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Synthesis and in vivo evaluation of Tc-99m-labeled cyclic *Ciso*DGRC peptide conjugates for targeting $\alpha_v\beta_3$ integrin expression

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ABSTRACT

Two $\alpha_v\beta_3$ integrin-binding peptide conjugates containing the cyclic *Ciso*DGRC motif, a linker, and a chelator to enable Tc-99m labeling via the *fac*-[^{99m}Tc(CO)$_3$]$^+$ core were synthesized. In vivo biodistribution studies in U87MG tumor-bear nude mice at 1 h post-injection revealed a profound effect of the linker on the clearance of the radiotracer from the blood stream. In vivo blocking studies demonstrated the selective binding to the tumors expressing $\alpha_v\beta_3$-integrin and other tissues. The HPLC analysis of urine samples collected upon necropsy showed no degradation indicating their metabolic stability. These results suggest that cyclic *Ciso*DGRC motif could be exploited as a new $\alpha_v\beta_3$-targeting vector by an appropriate selection of a linker between the peptide and the payload to obtain optimum pharmacokinetic properties.

Angiogenesis, the formation of new blood vessels, is a rate-limiting step for the growth of solid tumors.[1–3] Tumors produce many angiogenic factors, which are able to activate endothelial cells in established blood vessels and induce endothelial proliferation, migration, and new vessel formation through a series of sequential but partially overlapping steps.[4,5] The angiogenic process depends on vascular endothelial cell migration and invasion and is regulated by cell adhesion receptors.[4,5] Integrins are a family of proteins that facilitate the cellular adhesion and migration on extracellular matrix proteins in the intercellular spaces and basement membranes, and regulate cellular entry and withdrawal from the cell cycle.[6,7] Integrin $\alpha_v\beta_3$ serves as a receptor for extracellular matrix proteins such as vitronectin, fibronectin, etc., with exposed arginine-glycine-aspartate (RGD) tripeptide sequence.[8,9] Integrin $\alpha_v\beta_3$ is expressed at low levels on epithelial and mature endothelial cells, but it is highly expressed on activated endothelial cells in neovasculature of solid tumors such as glioblastomas, breast cancer, etc.[10,11] The restricted expression of integrin $\alpha_v\beta_3$ during tumor growth, invasion, and metastasis present an attractive molecular target for diagnosis and treatment of the rapidly growing and metastatic tumors.

Various tumor-homing peptides containing the asparagine-glycine-arginine (NGR) motif have been discovered by peptide-phage library panning in tumor-bearing mice.[12] The tumor-homing properties of these peptides rely on the interaction with aminopeptidase N (CD13), a membrane protease expressed by the tumor neovasculature.[13,14] Because of this property, a cyclic CNGRC peptide containing NGR motif has been exploited for targeted delivery of various therapeutic and diagnostic agents to tumor vessels, in an attempt to increase their antitumor activity or tumor localization.[12,15–19] Recent studies have shown that NGR motif can rapidly convert to isoaspartate-glycine-arginine (*iso*DGR) by asparagine deamidation, generating $\alpha_v\beta_3$ ligands capable of affecting endothelial cell functions and tumor growth.[20–24] Biochemical, NMR structure analysis, and $\alpha_v\beta_3$ docking studies showed that *iso*DGR, but not NGR and DGR, can fit into the RGD-binding pocket of $\alpha_v\beta_3$ integrin, recapitulating not only the canonical RGD/$\alpha_v\beta_3$ contacts but also establishing additional polar interactions.[20,22] Thus, *iso*DGR motif could be exploited as a new $\alpha_v\beta_3$-targeting vector alternative to traditional RGD motif.

Here we report radiosynthesis and preliminary in vivo evaluation of two new cyclic *Ciso*DGRC peptide conjugates containing a bis(2-pyridylmethyl)amino (BPy) chelator, radiolabeled via the *fac*-[^{99m}Tc(CO)$_3$]$^+$ core, in human glioblastoma U87MG tumor-bearing nude mice. The small size of the *fac*-[^{99m}Tc(CO)$_3$]$^+$ core allows labeling the peptides with high specific activities while retaining biological activity and specificity, and produces in vivo stable and kinetically inert complexes.[25,26]

The BPy chelators containing 3- and 6-carbon chains were synthesized according to the literature procedure.[27] The peptide conjugates were manually synthesized as shown in Figure 1 by solid phase peptide synthesis method employing traditional Fmoc chemistry and HBTU activation of carboxyl groups on the reactant

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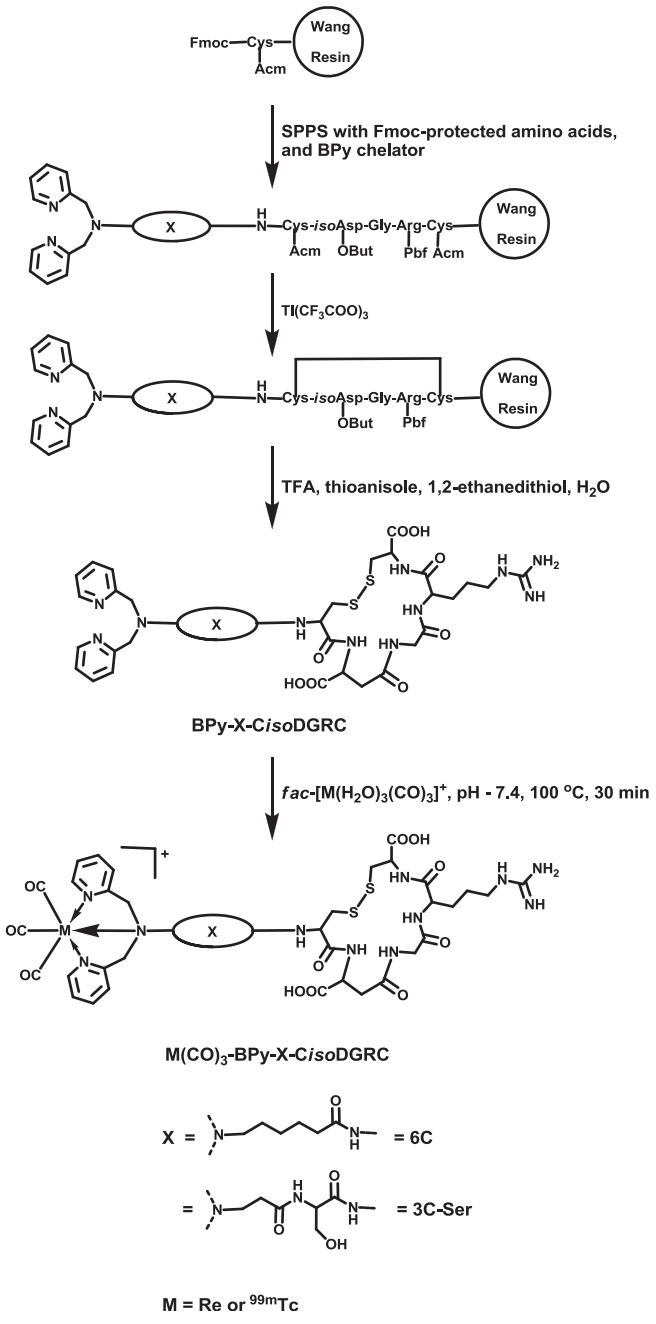

Figure 1. Synthesis and complexation of C*iso*DGRC conjugates with *fac*-[Re/^{99m}Tc(CO)$_3$]-core.

with the N-terminal amino group on the growing peptide anchored via the C-terminus to the resin. On-resin oxidation of cysteine thiols was carried out by treating the acetamidomethyl (acm)-protected cysteines with thallium (III) trifluoroacetate (2 equiv) in DMF (2 mL) for 2 × 60 min at room temperature. The peptide conjugates were obtained in an overall yield of 21–25% after HPLC purification. Cold *fac*-[Re(CO)$_3$]$^+$ analogs were synthesized by reacting BPy-X-C*iso*DGRC (2 mg) in water (500 μL) at 100 °C for 30 min with [Re(CO)$_3$(H$_2$O)$_3$]Br precursor (2 mg) prepared according to the procedure reported elsewhere.[28] Re(CO)$_3$-BPy-X-C*iso*DGRC were obtained in quantitative yield after HPLC purification. All peptides were characterized by electrospray mass spectrometry (Table 1).

The Tc-99m labeling was achieved by adding a solution of BPy-X-C*iso*DGRC (100 μg) in water (50 μL) to a vial containing 1 N HCl

Table 1
HPLC and mass spectrometry data.

Peptide	HPLC retention time (min:s)	Mass	
		Calculated	Observed
BPy3C-Ser-C*iso*DGRC	8:55	890.3	891
Re(CO)$_3$-BPy3C-Ser-C*iso*DGRC	13:33	1164.3	1161
^{99m}Tc(CO)$_3$-BPy3C-Ser-C*iso*DGRC	13:42	—	—
BPy6C-C*iso*DGRC	10:32	845.3	846.4
Re(CO)$_3$-BPy6C-C*iso*DGRC	14:37	1119.3	1116.3
^{99m}Tc(CO)$_3$-BPy6C-C*iso*DGRC	14:50	—	—

(25 μL), PBS (375 μL), and *fac*-[^{99m}Tc(H$_2$O)$_3$(CO)$_3$]$^+$ precursor (100 μL, ~2 mCi), prepared via the Isolink kit (Covidien, St. Louis, MO) as per the manufacturer's instructions, and heating the vial at 100 °C for 30 min. This method of Tc-99m labeling resulted in high radiochemical yields at relatively low-peptide concentrations, which is a characteristic advantage of the labeling biomolecules containing tridentate-chelating ligand systems with the *fac*-[^{99m}Tc(CO)$_3$]-core.[25–27,29] The radiochemical purity of the HPLC-purified product was found to be >98%. The HPLC retention times of ^{99m}Tc(CO)$_3$-BPy-X-C*iso*DGRC were matched to the corresponding cold Re analogs, which confirmed the radiolabeled product formation. The ^{99m}Tc(CO)$_3$-BPy-X-C*iso*DGRC eluted ~4.5 min after the unlabeled BPy-X-C*iso*DGRC under our HPLC conditions enabling collection of high-specific activity, NCA ^{99m}Tc(CO)$_3$-BPy-X-C*iso*DGRC.

The biodistribution of ^{99m}Tc(CO)$_3$-BPy-X-C*iso*DGRC in U87MG tumor-bearing nude mice was studied at 1 h post-injection. As seen in Figure 2, the linker showed profound effect on the clearance of ^{99m}Tc(CO)$_3$-X-C*iso*DGRC from the blood stream. ^{99m}Tc(CO)$_3$-BPy6C-C*iso*DGRC, the conjugate containing a hexanoic acid (6C) linker being relatively more hydrophobic of the two conjugates was cleared mostly through hepatobiliary pathway. The cumulative radioactivity in liver and intestine was >76.5% of injected dose (%ID). In contrast, ^{99m}Tc(CO)$_3$-BPy3C-Ser-C*iso*DGRC, which has a linker comprised of a propanoic acid and serine residue (3C-Ser), was cleared primarily through renal-urinary pathway. The cumulative accumulation of radioactivity in liver and intestine was <8.5%ID for ^{99m}Tc(CO)$_3$-BPy3C-Ser-C*iso*DGRC. In general, the presence of *fac*-[^{99m}Tc(CO)$_3$]$^+$ core and the BPy chelator combination makes a conjugate lipophilic in nature, but we observed that the presence of 3C-Ser linker sufficiently alters the in vivo characteristics. Thus, the typical high hepatic accumulation is shifted towards rapid renal clearance from the body resulting in lower background radioactivity. Although ^{99m}Tc(CO)$_3$-BPy6C-C*iso*DGRC is relatively more lipophilic as seen from HPLC retention times of the two conjugates (13:42 min vs 14:50 min), the clearance shift from hepatobiliary to renal route is more than expected.

In tumors, the radioactivity accumulation of 1.3 ± 0.46%ID/g and 0.57 ± 0.21%ID/g was observed for ^{99m}Tc(CO)$_3$-BPy6C-C*iso*DGRC and ^{99m}Tc(CO)$_3$-BPy3C-Ser-C*iso*DGRC, respectively (Fig. 2). The radioactivity cleared rapidly from the blood stream with only 0.84 ± 0.12%ID/g and 0.67 ± 0.30%ID/g remained in blood for ^{99m}Tc(CO)$_3$-BPy6C-C*iso*DGRC and ^{99m}Tc(CO)$_3$-BPy3C-Ser-C*iso*DGRC, respectively. Low tumor/blood ratios of about 1.5 and 0.9; and tumor/muscle ratios of about 4.6 and 3.2 were achieved for ^{99m}Tc(CO)$_3$-BPy6C-C*iso*DGRC and ^{99m}Tc(CO)$_3$-BPy3C-Ser-C*iso*DGRC, respectively. The SPECT imaging of ^{99m}Tc(CO)$_3$-BPy-X-C*iso*DGRC in U87MG tumor-bearing nude mice at 1 h post-injection (Fig. 3) confirmed the results of the biodistribution study. High uptake of radioactivity was seen in liver and intestine for ^{99m}Tc(CO)$_3$-BPy6C-C*iso*DGRC, in contrast, high accumulation of radioactivity was seen in bladder for ^{99m}Tc(CO)$_3$-BPy3C-Ser-C*iso*DGRC. No radioactivity uptake was seen in tumors due to the low tumor/blood ratios at the chosen imaging time.

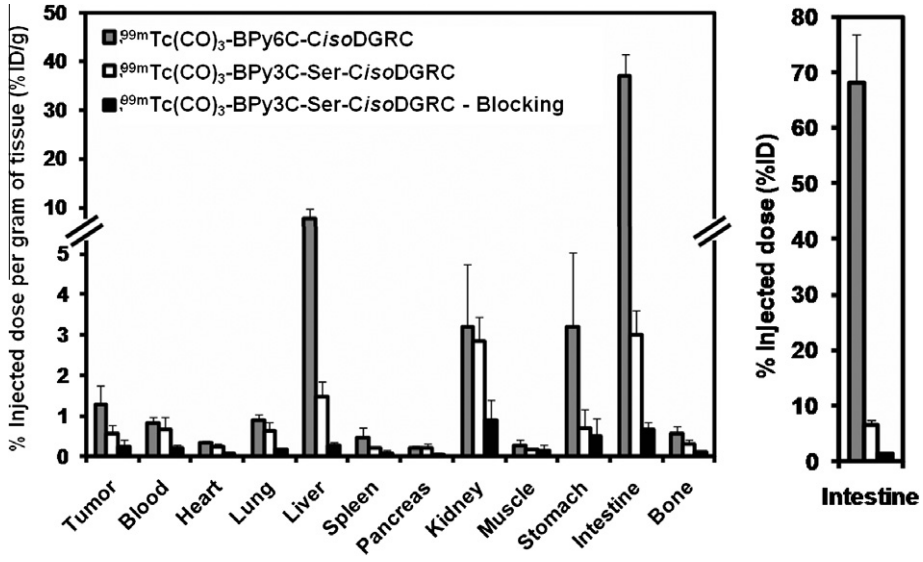

Figure 2. Biodistribution of $^{99m}Tc(CO)_3$-BPy-X-*Ciso*DGRC in U87MG tumor-bearing nude mice at 1 h post-injection. Error bar indicates SD ($n = 4$ for $^{99m}Tc(CO)_3$-BPy6C-*Ciso*DGRC; 6 for $^{99m}Tc(CO)_3$-BPy3C-Ser-*Ciso*DGRC; and 3 for blocking study).

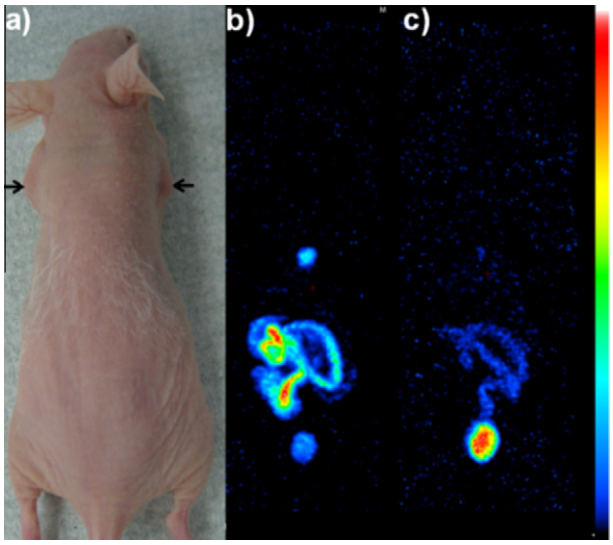

Figure 3. (a) Photograph of the U87MG tumor-bearing nude mouse used for imaging $^{99m}Tc(CO)_3$-BPy6C-*Ciso*DGRC (arrows indicate tumor); (b) and (c) are SPECT images of a U87MG tumor-bearing nude mice injected with $^{99m}Tc(CO)_3$-BPy6C-*Ciso*DGRC and $^{99m}Tc(CO)_3$-BPy3C-Ser-*Ciso*DGRC at 1 h post-injection, respectively.

To demonstrate the specificity of radioactivity uptake in tumors and other $\alpha_v\beta_3$-integrin expressing tissue, we performed a biodistribution study by co-injecting an excess (500 μg) of non-radioactive *Ciso*DGRC with $^{99m}Tc(CO)_3$-BPy3C-Ser-*Ciso*DGRC in U87MG tumor-bearing nude mice (Fig. 2). We observed that the tumor uptake reduced by about 50% to 0.24 ± 0.02%ID/g in presence of the excess peptide. Along with tumor, radioactivity accumulation in all other $\alpha_v\beta_3$-integrins expressing tissues (heart, lung, liver, kidney, and intestine) was also significantly blocked. These results clearly demonstrate that $^{99m}Tc(CO)_3$-BPy-X-*Ciso*DGRC binds selectively to the $\alpha_v\beta_3$-integrin present on the U87MG tumor cells and other tissues under in vivo conditions.

The HPLC analysis of urine samples collected during biodistribution studies from U87MG tumor-bearing nude mice injected with $^{99m}Tc(CO)_3$-BPy3C-Ser-*Ciso*DGRC at 1 h pi showed that the integrity of the radiolabeled peptide remained intact in both with and without co-injection of excess *Ciso*DGRC (Fig. 4). The metabolic stability shown by this conjugate may be due to the cyclic structure of the *Ciso*DGRC.

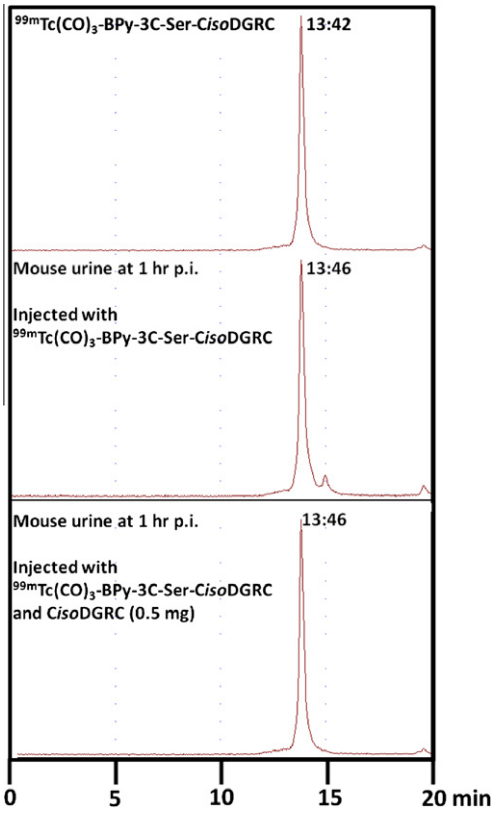

Figure 4. Radio-HPLC chromatograms. HPLC solvents consisted of water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (solvent B). A Sonoma C18 column (ES Industries, West Berlin, NJ) 10 μm, 100 Å, 4.6 × 250 mm was used with a flow rate of 1.5 mL/min. The HPLC gradient system began with an initial solvent composition of 95% A and 5% B for 2 min followed by a linear gradient to 50% A and 50% B in 15 min, after which the column was re-equilibrated.

In conclusion, the comparative evaluation of the cyclic C*iso*DGRC peptides, labeled via the *fac*-[^{99m}Tc(CO)$_3$]-core, showed that the in vivo characteristics of the conjugate containing a serine residue in the linker was more favorable in terms of clearance. Further improvements in the structure of the conjugate may improve its tumor uptake and make it an attractive candidate for the imaging of $\alpha_v\beta_3$-positive tumors. One strategy that has been successfully applied to improve tumor uptake of the radiolabeled cyclic RGD peptide conjugates is to have multiple RGD units in the conjugate.[30–33] Apparently, a multimeric preparation significantly enhances the binding affinity of the receptor–ligand interaction through the polyvalency effect.[30,34] Together with the choice of appropriate linker for desired pharmacokinetics, it may be possible to exploit multimerization approach for enhanced tumor uptake of the cyclic C*iso*DGRC motif via integrin $\alpha_v\beta_3$-targeting.

Acknowledgments

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