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# Preparation and characterization of $^{99m}Tc(CO)_3$–BPy–RGD complex as $\alpha_v\beta_3$ integrin receptor-targeted imaging agent

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**Abstract**

The aim of this study is to develop a novel arginine–glycine–aspartic acid (RGD) peptide-containing ligand for $^{99m}Tc$ labeling as $\alpha_v\beta_3$ integrin receptor-targeted imaging agent. BPy–RGD conjugate was successfully synthesized by coupling of 5-carboxylate-2,2′-bipyridine and c(RGDyK) peptide through EDC/SNHS in aqueous solution and was characterized by MADLI-TOF-MS ($m/z = 802.72$, $C_{38}H_{48}N_{11}O_9$). $^{99m}Tc(CO)_3$–BPy–RGD was prepared by exchange reaction between $[^{99m}Tc(H_2O)_3(CO)_3]^+$ and BPy–RGD. Final product was purified by HPLC and tested for octanol/water partition coefficient. Cell-binding assays of BPy–RGD and unmodified c(RGDyK) were tested in MDA-MB-435 cells ($^{125}$I-echistatin as radioligand). Preliminary biodistribution of the $^{99m}Tc(I)$-labeled radiotracer in orthotopic MDA-MB-435 breast tumor xenograft model was also evaluated. The BPy–RGD conjugate had good integrin-binding affinity (50% inhibitory concentration ($IC_{50}$) = 92.51 ± 22.69 nM), slightly lower than unmodified c(RGDyK) ($IC_{50}$ = 59.07 ± 11.03 nM). The hydrophilic radiotracer also had receptor-mediated activity accumulation in MDA-MB-435 tumor (1.45 ± 0.25 percentage of injected dose per gram (%ID/g) at 1.5 h postinjection (p.i.)), which is known to be integrin positive. After blocking with c(RGDyK), the tumor uptake was reduced from 0.71 ± 0.01%ID/g to 0.33 ± 0.18%ID/g at 4 h p.i. $^{99m}Tc(I)$ tricarbonyl complex of cyclic RGD peptide is a promising strategy for integrin targeting. Further modification of the bipyridine-conjugated RGD peptide by using more potent RGD peptides and fine tuning of the tether group between the RGD moiety and $^{99m}Tc(CO)_3^+$ core to improve the tumor targeting efficacy and in vivo kinetic profiles is currently in progress.


*Keywords:* $^{99m}Tc$-tricarbonyl; $\alpha_v\beta_3$ integrin; Cyclic RGD peptide

## 1. Introduction

Angiogenesis, the formation of new blood vessels from the pre-existing ones, is a fundamental process occurring during embryonic development, the reproductive cycle, and in pathological conditions, such as wound healing, diabetic retinopathy, muscular degeneration, rheumatoid arthritis, and psoriasis (Albelda and Buck, 1990; Chen et al., 2004a–h; Jain, 2003). Tumor growth and metastasis are particularly dependent on angiogenesis. Angiogenesis is a complex process involving extensive interplay between cells, soluble factors, and extracellular matrix (ECM) components. Integrin receptors are responsible for a wide range of ECM and cell–cell interactions, which have been well studied in many tumor types (Hynes, 1992). The vitronectin receptor $\alpha_v\beta_3$ is a member of the integrin superfamily of receptors and is highly expressed on activated endothelial cells and solid tumor cells but not present in resting endothelial cells and most normal organ systems (Eliceiri and Cheresh, 1999). The density of integrin $\alpha_v\beta_3$ receptors on the cell surface had been quantified for several solid tumor cells and the highest receptor density was found on U87MG glioblastoma cells ($(1.28 \pm 0.46) \times 10^5$ receptors/cell) (Zhang et al., 2005). Similar to several other integrins, $\alpha_v\beta_3$ recognizes the tripeptide sequence arginine–glycine–aspartic acid (RGD). The inhibition of $\alpha_v\beta_3$ is currently being evaluated as a new

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strategy for tumor-specific anticancer therapy. Antibody, peptide, and peptidomimetic antagonists of integrin $\alpha_v\beta_3$ have been shown to inhibit angiogenesis by selectively promoting apoptosis of vascular endothelial cells (Xiong et al., 2002). Encouraging experimental studies have already led to initial clinical trials evaluating the use of $\alpha_v\beta_3$ antagonists as antiangiogenic drugs in patients with various malignant tumors (Brower, 1999). These RGD peptides have also been useful in imaging sites of tumor angiogenesis (Zhang et al., 2005) and may provide a means to deliver molecularly guided radiotherapy to the vascular bed of tumors (Wu et al., 2005). We and others have labeled cyclic RGD peptides with $^{125}$I, $^{99m}$Tc, $^{111}$In, $^{90}$Y, $^{18}$F, and $^{64}$Cu for radionuclide imaging of $\alpha_v$-integrin expression, studying pharmacokinetic behavior of integrin antagonists, carrying out internal radiotherapy, and evaluating anti-integrin treatment response (Chen et al., 2004a–h; Haubner et al., 2004; Janssen et al., 2002a, b, 2004).

The aim of this study is to extend this effort and develop novel radiotracers for single photon emission computed tomography (SPECT) imaging of tumor integrin expression. Although with slightly lowered resolution and sensitivity as compared to positron emission tomography (PET), the widespread availability of SPECT instrument, readily available $^{99m}$Tc through inexpensive $^{99}$Mo–$^{99m}$Tc generator, and simple radiolabeling procedure promises SPECT to play an increasingly important role in molecular nuclear medicine imaging.

## 2. Materials and methods

### 2.1. Materials

Monomeric cyclic RGD peptide c(RGDyK) was synthesized as previously described (Chen et al., 2004a–h). All other reagents were of analytical grade and commercially available. $^{99m}$Tc-pertechnetate ($[^{99m}Tc]NaTcO_4$) was purchased as a solution in physiological saline from GE Healthcare (San Jose, CA). IsoLink™ labeling kits were obtained from Mallinckrodt (St. Louis, MO). Semi-preparative reversed-phase HPLC was accomplished by use of a Dionex P680 chromatography system with a UVD 170U detector and a solid-state radiation detector (model 105S, Carroll & Ramsey Associates). Purification was performed with a Vydac 218TP510 protein and peptide column (5 μm; $250 \times 10\,mm^2$). The flow rate was 5 mL/min. The mobile phase was changed from 95% solvent A (0.1% trifluroacetic acid (TFA) in water) and 5% solvent B (0.1% TFA in acetonitrile) (0–3 min) to 20% solvent A and 80% solvent B at 25 min. The analytic HPLC method was performed with the same gradient system but with a Vydac 218TP54 column (5 μm; $250 \times 4.6\,mm^2$) and a flow rate of 1 mL/min. The absorbance was monitored at 218 nm. Reversed-phase extraction $C_{18}$ Sep-Pak cartridges (Waters) were pre-treated with methanol and water before use.

### 2.2. Synthesis of bipyridine RGD conjugate

*6-Carboxy-2,2′-bipyridine (BPy-$CO_2H$)* (Fletcher et al., 2001): 6-Methyl-2,2′-bipyridine (0.5 g, 2.9 mmol) was suspended in 4 mL of water and then 2 g of $KMnO_4$ was added in four portions over 7 h, heated initially at 70 °C for 3 h and then at 90 °C for the subsequent 4 h. The mixture was hot filtered through qualitative P8-creped filter paper and washed with hot water ($3 \times 3$ ml). The solution was then slowly acidified with 1 M HCl to obtain a white precipitate at pH ca. 4–5. The precipitate was collected by filtration and dried in vacuo (Fletcher et al., 2001). This compound was characterized by HPLC and used for next reaction without further purification.

*Bipyridine c(RGDyK) conjugate (BPy–RGD)*: 16.0 mg (79.6 μmol) 6-carboxy-2,2′-bipyridine was dissolved in 0.8 mL $H_2O$. After adjusting pH to 5–5.5 by 0.1 N NaOH, 14.3 mg (65.9 μmol) of sodium *N*-hydroxysulfonosuccinimide (Sulfo-NHS, Sigma-Aldrich) and 15.5 mg (80.7 μmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, Fluka) were added. After reaction at 4 °C for 30 min, the pH of the reaction mixture was adjusted to 9 by 0.1 N NaOH and 5.2 mg (8.4 μmol) of c(RGDyK) dissolved in 0.5 mL of 0.1 M $Na_2HPO_4$ solution (pH = 9). After standing at room temperature for 2 h, HPLC was applied to purify the final conjugate, which was then characterized by MADLI-TOF-MS.

### 2.3. Radiolabeling of BPy–RGD conjugate

The Isolink kit vial (Mallinckrodt), containing the following lyophilized formulation: 8.5 mg sodium tartrate, 2.85 mg $Na_2B_4O_7 \cdot 10H_2O$, 7.15 mg of sodium carbonate, and 4.5 mg sodium boranocarbonate, in freeze-dried form was used for preparing the labeling precursor ($[^{99m}Tc(H_2O)_3(CO)_3]^+$). One milliliter of freshly eluted $^{99m}TcO_4^-$ from a commercial generator (about 370 MBq) was added to the vial and followed by incubation at 100 °C for 20 min. The pH was then adjusted to 6–7 with 1 N HCl. The purity of the reaction mixture was analyzed by reversed-phase HPLC at a flow rate of 1 mL/min. Labeling of the ligands (BPy–$CO_2H$ or BPy–RGD) was performed by mixing 10 μL of different concentrations of the ligands solution (resulting in an amount of 1–25 μM ligand per MBq $^{99m}$Tc activity) with 490 μL of the $[^{99m}Tc(CO)_3(OH_2)_3]^+$ precursor solution and heating for 20 min at different temperatures (25, 70, and 100 °C). The final product was purified by HPLC. The product fractions were collected, combined, and evaporated to remove the HPLC solvent. The activity was finally reconstituted in phosphate-buffered saline (pH = 7.4) and passed through a 0.22 μm Millipore filter into a sterile vial for in vitro and in vivo applications. The in vitro stability of $^{99m}Tc(CO)_3$–BPy–RGD conjugate was determined by measuring the radiochemical purity (RCP) after storage at room temperature for 6 h.