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Effect of the Bifunctional Chelate on the Biodistribution of ^{99m}Tc-labeled Cyclic RGD Peptide

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Abstract - A novel N_3S_1 chelate, Pro-Lys-Cys (PKC) to cyclic RGD to radiolabel with ^{99m}Tc was conjugated in an effort to decrease the high intestinal accumulation observed for ^{99m}Tc-labeled PGC-RGD. The target specificity of the resulting PKC-RGD was similar to that of PGC-RGD as determined by a cell binding assay and a competition binding assay. The ^{99m}Tc radiolabeling of PKC-RGD resulted in radiochemical yields of 98% under mild conditions at high specific activities. Biodistribution data in normal mice clearly showed a significant decrease in intestinal uptake at 2 h postinjection for the ^{99m}Tc-PKC-c (RGDyK) compared to the ^{99m}Tc-PGC-c (RGDyK) (from 19.65%ID·g^{-1} to 7.31%ID·g^{-1} for the GI tract). The ^{99m}Tc-PKC-c (RGDyK) biodistribution was also shown by a higher retention of radioactivity in the whole body, but with kidney accumulation over 8-fold higher than observed with ^{99m}Tc-PGC-c (RGDyK) at 2 h (12.62%ID·g^{-1} for PKC-RGD and 1.54%ID·g^{-1} for PGC-RGD, respectively). These results show that the biodistribution may be altered especially concerning lipophilicity resulting in renal rather than hepatobiliary excretion. This comparative study made it possible to explore the effects of lipophilicity on the biodistribution of ^{99m}Tc-labeled c (RGDyK) through the use of different tripeptide N_3S_1 chelators. Therefore, ^{99m}Tc-PKC-c (RGDyK) may be an attractive alternative for the *in vivo* imaging of integrin receptors.

Key words : ^{99m}Tc, N_3S_1, Cyclic RGD peptide, Integrin, Targeting

INTRODUCTION

Both N_3S_1 and N_2S_2 ligand systems have been used to radiolabel biologically active molecules, such as antibodies and peptides with ^{99m}Tc, and the characteristics of these labeling methods are well known (Pearson 1996; Haubner 2004; Cyr 2007; Liu 2008). In most cases, the chelator is conjugated to the biomolecules radiolabeled before or after conjugation. Some investigators have reported the synthesis of peptides which have a N_2S_2 or N_3S_1 chelating core incorporated during peptide synthesis (Liu 1999; Cyr 2007). This strategy would be more favorable for preparing the biologically active peptides containing an oxotechnetium coordination core using a single solid-phase synthesis route. However, as concerns the favorable pharmacokinetics of radiolabeled compounds, chelators need to be improved. The chelator moiety can have a profound influence on the biological properties or biodistribution, resulting from its lipophilicity and charge etc (Liu 1999; Decristoforo 2006).

The cyclic RGD is an antagonist for integrin which is potentially used for radiopharmaceuticals (Liu 2003; Chen 2004a; Temming 2005; Decristoforo 2006). Integrin is well known to overexpress in several cancer cells (Zitzmann 2002). This overexpression makes them attractive targets for tumor imaging with radionuclides. The typical approach

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used for receptor-mediated imaging is to make an appropriate BFCA that possesses a high binding affinity for the receptor of interest and desired biological behavior in vivo (Temming 2005; Liu 2008).

We recently described the synthesis and in vitro and in vivo evaluation of a novel tripeptide N_3S_1 chelator, Pro-Gly-Cys (PGC) and its RGD conjugate, PGC-c (RGDyK) radiolabeled with ^{99m}Tc (Lee 2010). The PGC used in this investigation was synthesized and incorporated with an integrin targeting RGD peptide as a test approach using a single solid phase synthesis route. However, this radiolabeled compound, ^{99m}Tc-PGC-c (RGDyK) had a modest tumor uptake and showed persistent radioactivity accumulation in the intestine. Therefore, for the achievement of favorable biological behavior in the biodistribution, the N_3S_1 chelating moiety needs to be improved.

There have been efforts to improve unfavorable biodistribution by modification of peptides or introduction of hydrophilic amino acid or the use of a variety of co-ligands. Cyr *et al*. designed a more hydrophilic peptide-derived chelator that was labeled with ^{99m}Tc via tripeptide N_3S_1 chelator and found clearly higher radioactivity in the kidney (Haubner 2004; Cyr 2007). Decristoforo *et al*. explored the use of different co-ligands for changing the lipophilicity in order to evaluate differences in the in vivo behavior of ^{99m}Tc-labeled peptides. An attempt to use an HYNIC-c (RGDfK) for labeling with different co-ligands resulted in favorable biokinetics with especially low intestinal excretion and low kidney retention (Decristoforo 1999, 2006).

The objective of the present study was to improve the biological behavior of chelator by changing its lipophilicity in which, while maintaining the simplicity of the peptide synthesis, a hydrophilic amino acid was to be incorporated in Pro-X-Cys backbone (PXC, X are designated for some hydrophilic amino acids). As a test of the approach, Pro-Lys-Cys (PKC) by substitution of L-glycine by L-lysine was synthesized for the preparation of PKC-c (RGDyK) (thus N_3S_1 chelator-RGD conjugate with L-lysine on the PXC backbone). After preparation of the synthesized chelators-RGD conjugates, the properties of the radiolabeling and a comparison of biodistribution for ^{99m}Tc-PGC-c (RGDyK) and ^{99m}Tc-PKC-c (RGDyK) were evaluated in vitro and in normal mice. We describe here the results of a comparison study of ^{99m}Tc-labeled RGD compounds in which two N_3S_1 chelators, PGC and PKC were incorporated, respectively.

EXPERIMENTAL

1. General

General experimental methods were followed as our previous report (Lee 2010). All the chemicals and reagents used in this study were analytically grade purchased from a commercial company and used without any further purification. Fmoc-protected amino acid, O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and N-Hydroxybenzotriazole (HOBt) were purchased from Anaspec (Frenont, CA, USA). The automated solid-phase synthesis was accomplished by the use of a Multiple Biomolecular Synthesizer (Peptron, Daejeon, Republic of Korea). The analytical and preparative RP-HPLC was performed on a SHIMAZU prominence HPLC by using a Shiseido capcell pak C-18 column. The wavelength used for UV detection was 220 nm for the analytical RP-HPLC. The LC/MS was performed by using the HP 1100 series. Radioactivity for the cell binding assay and biodistribution experiments was determined with a Wallac 1470 Wizard automated gamma counter (PerkinElmer Life Sciences). Sodium pertechnetate ($Na^{99m}TcO_4$) was obtained from a ^{99}Mo/^{99m}Tc generator (Samyoung Unitech, Daejeon, Republic of Korea) and radioactivity was measured by using an ionizing chamber (Atomlab 200, Bio-dex).

2. Synthesis of PKC-c (RGDyK) and PGC-c (RGDyK) derivatives

PKC-c (RGDyK) and PGC-c (RGDyK) are RGD peptide derivatives containing tripeptide chelator moieties consisting of Pro-Lys-Cyc (PKC) and Pro-Gly-Cyc (PGC), respectively. Synthetic peptides were prepared by solid phase synthesis as previously described (Lee 2010). Briefly, trityl resin conjugated Fmoc-Aspartic acid allylester was used as a solid support of the reaction. The linear sequence peptide was prepared by applying the consecutive method of coupling between amine and carboxylic acid. As the activating reagents to ensure efficient coupling, O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and N-hydroxybenzotriazole (HOBt) were applied. The Fmoc-protecting group was removed, followed by the sequential addition of Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(MTT)-OH, Fmoc-D-Tyr-OH. The Fmoc protecting groups were removed after every coupling step under standard conditions (20% piperidine in DMF). Cyclization of RGDyK was ac-

complished by the reaction between amine of tyrosine and carboxylic acid of aspartic acid by applying a conventional amide coupling method after deprotection of the allyl group of the aspartic acid using tetrakis (triphenylphosphine) palladium (0) and 1,3-dimethylbarbituric acid in dichloromethane (DCM). To introduce the N_3S_1 chelating moiety (PKC and PGC), the c (RGDyK) sequence was elongated by the sequential additions of Fmoc-Cys(Trt)-OH, Fmoc-Lys-OH for PKC (or Fmoc-Gly-OH for PGC) and Fmoc-Pro-OH in ε-amine of lysine. After the last assembly step, the resulting peptides, PKC-c (RGDyK) and PGC-c (RGDyK) were cleaved from the polymeric support by treatment with 90% trifluoroacetic acid (TFA) containing 2.5% triisopropylsilane (TIS), 2.5% ethanedithiol (EDT), 2.5% thioanisole, and 2.5% deionized water (TFA : TIS : EDT : Thioanisole : H_2O = 90 : 2.5 : 2.5 : 2.5 : 2.5). The crude products were purified by Shimadzu HPLC using capcell pak C18 column, a mobile phase with 0.1% TFA/water (A) and 0.1% TFA/acetonitrile (B), and gradient conditions with 0∼10% B in 2 min, 10∼40% B in 10 min and 40∼70% B in 2 min at a flow rate 1 ml · min^{-1}.

3. Radiolabeling with ^{99m}Tc

Radiolabeling of PKC-c (RGDyK) and PGC-c (RGDyK) with ^{99m}Tc was followed as our previous report (Lee 2010). PKC-c (RGDyK) and PGC-c (RGDyK) were dissolved in 1 ml of distilled water to give a concentration of 10^{-3} M (10^{-6} mol · ml^{-1}). The labeling of compounds with ^{99m}Tc was performed by transchelation with ^{99m}Tc-glucoheptonate (^{99m}Tc-GH) using a kit vial formulation. Briefly, the glucoheptonate (GH) kit vial containing 20 mg GH and 0.45 mg stannous chloride ($SnCl_2$) was dissolved with 400 μl of sodium ^{99m}Tc pertechnetate ($Na^{99m}TcO_4$) from a generator containing up to 370 MBq, and was allowed to stand for 10 min at room temperature for preparing the ^{99m}Tc-GH. The reaction solution was analyzed by ITLC on silica gel strips with saline or MEK as the mobile phase to check the amount of colloid formation. In the saline as a mobile phase, free $^{99m}TcO^{4-}$ and ^{99m}Tc-GH migrate at Rf 1.0, and colloid remains at Rf 0.0. In the MEK, free $^{99m}TcO^{4-}$ migrates at Rf 1.0, and ^{99m}Tc-GH and colloid remains at Rf 0.0, respectively. A 200 μl aliquot (185 MBq) of ^{99m}Tc-GH (radiochemical purity: >99%) from this vial was then added to various concentrations of the peptide solution (10^{-7} mol, 10^{-8} mol and 10^{-9} mol) in 300 μl of 50 mM sodium acetate buffer (pH 5.5) and the reaction was allowed to stand for 20 min at room temperature. The radiochemical purity (RCP) and the efficiency of the radiolabeling yield of compounds were determined by RP-HPLC on Waters HPLC system equipped with Waters 2695 pump, UV detector (Waters 2487), RI detector (In/US γ-detector system), and an X-terra C-18 column (5 μm, 4.6 × 250 mm) at a flow rate of 1.0 ml · min^{-1} with a gradient mobile phase. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B). The elution profile was; solvent (A) 100% for 4 min, 100% to 50% for 3 min, 50% to 30% for 5 min, 30% for 8 min and 30% to 100% for 2 min.

4. In vitro evaluation of radiolabeled compounds

4.1 Stability

The in vitro stability study was performed by incubation in an aqueous solution at 37°C for 24 h. Stability in the human serum was measured in parallel. After incubation, plasma samples were precipitated with methanol and centrifuged (13,000 rpm, 5 min). The supernatant was filtered and the aliquot was analyzed by RP-HPLC with γ-detector to determine the stability of this radiolabeled compound.

4.2 Log P (n-octanol/saline)

The log P value was determined by using a slight modification of the method described elsewhere (Decristoforo 2006). 100 μl of aliquot of the radiolabeled compound was added to 1 ml of octanol/water in an eppendorf tube. The solution was mixed for 5 min and centrifuged for 5 min. 100 μl aliquots of both the n-octanol phase and the saline phase, respectively were as transferred and measured the radioactivity using a Wallac 1470 Wizard automated gamma counter (PerkinElmer Life Sciences). The Log value of the partition coefficient P (n-octanol/saline) was calculated. The experiments were performed in triplicate.

4.3 Binding affinity

The affinity of PKC-c (RGDyK) and PGC-c (RGDyK) was determined using a competitive binding assay. ^{99m}Tc-PKC-c (RGDyK) was used as the radioligand in this assay. Briefly, $\alpha_v\beta_3$ positive U87MG cells were grown in 75 T-flask until 80∼90% confluence. Cells were harvested with trypsin and washed with a PBS buffer, and 10^6 cells were transferred to eppendorf tubes. After incubation with radiolabeled RGD (37 kBq) and appropriate dilutions of nonlabeled c (RGDyK), PKC-c (RGDyK) and PGC-c (RGDyK) in 1 ml of the binding buffer [cell binding buffer composition; 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride (Tris-HCl),

pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1% (w/v) BSA] at 37°C for 1 h, the incubation was interrupted by centrifugation followed by removal of the supernatant and rapid washing three times with a cell binding buffer. Thereafter, the cells were collected to measure the radioactivity with a γ-counter. The IC_{50} values of the RGD peptides were calculated by non-linear regression using GraphPad Prism software (GraphPad Prism 4.0 software, San Diego, CA, USA). Each point represents a result of the average of triplicate.

5. In vivo biodistribution studies

The biodistribution experiments for the radiolabeled RGD derivatives, ^{99m}Tc-PGC-c (RGDyK) and ^{99m}Tc-PKC-c (RGDyK) were performed with female normal mice (provided by OrientBio, Republic of Korea). The stock solution of the ^{99m}Tc-labeled RGD used in the animal studies was prepared for 2×10^{-8} mol · ml^{-1} with an activity of 370 MBq · ml^{-1}. Solutions for injection were prepared by the dilution of the stock solution to obtain a solution of 3.7 MBq · ml^{-1}. The mice were administered ^{99m}Tc-labeled peptides intravenously via the tail vein at an injection dose of 370 kBq/100 μl. At 30 min, 2 h, 4 h and 24 h, the animals (n = 5) were sacrificed. Blood and major organs (liver, intestine, kidney, spleen, heart, lung and stomach) were subsequently dissected and counted in a γ-counter. Percent injected dose (%ID) and percent injected dose per gram of organ (%ID · g^{-1} organ) were calculated from standard control activity with appropriate decay corrections. All groups consisted of four or five mice. All mean values are given as ± SD.

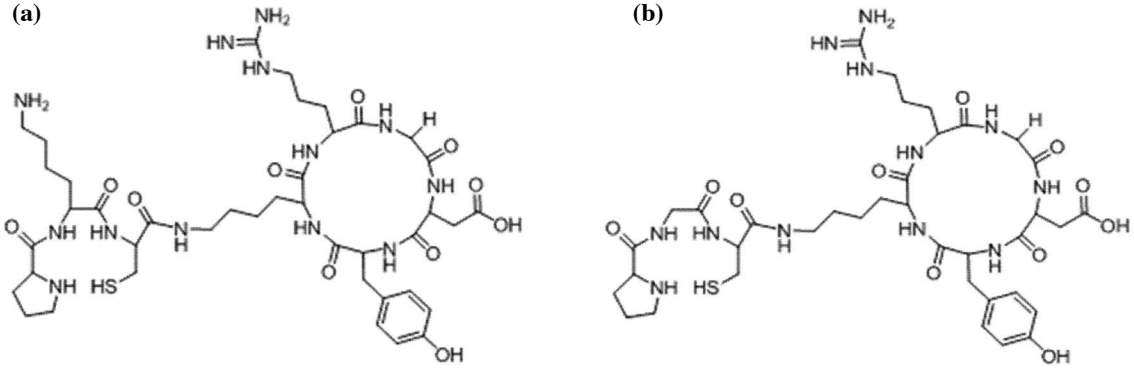
RESULTS

1. Preparation of PKC-c (RGDyK) and PGC-c (RGDyK)

Novel tripeptide N_3S_1 chelators, Pro-Lys-Cys (PKC) and Pro-Gly-Cys (PGC) were conjugated to an RGD peptide at the ε-amino group of the lysine in c (RGDyK) during peptide synthesis (Fig. 1). The final peptides were cleaved from the resin with simultaneous removal of all the protecting groups. The crude products were purified by a Shimadzu HPLC using a capcell pak C18 column in which PKC-c (RGDyK) and PGC-c (RGDyK) typically eluted at 6.0 min and 5.9 min, respectively. The purified products were analyzed by a LC/MS (HP 1100). A major ion peak for the LC/MS was found at M + 1 (*m/z*): 948.4 for PKC-c (RGDyK) and 877.4 for PGC-c (RGDyK), respectively.

2. Radiolabeling and characterization

All peptides were labeled at high specific activities (up to 37 GBq · μmol^{-1}). The labeling yield, stability and log P value for the ^{99m}Tc-PKC-c (RGDyK) and ^{99m}Tc-PGC-c (RGDyK) were evaluated using an RP-HPLC and gamma-counter. All the radiolabeled compounds revealed a high labeling yield (≥ 98%) at 10^{-8} mol. As shown in Fig. 2, ^{99m}Tc-PKC-c (RGDyK) and ^{99m}Tc-PGC-c (RGDyK) were identified at 10.02 min and 10.28 min, respectively. All ^{99m}Tc-labeled peptides showed high stability for up to 24 h. When the labeling compounds were incubated at 37°C for 24 h, above 90% of ^{99m}Tc remained chelated with PKC-c (RGDyK) and PGC-c (RGDyK). The Log P values of the ^{99m}Tc-PKC-c (RGDyK)



PKC-c (RGDyK) **PGC-c (RGDyK)**

Fig. 1. Structures of novel N_3S_1 chelator-conjugated RGD derivatives. (a) PKC-c (RGDyK), Pro-Lys-Cys-c (RGDyK). (b) PGC-c (RGDyK), Pro-Gly-Cys-c (RGDyK). The novel tripeptide N_3S_1 chelators comprised of amino acids, Pro-Lys-Cys (PKC) and Pro-Gly-Cys (PGC).

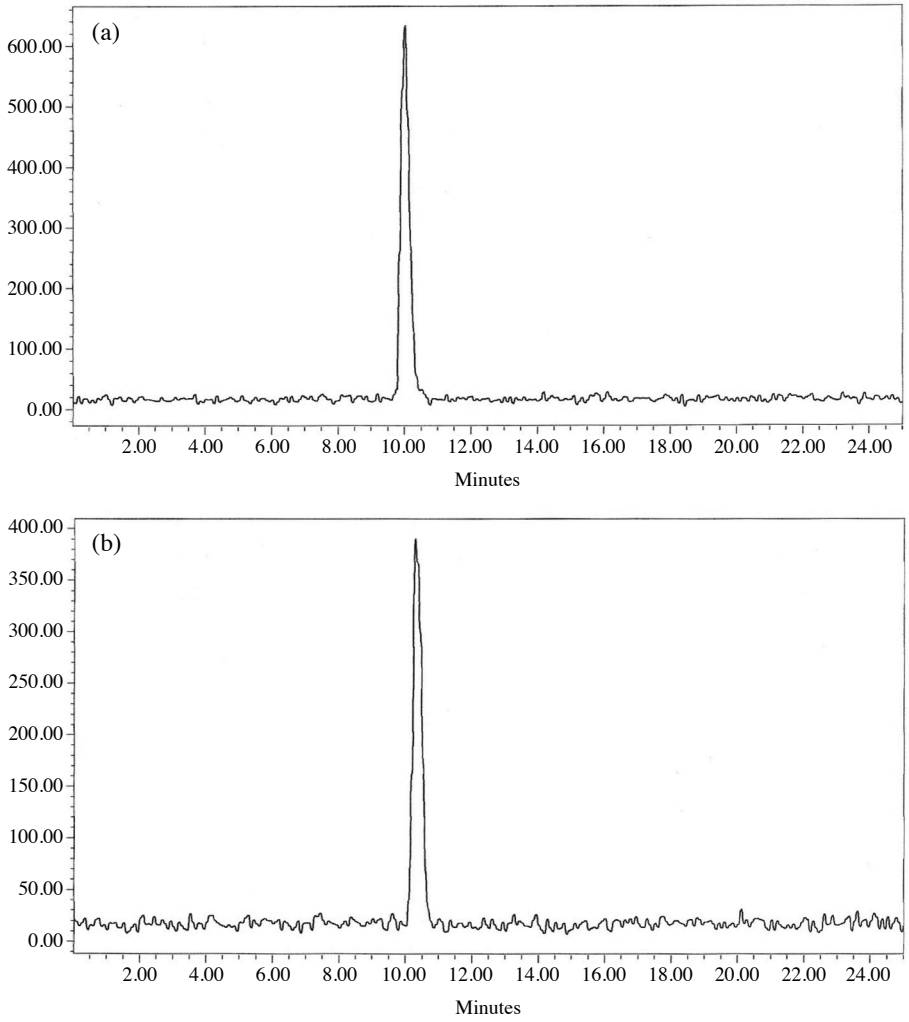


Fig. 2. γ-HPLC profile of ^{99m}Tc-labeled peptides. PGC and ^{99m}Tc-PGC-c (RGDyK). (a) ^{99m}Tc-PKC-c (RGDyK) (10.02 min), (b) ^{99m}Tc-PGC-c (RGDyK) (10.28 min).

Table 1. Characteristics for two ^{99m}Tc-labeled RGD peptides

Compound	T_R (min)[a]	Log P[b]	IC_{50} (nM)[c]
^{99m}Tc-PKC-c (RGDyK)	10.02	−3.48	4.98
^{99m}Tc-PGC-c (RGDyK)	10.28	−2.90	5.93

a: Retention time in γ-HPLC
b: The Log value of the partition coefficient P (n-octanol/saline)
c: Concentration of 50% binding inhibition of the RGD peptides

and ^{99m}Tc-PGC-c (RGDyK) were −3.48 and −2.90, respectively. These data are summarized in Table 1.

3. Binding affinity

The affinity of PKC-c (RGDyK), PGC-c (RGDyK) and c (RGDyK) for the $\alpha_v\beta_3$ positive U87MG cells was determined in competitive binding assays. The binding of the ^{99m}Tc-PKC-c (RGDyK) to the U87MG cells was competed by non-labeled c (RGDyK), PKC-c (RGDyK) and PGC-c (RGDyK) in a concentration-dependent manner. The IC_{50} values were 2.26 nM for c (RGDyK), 4.98 nM for PKC-c (RGDyK) and 5.93 nM for PGC-c (RGDyK), respectively.

4. In vivo biodistribution studies with normal mice

The biodistribution behaviors for the ^{99m}Tc-PKC-c (RGDyK) and ^{99m}Tc-PGC-c (RGDyK) were measured at 30 min, 2 h, 4 h and 24 h after administration. The results of the biodistribution studies in normal mice are summarized in Table 2 and Fig. 3. Compared to the results of the biodistribution for the ^{99m}Tc-PGC-c (RGDyK), significant changes in pharmacokinetic behavior for the ^{99m}Tc-PKC-c (RGDyK) were found, resulting in a decrease of dominant hepatobiliar excretion. Biodistribution results in normal mice clearly

Table 2. Biodistribution of ^{99m}Tc-PKC-c (RGDyK) and ^{99m}Tc-PGC-c (RGDyK) in normal mice (n = 5) at 30 min, 2 hr, 4 hr and 24 hr postinjection, respectively. Data are expressed as %ID · g^{-1} organ. The mice were administered ^{99m}Tc-labeled peptides intravenously via the tail vein injection dose of 370 kBq/100 µl

Organ	30 min		2 hr		4 hr		24 hr	
	[a]PKC	[b]PGC	PKC	PGC	PKC	PGC	PKC	PGC
Blood	0.94 ± 0.49	0.22 ± 0.03	0.22 ± 0.04	0.11 ± 0.01	0.12 ± 0.03	0.07 ± 0.02	0.04 ± 0.01	0.03 ± 0.01
Liver	5.63 ± 0.49	0.93 ± 0.24	3.36 ± 0.82	0.48 ± 0.17	1.37 ± 0.25	0.26 ± 0.09	0.35 ± 0.05	0.12 ± 0.01
Kidney	14.06 ± 2.27	2.36 ± 0.40	12.62 ± 1.32	1.54 ± 0.20	8.02 ± 0.93	1.17 ± 0.25	2.27 ± 0.15	0.46 ± 0.10
Spleen	2.25 ± 0.19	0.35 ± 0.07	1.81 ± 0.23	0.21 ± 0.06	1.24 ± 0.18	0.15 ± 0.04	0.38 ± 0.03	0.06 ± 0.02
Heart	1.24 ± 0.23	0.28 ± 0.08	0.86 ± 0.04	0.13 ± 0.03	0.56 ± 0.05	0.10 ± 0.03	0.13 ± 0.02	0.03 ± 0.00
Sm Int	10.83 ± 2.18	15.29 ± 5.05	4.00 ± 1.77	3.00 ± 1.67	1.70 ± 0.17	0.41 ± 0.10	0.34 ± 0.05	0.11 ± 0.02
Lg Int	9.48 ± 2.40	76.98 ± 35.92	19.71 ± 3.77	88.16 ± 6.49	7.34 ± 2.81	14.93 ± 7.93	0.46 ± 0.06	0.22 ± 0.07
Lung	2.72 ± 0.50	0.65 ± 0.14	1.75 ± 0.19	0.34 ± 0.10	1.07 ± 0.16	0.27 ± 0.10	0.34 ± 0.06	0.11 ± 0.03
Stomach	3.47 ± 1.78	1.32 ± 0.42	2.42 ± 0.36	0.61 ± 0.13	1.39 ± 0.30	0.34 ± 0.22	0.38 ± 0.11	0.09 ± 0.05
[c]GI tract	6.40 ± 1.47	26.69 ± 11.39	7.31 ± 1.51	19.65 ± 6.31	2.94 ± 0.79	3.66 ± 2.21	0.38 ± 0.05	0.14 ± 0.04

a, ^{99m}Tc-PKC-c (RGDyK); b, ^{99m}Tc-PGC-c (RGDyK); c, GI tract (Stomach, Liver and Intestine)

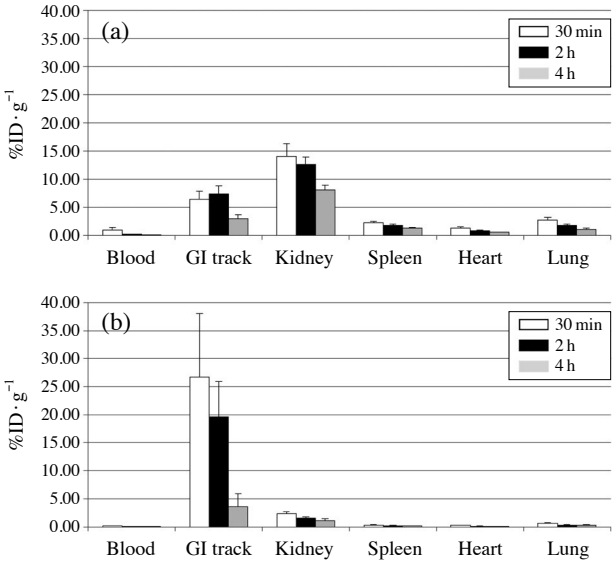


Fig. 3. Biodistribution of (a) ^{99m}Tc-PKC-c (RGDyK) and (b) ^{99m}Tc-PGC-c (RGDyK) at 30 min, 2 h and 4 h after administration to normal mice (n = 5). The mice were administered ^{99m}Tc-labeled peptides intravenously via the tail vein injection dose of 370 kBq/100 µl.

showed a significant decrease in intestinal accumulation at 2 h postinjection for the ^{99m}Tc-PKC-c (RGDyK) compared to the ^{99m}Tc-PGC-c (RGDyK) (from 19.65%ID · g^{-1} for PGC-c (RGDyK) to 7.31%ID · g^{-1} for PKC-c (RGDyK) in the GI tract). On the contrary, a significant increase in renal excretion at 2 h for the ^{99m}Tc-PKC-c (RGDyK) was found (12.62 %ID · g^{-1} for PKC-c (RGDyK); 1.54%ID · g^{-1} for PGC-c (RGDyK) in the kidney at 2 h). ^{99m}Tc-PGC-(cRGDyK) was rapidly cleared from the blood and the remaining major organs (mostly below 1%ID · g^{-1} at 2 h), but uptake in the GI tract revealed high values: 26.69%ID · g^{-1} at 30 min and 19.65%ID · g^{-1} at 2 h. As shown in Table 2, clearance of radioactivity in the blood and the remaining organs was more rapid for the ^{99m}Tc-PGC-c (RGDyK) compared to that of the ^{99m}Tc-PKC-c (RGDyK). The ^{99m}Tc-PKC-c (RGDyK) showed substantially higher radioactivity in the remaining organs than that for the ^{99m}Tc-PGC-c (RGDyK) indicating more significant retention in the blood without major hepatobiliary excretion (Table 2).

DISCUSSION

Many investigators have been interested in the influence of chelators on pharmacokinetics (Vanderheyden 2006; Cyr 2007; Decristoforo 2007). These studies show that the choice of chelator is an important factor in the in vitro and in vivo properties of radiolabeled compounds. These differences might result from charge, lipophilicity and conformational effect, which may interfere with the biological affinity or result in unfavorable biological behavior. There have been several strategies to change the behavior of ^{99m}Tc-labeled compounds by incorporating various chelators or additional modification with glucosamic moiety or PEGylation (Haubner 2001; Chen 2004b; Cyr 2007). It has been reported that modification through replacement of amino acid by some hydrophilic residue is possible to tune the biological behavior of ^{99m}Tc-labeled compounds (Haubner 2004; Cyr 2007).

Previously, a novel tripeptide N_3S_1 chelator, PGC and its RGD conjugate for ^{99m}Tc were designed and evaluated both in vitro and in vivo (Lee 2010). In this study, the ^{99m}Tc-PGC-c (RGDyK) showed predominant intestinal uptake compared

to other organs. Actually, N_xS_{4-x} chelators often have high lipophilicity resulting in high hepatobiliary excretion of the radiolabeled compound (Liu 1999). The ^{99m}Tc-labeled PGC-ethylamide (backbone for chelating core structure of PGC-c (RGDyK)) had higher lipophilicity (Log P: -0.35) compared to -2.90 for the ^{99m}Tc-PGC-c (RGDyK). This leads to high liver and intestinal uptake. To be sure, a lower lipophilicity of the radiolabeled compound results in less accumulation for hepatobiliary excretion (80.00 ± 8.87 for ^{99m}Tc-PGC-ethylamide; $22.16 \pm 5.66\%ID \cdot g^{-1}$ for ^{99m}Tc-PGC-c (RGDyK) at 2 h) (Lee *et al*. 2010). Haubner (2004) and Decristoforo (2006) have recently reported that an N_3S_1 type ligand, DKCK and EDDA/HYNIC for labeling a cyclic RGD showed higher hydrophilicity (Log P: < -3.5 for EDDA/HYNIC) compared to -3.2 of [18F]Galacto-RGD. The ^{99m}Tc labelled EDDA/HYNIC-RGD showed low intestinal excretion and low kidney retention. The ^{99m}Tc-DKCK-RGD showed predominant renal elimination of the tracer. From this result, a novel tripeptide N_3S_1 chelator (PXC) derivative, PKC-c (RGDyK) was designed and evaluated for the achievement of favorable biokinetics in the biodistribution.

In this study, conjugates of c (RGDyK) with novel tripeptide N_3S_1 chelators, PXC comprising of Pro-X-Cys (PKC and PGC containing, respectively, L-lysine and L-glycine in the X position of PXC) were prepared and labeled with ^{99m}Tc to compare the influence of the bifunctional chelate on the biological behavior of ^{99m}Tc-labeled c (RGDyK). Two novel tripeptide N_3S_1 chelators, PKC and PGC were covalently coupled with c (RGDyK) at the ε-amino group of the lysine during solid-phase peptide synthesis, respectively. We expected minor alterations in biological affinity of the c (RGDyK) since affinity studies of modified c (RGDyK) indicate that conjugation at the ε-amino group of the lysine of the c (RGDyK) have minimal effects on receptor binding and biological activity (Haubner 2004). Since hydrophilic L-lysine substitution in the L-glycine position of PGC contribute to decrease the lipophilicity of N_3S_1 chelating core, it is expected to increase the extent of renal excretion (Verbeke 2000; Haubner 2004; Cyr 2007).

Labeling of the c (RGDyK) derivatives was performed using the same conditions and it is possible to radiolabel with high specific activities (about 20 $GBq \cdot \mu mol^{-1}$). The influence of chelator derivatives and subsequent labeling of the peptide-conjugates on the affinity for receptor was assessed using an in vitro binding affinity assay as described above. In literature, IC_{50} values are usually calculated from displacement assays of ^{125}I-echistatin using nonlabeled peptide derivatives. However, we preferred to investigate the binding study as described by previous report (Dijkgraaf 2007). As a consequence, a high binding affinity was determined for PKC-c (RGDyK) with an IC_{50} value of 4.98 nM and for PGC-c (RGDyK) with an IC_{50} value of 5.93 nM compared to c (RGDyK) with 2.26 nM in the same assay. The values obtained in this study show that the binding affinity is not affected by chelator derivatives and labeling. In vitro cell uptake values were significantly decreased when cells were blocked with excess cold c (RGDyK).

The in vitro stability of the radiolabeled compounds was sufficient for further investigation such as in vitro and in vivo evaluation. When the labeling compounds were incubated in aqueous solution at 37°C for 24 h, above 90% of the ^{99m}Tc remained chelated with PKC-c (RGDyK) and PGC-c (RGDyK).

The biodistribution of the ^{99m}Tc-labeled derivatives, ^{99m}Tc-PKC-c (RGDyK) and ^{99m}Tc-PGC-c (RGDyK) was evaluated in normal mice to establish the influence of hydrophilic L-lysine substitution in the N_3S_1 chelator core on the extent of renal excretion (Table 2 and Fig. 3). The biodistribution studies in normal mice clearly demonstrated the influence of the lipophilicity of the N_3S_1 chelating core on the biological behavior of the ^{99m}Tc-labeled RGD compound. Substitution of L-glycine by L-lysine for PKC-c (RGDyK) results in a clearly increased renal excretion and decrease in the intestinal uptake of the ^{99m}Tc-labeled compound ($7.31\%ID \cdot g^{-1}$ for GI track and $12.62\%ID \cdot g^{-1}$ for kidney in normal mice at 2 h). This result might be explained by the fact that the decreased lipophilicity of PKC-c (RGDyK) (Log P: -3.48) leads to renal excretion compared to the hepatobiliary excretion of PGC-c (RGDyK) (Log P: -2.90). This result showed that conventional solid-phase preparation of tripeptide chelator is a powerful strategy for improving pharmacokinetics.

Several reports have appeared comparing properties such as labeling efficiency, stability, characterization and biodistribution of ^{99m}Tc labeled peptides on the influence of chelator (Haubner 2004; Vanderheyden 2006; Cyr 2007; Decristoforo 2007). In the comparison, the biodistribution may depend upon the choice of chelator, and it may be possible to be optimized especially concerning lipophilicity resulting in renal rather than hepatobiliary excretion. A recent comparison study was also reported, in which systematic changes in the N_3S_1 chelator amino sequence during peptide synthesis may contribute to achieve favorable biodistribution, such as decreas-

ed liver and GI uptake and increased renal excretion (Cyr 2007). Therefore, these comparative studies underline that peptide labeling strategies using ^{99m}Tc must be selected and optimized by changes in the chelator region.

The influence of the chelator on biodistribution was examined. The results of this study make it possible to compare the biological behavior of radiolabeled compounds, and provide certain information for the improvement of the chelator. Furthermore, it is also demonstrated that lipophilicity and biodistribution for the radiolabeled compound can be tuned, implying application of this novel tripeptide chelator system for radiopharmaceutical design. PKC-c (RGDyK) conjugate will be radiolabeled with ^{99m}Tc, and evaluated in vitro and in vivo with an integrin positive tumor model for further investigations.

CONCLUSION

The results of this study have provided that the c (RGDyK) peptide for integrin receptor can be labeled with ^{99m}Tc using an tripeptide N_3S_1 chelating system with highest labeling yield. Hydrophilic lysine substitution in X position of PXC resulted in similar integrin receptor specificity, reduced intestinal accumulation, and increased renal excretion. The property of the N_3S_1 chelator clearly influences the biodistribution of the ^{99m}Tc-labeled compound. By decreasing the lipophilicity of the conjugate, fast blood clearance and the elimination of the ^{99m}Tc-labeled through the hepatobiliary route decreased, and a high radioactivity was excreted through the renal system. Further experiments are needed to evaluate the potential of this novel chelating system for tumor imaging and the consequences of modification in the biodistribution.

Abbreviation

Fmoc: 9-Fluorenylmethoxycarbonyl
Allyl: Allyl
Pbf: 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
Mtt: 4-Methyltrityl
Trt: Trityl
HBTU: O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate
HOBt: N-Hydroxybenzotriazole
DIPEA: Diisopropylethylamine
DMF: Dimethylformamide
DCM: Dichloromethane
DIC: 1,3-Diisopropylcarbodiimide
TFA: Trifluoroacetic acid
TIS: Triisopropylsilane
EDT: Ethanedithiol

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