy analogs. The  $\text{IC}_{50}$  values for integrin affinity were determined to be 2.38  $\mu\text{M}$  for cRGDy and 3.42  $\mu\text{M}$  for cMBP-click-cRGDy on U87MG cells. A cRGDy and cMBP-click-cRGDy shows similar binding affinities for integrin. The  $\text{IC}_{50}$  values for the c-Met affinity of cMBP GGG-SC and cMBP-click-cRGDy were 1.53 and 3.84  $\mu\text{M}$ , respectively ( $n = 2$ ). The comparable  $\text{IC}_{50}$  values from these two sets of experiments suggest that the cMBP-click-cRGDy peptide possesses comparable c-Met and integrin  $\alpha_v\beta_3$  receptor-binding affinities as the corresponding monomer. A blocking study with  $^{125}\text{I}$ -cMBP-click-cRGDy in U87MG cells showed that  $^{125}\text{I}$ -cMBP-click-cRGDy binding was significantly inhibited by cMBP-GGG and cRGDy (Fig. 3).

**Biodistribution studies.** The biodistribution of  $^{125}\text{I}$ -cMBP-click-cRGDy was determined in a U87MG xenograft model (Table 1). The tumor uptake was  $5.34 \pm 0.49\%$ ID/g at 30 min, and decreased over time to  $4.60 \pm 2.08\%$ ID/g at 1 h and  $3.13 \pm 0.27\%$ ID/g at 4 h. Kidney accumulation peaked at 30 min ( $40.67 \pm 4.67\%$ ID/g), quickly cleared by 4 h ( $15.83 \pm 1.56\%$ ID/g), and exhibited a higher signal intensity than  $^{125}\text{I}$ -cMBP-GGG-SC.<sup>(14)</sup> The biodistribution of both  $^{125}\text{I}$ -cMBP-click-cRGDy and  $^{125}\text{I}$ -cMBP-GGG-SC showed similar patterns at 30 min, but  $^{125}\text{I}$ -cMBP-click-cRGDy was eliminated sooner from the whole body by 4 h compared to  $^{125}\text{I}$ -cMBP-GGG-SC (Fig. 4a,b). Tumor-to-blood (T/B), tumor-to-kidney (T/K), tumor-to-liver (T/L) and tumor-to-muscle (T/M) ratios of  $^{125}\text{I}$ -cMBP-click-cRGDy at 30 min post-injection were 2.34, 0.13, 1.99, and 5.18, and at 4 h post-injection, they were 10.07, 0.33, 6.75, and 11.12, respectively. However, the T/B, T/K, T/L, and T/M ratios of  $^{125}\text{I}$ -cMBP-GGG-SC at 30 min post-injection were 1.26, 0.19, 5.23, and 2.08, and at 4 h post-injection, they were 2.23, 0.86, 2.36, and 1.71, respectively (Fig. 4c,d).<sup>(14)</sup>

**Single photon emission computed tomography/CT.** Representative SPECT/CT images of  $^{125}\text{I}$ -cMBP-click-cRGDy are illustrated in Figure 4. The static pin-hole image shows tumoral uptake and kidney and bladder activity at 1 h (Fig. 5a). Tumor uptake was clearly visualized by CT and CT fusion imaging (Fig. 5b,c). The planar image of  $^{125}\text{I}$ -cMBP-click-cRGDy at 4 h shows higher tumoral uptake than the 1 h image, and also shows thyroid uptake (Fig. 5d). For the *in vivo* inhibition studies,  $^{125}\text{I}$ -cMBP-click-cRGDy and free cRGDy were co-injected into the U87MG tumor mouse. As shown in





**Fig. 5.** Pin-hole planar and reconstructed co-registered transverse single photon emission computed tomography (SPECT)/computed tomography (CT) images of iodine 125 ( $^{125}\text{I}$ )-mesenchymal-epithelial transition factor-binding peptide (cMBP)-click cRGDyK in an U87MG xenograft mouse at 1 h. Pinhole gamma camera image (a), reconstructed, co-registered coronal slice image (b), and maximum-intensity-projection SPECT/CT fusion image (c) of  $^{125}\text{I}$ -cMBP-click-cRGDyK. Planar image of  $^{125}\text{I}$ -cMBP-click-cRGDyK in the U87MG xenograft mouse at 4 h (d). Inhibition image at 4 h post-co-injection of  $^{125}\text{I}$ -cMBP-click-cRGDyK and free cRGDyK (48 nmol) in the U87MG mouse (e). Free cMBP 3 glycine-a single name of amino acids (SC) (0.16  $\mu\text{mol}$ ) inhibition image at 4 h (f). B, bladder; K, kidney; Thy, thyroid, T, tumor.

Figure 5e, tumoral uptake was significantly decreased by free cRGDyK at 4 h post-injection. The image-based region of interest (ROI) ratios for both the tumor and non-tumor regions in  $^{125}\text{I}$ -cMBP-click-cRGDyK-injected mice at 30 min and 4 h post-injection were  $24 \pm 8$  and  $4 \pm 3$ , and  $30 \pm 7$  and  $4 \pm 2.9$  (mean of count/pixel), respectively. The ROI ratios in inhibited mice at 30 min and 4 h post-injection were  $14 \pm 5$  and  $12 \pm 3$ , and  $12 \pm 4$  and  $6 \pm 2$  (mean of count/pixel), respectively. Moreover, whole body activity was quickly eliminated through the kidney, but accumulated in the bladder, from 10 min post-injection due to the residual receptor presence of free cRGDyK in the whole body. Iodine-125-cMBP-click-cRGDyK and free cMBP-GGG-SC were co-injected into the U87MG tumor mouse. The ROI ratios at 30 min and 4 h post-injection were  $46 \pm 6.7$  and  $19 \pm 5.9$ , and  $23 \pm 6.5$  and  $16 \pm 3.8$  (mean of count/pixel), respectively (Fig. 5f).

## Discussion

Since U87MG tumor cells express both c-Met and integrin  $\alpha_v\beta_3$ , we explored whether a dual c-Met- and integrin  $\alpha_v\beta_3$ -targeting approach would allow us to develop improved imaging probes over  $^{125}\text{I}$ -cMBP-GGG-SC that only recognizes one receptor type. In a previous study, cMBP was conjugated with two types of linkers, such as GGG and 8-aminooctanoic acid (AOC). The tissue distribution of three different peptides,  $^{125}\text{I}$ -cMBP,  $^{125}\text{I}$ -cMBP-GGG-SC, and  $^{125}\text{I}$ -cMBP-AOC-C, were determined in U87MG-xenografted mice.<sup>(14)</sup>

As determined by a biodistribution study,  $^{125}\text{I}$ -cMBP-GGG-SC exhibited the highest T/B at 4 h. However, static pin-hole images of  $^{125}\text{I}$ -cMBP-GGG-SC showed a relatively low tumor uptake and high body background activity at 1 and 4 h, and even higher pancreatic and renal activities at all time points. Therefore, a modification of cMBP-GGG-SC through heterodimerization of two ligands, one targeting c-Met and the other targeting integrin, was needed to improve the targetability for an *in vivo* cancer model.

Peptide modifications to be used as imaging agents can be achieved by various methods. In order to improve binding affinity and receptor selectivity and to avoid the fast degradation of peptides *in vivo*, several researchers introduced cyclization methods.<sup>(17)</sup> Heteromultimerization and homomultimerization of ligands with multitargeting properties have also been developed by many researchers.<sup>(2-6)</sup> These approaches have successfully improved tumor-targeting efficacy and pharmacokinetics compared to single analog methodologies. We therefore synthesized radiolabeled  $^{125}\text{I}$ -cMBP-click-cRGDyK (Fig. 1). The linking groups in small peptide receptor-targeted radiopharmaceutical designs have largely been viewed as merely convenient ways to adjust blood retention and overall clearance of radiopharmaceuticals, without making substantial alterations to the targeting vector. In this study, two different peptides were connected, forming a 1,2,3-triazole ring between an alkyne and an azide terminal.

Our data from the receptor-binding assays demonstrated that cMBP-click-cRGDyK is similar to cMBP-GGG-SC for c-Met

binding and to cRGDyk for integrin  $\alpha_v\beta_3$  binding (Fig. 2). However, the binding affinity of cMBP-click-cRGDyk for both receptors was not improved. As shown in Table 1,  $^{125}\text{I}$ -cMBP-click-cRGDyk tends to have a faster washout than  $^{125}\text{I}$ -cMBP-GGG-SC, but  $\log P$  values of the two radiolabeled compounds were similar ( $-2.40 \pm 0.05$  and  $-2.75 \pm 0.05$ , respectively). In another previous heterodimer study,  $^{18}\text{F}$ -FB-BBN-RGD tended to have a slower washout than  $^{18}\text{F}$ -FB-BBN, which might be the result of enhanced, effective binding due to dual targeting.<sup>(7)</sup> In comparing our results, although  $^{125}\text{I}$ -cMBP-click-cRGDyk and  $^{125}\text{I}$ -cMBP-GGG-SC showed similar binding affinity and hydrophilicity,  $^{125}\text{I}$ -cMBP-click-cRGDyk tended to have a faster clearance rate *in vivo* than  $^{125}\text{I}$ -cMBP-GGG-SC.

The tumoral uptake decreased with time compared to  $^{125}\text{I}$ -cMBP-GGG-SC ( $6.53 \pm 2.29$ ,  $6.85 \pm 1.89$ , and  $7.05 \pm 1.24$  at 30 min, 2 and 4 h). Moreover, high kidney uptake appeared in three time points compared to  $^{125}\text{I}$ -cMBP-GGG-SC ( $27.54 \pm 5.11$ ,  $9.54 \pm 1.91$ , and  $6.18 \pm 1.92$  at 30 min, 2 and 4 h), slowly decreasing from  $40.67 \pm 4.67$  to  $15.83 \pm 1.56$ . As shown Figure 4(c,d), the T/B, T/L, and T/M ratios of  $^{125}\text{I}$ -cMBP-click-cRGDyk were higher than those of  $^{125}\text{I}$ -cMBP-GGG-SC. While the initial tumor accumulation of  $^{125}\text{I}$ -cMBP-click-cRGDyk was less than  $^{125}\text{I}$ -cMBP-GGG-SC, the T/B, T/L, and T/M ratios increased with time due to the relatively low activities in major organs, such as the heart, lung, blood, liver, intestines, muscles, and spleen. Iodine-125-cMBP-click-cRGDyk demonstrated a quicker clearance rate than  $^{125}\text{I}$ -cMBP-GGG-SC (blood activity:  $4.22 \pm 0.75$ ,  $2.77 \pm 0.38$  and  $2.39 \pm 0.50$  at 30 min, 2 and 4 h) and lower background activity.

The imaging quality of  $^{125}\text{I}$ -cMBP-click-cRGDyk was evaluated in a U87MG tumor xenografted model. The quality was also improved by  $^{125}\text{I}$ -cMBP-click-cRGDyk compared to  $^{125}\text{I}$ -cMBP-GGG-SC in the liver, heart, pancreas, and kidneys at 1 and 4 h (Fig. 5). However, thyroid uptake was observed in the 4 h post-injection image (Fig. 5d), but this might be due to the deiodination of  $^{125}\text{I}$ -cMBP-click-cRGDyk. In a previous study, the labeling stability of  $^{125}\text{I}$ -cMBP-GGG-SC was stable, and cMBP-GGG-SC did not internalize into the U87MG cells; thyroid uptake did not appear in the *in vivo* study. However, as labeled radioiodine on the tyrosine ring of RGD peptides was

found to be unstable *in vivo*,<sup>(18,19)</sup>  $^{125}\text{I}$ -cMBP-click-cRGDyk was thought to be internalized like  $^{125}\text{I}$ -cMBP-AOC-C and then deiodinated. Taking this into consideration, a direct comparison between two compounds with radioiodine activity might be difficult to achieve, since there is the possibility of underestimating cMBP-click-cRGDyk binding with integrin due to deiodination from the heterodimer compound, compared to cMBP-GGG-SC for c-Met only. When labeling efficiency and the stability of  $^{125}\text{I}$ -cRGDyk was estimated, the *in vitro* labeling stability of  $^{125}\text{I}$ -cRGDyk quickly decreases with time. Consequently,  $^{125}\text{I}$ -cMBP-click-cRGDyk might demonstrate faster clearance than  $^{125}\text{I}$ -cMBP-GGG-SC.

Moreover, although the tumor volumes used in this study were smaller than those of  $^{125}\text{I}$ -cMBP-GGG-SC, they were well visualized. For the identification of integrin receptor targetability, free cRGDyk (48 nmol) and cMBP-GGG-SC were co-injected, and images were acquired (Fig. 5e). These inhibition results suggest that  $^{125}\text{I}$ -cMBP-click-cRGDyk was specifically taken up by integrin  $\alpha_v\beta_3$  and the c-Met receptor.

In conclusion, we successfully developed a heterodimeric peptide that binds to both the c-Met receptor and integrin  $\alpha_v\beta_3$  using click reaction methodology. Dual integrin and/or c-Met receptor recognition showed slightly improved tumor-targeting efficacy and imaging quality compared to  $^{125}\text{I}$ -cMBP-GGG-SC single analogs, despite high renal clearance activity and decreasing tumoral uptake over time. However, additional experiments are necessary to improve clinical pharmacokinetics, such as decreased kidney uptake and prolonged tumor uptake.

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## Disclosure Statement

The authors have no conflict of interest.

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