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In Vitro and In Vivo Evaluation of a Novel $^{99m}Tc(CO)_3$-Pyrazolyl Conjugate of *cyclo*-(Arg-Gly-Asp-D-Tyr-Lys)

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Radiolabeled peptides containing the Arg-Gly-Asp amino acid sequence (single letter code = RGD) have been studied extensively to target integrin receptors upregulated on tumor cells and neovasculature. Integrins are cell surface transmembrane glycoproteins that exist as $\alpha\beta$ heterodimers. The $\alpha_v\beta_3$ integrin is known to be overexpressed in many tumor types and is expressed at lower levels in normal tissues. Furthermore, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subtypes are expressed in neovasculature during angiogenesis. Thus, there is some impetus to image angiogenesis and tumor formation in vivo using RGD-based peptide targeting vectors. In this study, we report the design and development of a new cyclic RGD analogue *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] (PZ = 3,5-Me_2-pz$(CH_2)_2$N$((CH_2)_3$COOH)-$(CH_2)_2NH_2$) that can be radiolabeled with the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ metal aquaion. Radiochemical evaluation of this new conjugate in vitro indicated a facile radiosynthesis of the new ^{99m}Tc-RGD conjugate with high radiolabeling yields (≥95%) and high specific activities. In vitro internalization and blocking assays in $\alpha_v\beta_3$ receptor-positive, human M21 melanoma cancer cells showed the ability of this conjugate to target the integrin receptor with high specificity and selectivity. In vivo pharmacokinetic studies in normal CF-1 mice showed rapid clearance from blood with excretion primarily via/through the renal−urinary system. In vivo accumulation of radioactivity in mice bearing either $\alpha_v\beta_3$ receptor-positive or negative human melanoma tumors showed receptor specific uptake of tracer with accumulations of 2.50 ± 0.29 and 0.71 ± 0.08% ID/g in $\alpha_v\beta_3$ integrin positive (M21) and negative (M21L) tumors at 1 h postinjection (p.i.), respectively.

INTRODUCTION

Radiolabeled peptides containing the RGD amino acid sequence have been studied extensively to develop site-directed targeting vectors for integrin receptors upregulated on tumor cells and neovasculature (*1*, *2*). The RGD consensus sequence, Arg-Gly-Asp, appears in several proteins of the extracellular matrix, including vitronectin, fibronectin, fibrinogen, von Willebrand factor, thrombospondin, and osteopontin (*3*). Integrin recognition of the canonical RGD sequence plays a prominent role in many cell−cell and cell−extracellular matrix (ECM) interactions. Integrins are cell surface transmembrane glycoproteins that exist as $\alpha\beta$ heterodimers, and at least 24 different combinations of $\alpha\beta$ heterodimers are known (*4*). The integrins of most interest in cancer imaging and therapy contain the α_v subunit, particularly the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subtypes. The $\alpha_v\beta_3$ integrin is known to be overexpressed in many tumor types and expressed at lower levels in normal tissues (*5*). Both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subtypes are expressed in neovasculature during angiogenesis (*5*). Therefore, the impetus to image/treat angiogenesis and tumor formation in vivo using RGD-based peptide targeting vectors is of significant interest.

RGD peptide conjugates used to target integrin receptors are generally linear, disulfide-cyclized, or head-to-tail cyclized constructs (*6*−*18*). However, the most extensive class of RGD analogues being studied utilizes the head-to-tail cyclized RGD derivatives. These analogues represent the most promising class of RGD-based imaging agents (*2*). Early investigations into the effect of head-to-tail cyclization on RGD affinity for the $\alpha_v\beta_3$ integrin led to the identification of *cyclo*-(RGDfV) (*19*), an $\alpha_v\beta_3$ antagonist with a low nanomolar IC_{50}. Further characterization led to the observation that a bulky hydrophobic residue was required in position 4 for maximum affinity, while position 5 was tolerant of a range of substitutions (*12*). For example, the insertion of either a D- or L-tyrosine into position 4 or 5 yielded RGD peptide conjugates with available sites for radioiodination. The resulting ^{125}I-labeled peptides were tested in vitro and in vivo to examine the feasibility of using cyclic RGD peptides as in vivo imaging agents (*12*). These conjugates displayed rapid

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blood clearance through hepatobiliary excretion and tumor uptake at 1 h p.i. (post intravenous administration) of 1.30 ± 0.13 %ID/g in M21 melanoma human tumor xenografts (*12*). Further attempts to improve the pharmacokinetics of these RGD constructs of this type resulted in the synthesis and in vitro/in vivo evaluation of a glycosylated analogue via the attachment of a carbohydrate moiety to the ϵ amino group of Lys5 (*13*). The resulting molecule showed decreased uptake in liver and intestine and increased tumor uptake and residualization, with 2.05 ± 0.55 %ID/g in M21 melanoma human tumor xenografts. Similar results have been reported using an ^{18}F-labeled galacto-RGD analogue, an agent that has already been evaluated in human patients (*14*, *15*).

New and exciting methods of attaching radiometals to specific biomolecular targeting vectors such as RGD continue to be of significant interest. $\alpha_v\beta_3$-Targeted cyclic pentapeptides include a lysine residue in position 5 for the purpose of appending a diverse array of radiolabels. For example, PET tracers have been synthesized by appending a PEG-[^{18}F]fluorobenzoate domain (*16*). Furthermore, metal chelators such as DTPA and DOTA have also been conjugated to RGD via a Lys5 residue to coordinate a wide range of radionuclides useful for imaging and therapy (*17*, *18*). The availability of Isolink for kit preparation of the *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ synthon (Isolink, Tyco Healthcare, St. Louis, MO) offers a new and exciting approach toward production of ^{99m}Tc-based RGD conjugates. Alberto and co-workers have well-established the macroscopic and tracer level chemistry of Tc(I) and Re(I) tricarbonyl complexes containing the *fac*-[M(CO)$_3$]$^+$ moiety (*20*−*27*). They have demonstrated the effectiveness of using ligand frameworks with a host of donor atoms (i.e., P, S, and N) to effectively stabilize the metal center to produce in vivo stable and kinetically inert complexes (*28*−*34*). Recently, Alves et al. have reported the design of a tridentate metal chelator that effectively coordinates the *fac*-[M(CO)$_3$]$^+$ metal center (M = Tc or Re) (*35*−*39*). This new ligand framework, 3,5-Me$_2$-pz(CH$_2$)$_2$N((CH$_2$)$_3$COOH)(CH$_2$)$_2$NH$_2$ (PZ), has been shown to produce in vitro and in vivo stable complexes of ^{99m}Tc. Furthermore, ^{99m}Tc-bombesin conjugates of 3,5-Me$_2$-pz(CH$_2$)$_2$N((CH$_2$)$_3$COOH)(CH$_2$)$_2$NH$_2$ demonstrated high selectivity and affinity for the GRP receptor, subtype 2 (*39*). As part of an International Atomic Energy Agency Co-ordinated Research Project, "Development of ^{99m}Tc-based Small Biomolecules Using Novel ^{99m}Tc Cores", there is some interest in conjugation of new ligand frameworks to Lys5 of cyclic RGD for the design and development of a novel, cyclized ^{99m}Tc-RGD analogue. The participating countries herein report the design and development of a new cyclic RGD analogue, *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] (PZ = 3,5Me$_2$-pz(CH$_2$)$_2$N((CH2)$_3$COOH)(CH$_2$)$_2$NH$_2$), that can be radiolabeled with the *fac*-M(CO)$_3$ metal center (M = Tc or Re). In this study, we report the synthesis and radiolabeling of *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)], in vitro internalization, and blocking assays of this conjugate in $\alpha_v\beta_3$ receptor-positive, human M21 melanoma cancer cells and in vivo pharmacokinetic studies of this conjugate in normal CF-1 mice. Furthermore, in vivo evaluation of this conjugate in mice bearing either $\alpha_v\beta_3$ receptor-positive or negative human M21 melanoma tumors will be discussed.

EXPERIMENTAL PROCEDURES

Materials. *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] was synthesized by Biosynthan (Gesellschaft fur Bioorganische Synthese mbH, Berlin, Germany). 3,5Me$_2$-pz(CH$_2$)$_2$N((CH2)$_3$COOH)(CH$_2$)$_2$NH−BOC was synthesized as previously reported (*38*). All other reagents were purchased from either Fisher Scientific (Chicago, IL) or Sigma-Aldrich (St. Louis, MO) and used without further purification. ^{125}I-Echistatin was purchased from Amersham-Pharmacia Biotech (Vienna, Austria). $\alpha_v\beta_3$ integrin receptors were purchased from Chemicon (Temecula, CA). ^{99m}Tc, in the form of [^{99m}TcO$_4$]$^-$, was eluted from commercially available ^{99}Mo/^{99m}Tc generator systems. Isolink radiolabeling kits were provided by Tyco Healthcare International (Petten, The Netherlands).

Methods. *1. Peptide Synthesis. cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ-BOC)] was synthesized by the reaction of 3,5Me$_2$-pz-(CH$_2$)$_2$N((CH2)$_3$COOH)(CH$_2$)$_2$NH−BOC, (2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate (TNTU), and diisopropylethylamine (DIEA) (1:1.2:1.2) in *N*,*N*,-dimethyl formamide (DMF) for 1 h. Two equivalents of the corresponding HONB-ester (hydroxy-5-norbornene-2,3-dicarboxylimide) solution was added to 1 equiv of the unprotected cyclopeptide, *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys], in DMF. The pH of the solution was adjusted to 8 by addition of *N*-methylmorpholine (NMM), and the reaction mixture was allowed to stir at room temperature for 48 h.

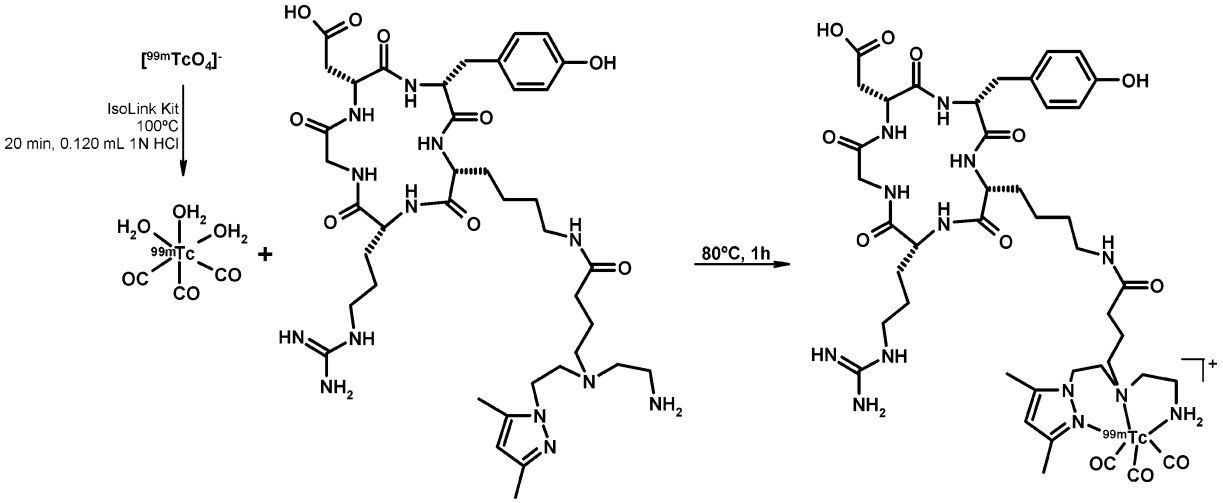
The crude, BOC-protected peptide conjugate was purified by preparative reversed phase-high performance liquid chromatography (RP-HPLC) utilizing a Shimadzu LC-8A system equipped with a SPD-6A tunable absorbance detector calibrated to 220 nm. HPLC solvents consisted of H$_2$O containing 0.05% trifluoroacetic acid (TFA, solvent A) and acetonitrile/water (80:20 containing 0.05% trifluoracetic acid (solvent B). An Ultrasep C-18 column (10.0 μm, 100 Å, 20 × 250 mm, SEPSERV GmbH, Berlin) was used with a flow rate of 15.0 mL/min. The HPLC gradient system began with a solvent composition of 95%A and 5%B and followed a linear gradient of 2.5%B/min. MALDI-MS of the BOC protected cyclic peptide was performed confirming the chemical constitution of the purified conjugate (calculated mass: 970.2; experimental mass: 970.4).

The cyclic RGD analogue, *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys-(PZ)], was obtained by reaction of *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ-BOC)] with TFA/water (95:2) for 1 h. Purification by preparative HPLC was performed, and the final purified analogue was analyzed by MALDI-MS (calculated mass: 870.2; experimental mass: 870.1).

2. Radiolabeling of cyclo-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] Conjugate. To 0.2 mL of 2.5 × 10^{-4} M aqueous solution of *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] was added 0.8 mL of [^{99m}Tc-(H$_2$O)$_3$(CO)$_3$]$^+$ prepared via the Isolink kit (Scheme 1). The solutions were allowed to incubate at 75 °C for 1 h. Quality control (radiochemical yield and radiochemical purity) of the products was determined by RP-HPLC. Analysis of radiolabeled and non-radiolabeled compounds was performed on a Waters 600S controller equipped with a Waters 626 pump, Waters 2487 dual wavelength absorbance detector, an Eppendorf CH-30 column heater, an in-line EG&G ORTEC NaI solid scintillation detector, and a Hewlett-Packard 3395 integrator. HPLC analysis of the new radiolabeled RGD conjugate was performed using an analytical C-18 reversed phase column (Phenomenex, 50 × 4.6 mm, 5 μm). The mobile phase consisted of a linear gradient system, with solvent A corresponding to 100% water with 0.1% trifluoroacetic acid and solvent B corresponding to 100% acetonitrile with 0.1% trifluoroacetic acid. The mobile phase began with solvent compositions of 95%A/5%B. At time = 25 min, the solvent compositions were 30%A/70%B. Solvent compositions of the mobile phase remained at 30%A/70%B for 1 min before being changed to 20%A/80%B. At time = 28 min, the solvent composition was changed to 95%A/5%B for column re-equilibration. The flow rate of the mobile phase was 1.5 mL/min. Product purification and final preparation of the labeled species were performed by collecting the samples off the chromatographic system, removal of solvent via a nitrogen stream, and reconstitution in normal saline.

3. In Vitro Stability in Human Blood. To 1 mL of whole human blood, collected in heparinized polypropylene tubes, was

Scheme 1. Radiosynthesis of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$



added a solution of the [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate (∼9.25 MBq). This mixture was incubated at 37 °C. Samples were taken at 5 min, 45 min, and 4 h postincubation, and centrifuged for 15 min at 2000 rpm at 4 °C. The plasma was separated and ethanol was added in a 2:1 (v/v) ratio. The samples were centrifuged at 3000 rpm (15 min, 4 °C), filtered through a Millex GV filter (0.22 μm), and the supernatant was analyzed by RP-HPLC. HPLC analyses of the ^{99m}Tc-conjugates were performed on a Shimadzu C-R4A chromatography system equipped with a Berthold - LB 505 gamma detector and a tunable absorption UV detector. Separations were achieved on an analytical C-18 reversed phase column (Nucleosil, 250 × 4.0 mm, 10 μm, 100 Å). The mobile phase consisted of a linear gradient system, with solvent A corresponding to 0.1% trifluoroacetic acid in water and solvent B corresponding to 0.1% trifluoroacetic acid in acetonitrile. The mobile phase (linear gradient) began with solvent compositions of 100%A/0%B. At time = 9 min, the solvent compositions were 75%A/25%B. Solvent compositions were changed linearly to 0%A/100%B at time = 20 min. At time = 22 min, the solvent composition was changed to 100%A/0%B for column re-equilibration. The detection wavelength was 220 nm, and the flow rate was 1 mL/min.

4. Binding Affinity of cyclo-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] to $\alpha_v\beta_3$ Integrin Receptors. The in vitro binding affinity of the pyrazolyl−RGD conjugate was determined and compared directly to *cyclo*-[Arg-Gly-Asp-D-Phe-Val] using ^{125}I-echistatin as the radioligand as described by Orlando and Cheresh (*40*). Briefly, 96-well plates were coated with $\alpha_v\beta_3$ integrin receptors (Chemicon, Temecula, CA) and incubated in the presence of varying amounts of competing ligand (0.05 nM to 50 μM RGD peptide) for 3 h with 370 Bq/well (0.05 nM) ^{125}I-Echistatin (Amersham Biosciences, Piscataway, NJ). After incubation, wells were washed three times with 25 mM Tris-HCl containing 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4, and bound ^{125}I-echistatin was solubilized with hot 2 N NaOH. Radioactivity in the resulting suspension was measured by a γ-counter (Wallac Wizard). IC_{50} values were calculated by fitting the percent inhibition values using ORIGIN software (Northhampton, MA).

5. In Vitro Internalization Analysis of [^{99m}Tc(CO)$_3$−cyclo-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in $\alpha_v\beta_3$ Positive M21 Melanoma Cells. $\alpha_v\beta_3$ positive M21 or $\alpha_v\beta_3$ negative M21L human melanoma cells (*41*) were grown in culture until a sufficient number of cells were available. For internalization experiments, cells were determined to be at a concentration of 2×10^6 cells/mL in RPMI 1640 containing 1% glutamine and 1% BSA. One milliliter of this solution was pipetted into each separate tube for internalization assays. [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate (≥100000 cpm, ∼1 nM) was added to the cellular media, and the solutions were incubated at 37 °C for 90 min (n = 3) in presence of either PBS/0.5%BSA buffer (150 μL; regular assay) or 10 μM *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys] (RGDyK) in PBS/0.5%BSA buffer (150 μL; nonspecific binding assay). Following the 90 min incubation period, the solutions were centrifuged, media was removed, and cells rinsed with cold TRIS buffered saline (×2). Thereafter, the cells were twice incubated at ambient temperature in 50 mM acetate buffer (pH = 4.2) for 15 min, a period sufficient to remove membrane bound radioligand. The supernatant was collected (membrane bound radioligand fraction), and the cells were washed with 50 mM acetate buffer. Cells were lysed by treatment in 1 N NaOH, and cell-associated radioactivity was collected (internalized radioligand fraction). Protein content in the NaOH fraction was determined using spectrophotometric techniques according to the Bradford method (*42*). The internalized and non-internalized fractions were determined by measuring radioactivity, and the internalized fraction was expressed as a percent of total activity per milligram of protein.

6. In Vivo Evaluation of [^{99m}Tc(CO)$_3$−cyclo-[Arg-Gly-Asp-D-Tyr-Lys(L)]] in Normal Mouse Models. All animal studies were conducted in accordance with the highest standards of care as outlined in the NIH guide for Care and Use of Laboratory Animals and the Policy and Procedures for Animal Research at the Harry S. Truman Memorial Veterans' Hospital. The animals were fed autoclaved rodent chow (Ralston Purina Company, St. Louis, MO) and water ad libitum. The biodistribution studies of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys-(PZ)]]$^+$ conjugate were determined in normal, CF-1 mice (female, 4−5 weeks old, ∼20 g). The mice were injected with 185 kBq (∼1×10^{-5} μg, specific activity ∼ 5.2×10^8Ci/mol) of HPLC-purified conjugate in 50 μL of isotonic saline via the tail vein. The mice were euthanized by cervical dislocation, and the tissues and organs were excised from the animals following at 1, 4, and 24 h p.i. Subsequently, the tissues and organs were weighed and counted in a NaI well counter, and the percent injected dose (%ID) and %ID/g of each organ or tissue was calculated. The %ID in whole blood was estimated assuming a whole-blood volume of 6.5% the total body weight.

In vivo stability of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate was determined at 1 h p.i. by assessing aliquots of urine, murine serum, and liver homogenate via RP-HPLC analysis using a method previously described herein (vide infra, in vitro serum stability). Urine: The urine was collected at the time of sacrifice and filtered through a Millex GV filter (0.22 μm), after which RP-HPLC was used to assess the degree

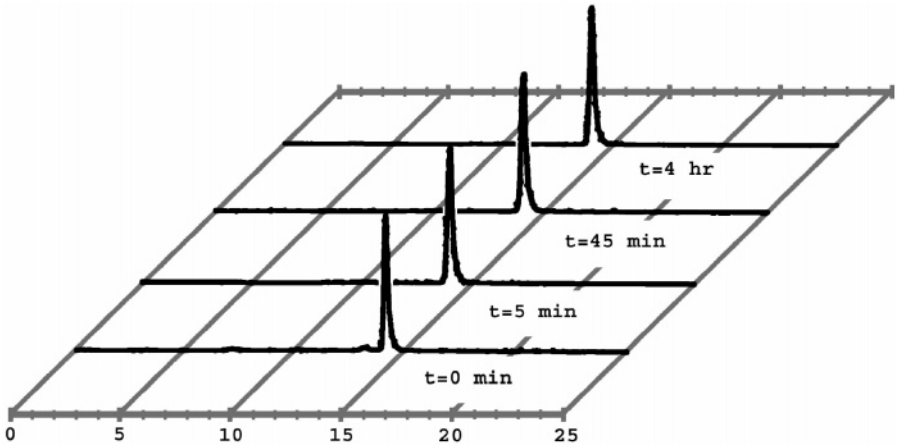


Figure 1. RP-HPLC stability profile of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in whole blood (t_R = 14.2 min).

of in vivo stability. Serum: Blood collected from mice was immediately centrifuged for 15 min at 3000 rpm at 4 °C, and the serum was separated. Aliquots of 100 μL of serum were treated with 200 μL of ethanol for protein precipitation. Samples were centrifuged at 4000 rpm for 15 min at 4 °C, the supernatant was collected and passed through a Millex GV filter (0.22 μm), and the degree of in vivo stability was assessed by RP-HPLC. Liver homogenate: After radiopharmaceutical administration, animals were kept for 1 h on normal diet ad libitum. Immediately upon sacrifice, the liver was excised, rinsed, and placed in chilled 50 mM TRIS/0.2 M sucrose buffer (pH = 7.4), wherein it was homogenized. The liver homogenate was treated with ethanol in a 2:1 EtOH/homogenate v/v ratio. The sample was then centrifuged at 25 000 rpm for 15 min at 4 °C and filtered through Millex GV filter (0.22 μm), and the activity was measured. Aliquots (in duplicate) of the filtrate were analyzed by RP-HPLC as previously described. Taking into account the radionuclide decay, the recovery of the radioactivity was found to be between 70 and 80% (activity of the filtrate/total liver activity).

7. In Vivo Evaluation of [^{99m}Tc(CO)$_3$−cyclo-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in $\alpha_v\beta_3$ positive and $\alpha_v\beta_3$ negative M21 Human, Melanoma-Bearing, Nude Mouse Models. All animal experiments were conducted in compliance with the Austrian animal protection laws and with approval of the Austrian Ministry of Science. Tumor uptake studies were performed in nu/nu mice (female, 6−9 weeks old, ∼20 g, Charles River). For the induction of tumor xenografts, M21 and M21L cells were subcutaneously injected into the left and right flanks of the mouse at a concentration of 5×10^6 cells per mouse. Tumors were allowed to grow to sizes of ∼0.3−0.6 cm^3 prior to initiation of the study. Animals were intravenously injected with 100 μL (925 kBq, ∼1 μg of peptide conjugate, specific activity ∼2.6×10^4 Ci/mol) of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate via the tail vein and were maintained on normal diet ad libitum. The mice were sacrificed by cervical dislocation at 1 and 4 h p.i. Tissues and organs were collected, weighed, and counted in a gamma counter, and the percent injected dose (%ID/organ) and %ID/g of each organ or tissue was calculated. Tumor-to-organ and tumor-to-blood ratios were also determined.

RESULTS

PZ-BOC (3,5Me$_2$-pz(CH$_2$)$_2$N((CH2)$_3$COOH)(CH$_2$)$_2$NH−BOC) was synthesized as previously reported (*35*−*38*) and provided to Biosynthan for conjugation to *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys]. The cyclic peptide, *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys-(PZ)], was synthesized by standard solid-phase synthetic methods, purified by preparative RP-HPLC, and characterized by MALDI-MS (calculated mass: 870.2; experimental mass: 870.1).

[^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ was produced in very high yield (≥90%, specific activity ∼ 6×10^6 Ci/mol) upon addition of [^{99m}Tc(H$_2$O)$_3$(CO)$_3$]$^+$ (prepared via the Isolink radiolabeling kit) to a vial containing 2.5×10^{-4}M *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] (Scheme 1). The radiochemical yield of the new ^{99m}Tc-conjugate was monitored by RP-HPLC. The HPLC chromatographic profile for [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ is demonstrated in Figure 1. The chromatogram shows a single peak (t_R= 14.2 min) corresponding to the new radiometallated conjugate. Pertechnetate had a retention time of ∼2.9 min under identical HPLC conditions. Nonmetallated *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(L)] eluted as a single species with a retention time of 8.8 min. This allowed for easy separation of metalated from nonmetallated conjugate, making it possible to collect very high specific activity [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys-(PZ)]]$^+$.

To assess the in vitro integrity of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate, stability assays in human blood were performed. Figure 1 shows the RP-HPLC stability profile of the new conjugate at various timepoints (0 min, 5 min, 45 min, and 4 h) postincubation with human blood. From this study, we can assess that [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate is ≥98% stable at 4 h postincubation in whole blood.

In vitro binding affinity of cyclo[Arg-Gly-Asp-D-Tyr-Lys-(PZ)] for the $\alpha_v\beta_3$ receptor was assessed by competitive binding assays utilizing ^{125}I-Echistatin as the binding displacement ligand (*40*). *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] showed very high binding affinity for the $\alpha_v\beta_3$ receptor with an IC$_{50}$ value of 3.01 nM (Figure 2). Comparatively, *cyclo*-[Arg-Gly-Asp-D-Phe-Val] showed an IC$_{50}$ value of 3.68 nM.

To evaluate the biological potential of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate, the degree of internalization in human, M21 melanoma cells was determined. This study showed approximately 1.0 ± 0.09% cellular uptake/mg of protein of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys-(PZ)]]$^+$ in M21 cells at 90 min postincubation. Blocking studies, in which excess *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys] was incubated concurrently with [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys-(PZ)]]$^+$, demonstrated the in vitro specificity of conjugate for $\alpha_v\beta_3$ receptor-expressing cells. The presence of 10 μM *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys] reduced cellular uptake/mg of protein to 0.34 ± 0.04% for [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ at 90 min postincubation. Comparably, cellular

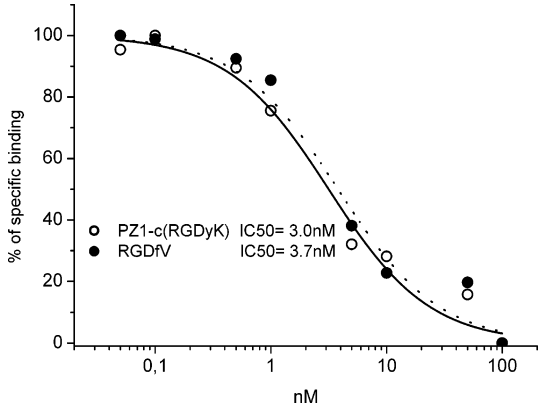


Figure 2. Competitive binding assays of *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] and *cyclo*-[Arg-Gly-Asp-D-Phe-Val] versus ^{125}I-echistatin in $\alpha_v\beta_3$ integrin receptors. IC_{50}s = 3.01 (*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys-(PZ)]) and 3.68 nM (*cyclo*-[Arg-Gly-Asp-D-Phe-Val]), respectively.

uptake/mg protein of $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in M21L receptor-negative cells was only 0.37 ± 0.11%.

Biodistribution studies in normal CF-1 mice are summarized in Table 1. These studies showed the $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate to be a hydrophilic species that effectively cleared the bloodstream at 1 h p.i. For example, only 0.23 ± 0.06% ID/g of the administered dose remained in whole blood at 1 h p.i. Approximately 75% of the injected dose was excreted at 1 h p.i. via the renal−urinary pathway for this ^{99m}Tc-RGD conjugate. Hepatobiliary uptake and excretion was minimal for this conjugate. For example, only 4.25 ± 0.70 and 2.03 ± 0.20% ID/g of $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ accumulated in intestines and liver tissue at 1 h p.i, respectively. Some residualization of radioactivity in normal kidney is evident for the $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate (3.11 ± 0.32% ID/g, 1h p.i.). However, this radioactivity appears to wash out significantly over time. To demonstrate the effective in vivo stability or kinetic inertness of $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate, urine, serum, and liver samples were acquired and analyzed by RP-HPLC at 1 h p.i. The samples were homogenized and reinjected onto the HPLC to verify product integrity. The conjugate showed remarkable in vivo stability as ≥98% of the conjugates were in the form of $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ at 1 h post-intravenous administration (Figure 3).

Table 2 summarizes the results of biodistribution studies of $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in nude mice bearing xenografted human, M21, melanoma tumors at 1 and 4 h post-intravenous injection. High tumor uptake and retention of $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys-(PZ)]]$^+$ in M21 melanoma tumors demonstrates effective selectivity and affinity of this new RGD conjugate for the $\alpha_v\beta_3$ receptor. For example, accumulation of radioactivity in tumor tissue was 2.50 ± 0.29 and 1.62 ± 0.44% ID/g at 1 and 4 h p.i., respectively. To demonstrate the effectiveness of $[^{99m}Tc(CO)_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ for selectively targeting the $\alpha_v\beta_3$ receptor, biodistribution studies were also performed in nude mice bearing xenografted receptor-negative, M21L, melanoma tumors (Table 2). Accumulation of radioactivity in M21L tumors showed markedly less tumor uptake at 1 h p.i as compared to the receptor-positive M21 cell line. For example, tumor accumulation in this receptor-negative cell line was only 0.71 ± 0.08% ID/g at 1 h p.i. Tumor-to-blood and tumor-to-muscle ratios in the receptor-positive M21 cell line were ∼2.6 and 2.4 at 1 h p.i, respectively. Conversely, these values were only 0.78 and 0.95 in receptor-negative M21L xenografted tumors. In all of these studies (i.e., tumor-bearing and non tumor-bearing mice), there is no significant uptake or retention of radioactivity in the stomach indicating that there is minimal, if any, in vivo dissociation of the ^{99m}Tc metal center from this tridentate ligand framework to produce $[^{99m}TcO_4]^-$.

DISCUSSION

Significant effort into the design and development of specific targeting vectors for imaging angiogenesis have provided valuable insight in identifying structural features that produce kinetically inert conjugates having very high affinity and selectivity for the $\alpha_v\beta_3$ integrin, a mediator of endothelial cell migration during blood vessel formation (*1*). These studies indicate that head-to-tail cyclized derivates of the general type *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys] are easily functionalized for radiolabeling at Lys5 producing antagonistic conjugates with nanomolar affinity for the $\alpha_v\beta_3$ integrin receptor (*6*−*18*). These studies have demonstrated the effectiveness of targeting angiogenesis factors for diagnosis and potential therapy of specific human tumors.

Typically, RGD peptide-based targeting vectors for integrin receptors are either linear, disulfide-cyclized, or head-to-tail cyclized constructs (*6*−*18*). However, increasing attention has recently focused on the development of dimeric or multimeric cyclic RGD peptide constructs. Multimerization is expected to increase the apparent affinity of targeting vectors for their cognate receptors due to avidity effects. In one instance, a dimeric *cyclo*-(RGDfK) construct was synthesized by bridging two lysine ϵ amino groups with a HYNIC-glutamic acid moiety (*43*). This peptide, when labeled with ^{99m}Tc, showed high receptor-mediated tumor uptake of 5.8 ± 0.7%ID/g at 1 h pi in an OVCAR-3/nude mouse in vivo model. The corresponding monomer showed tumor uptake of 5.2 ± 0.6%ID/g. However, at all timepoints, kidney retention of the dimer was significantly higher as compared to the monomer (*43*). In a second study, *cyclo*-(RGDfE) multimers were formed using variable-length PEG spacers linked to a diaminopropionic acid/lysine backbone (*44*). Monomeric, dimeric, and tetrameric forms were synthesized and labeled with ^{18}F. Biodistribution of the dimer was comparable to that of a monomeric, glycosylated ^{18}F-labeled RGD analogue (*44*).

Haubner and co-workers have used a tetrapeptide sequence (H-Asp-Lys-Cys-Lys-OH) as a chelating system for labeling a *cyclo*-RGD derivative (DKCK-RGD) with ^{99m}Tc (*45*). Gamma camera images at 4 h p.i. clearly demonstrated tumor-specific uptake of conjugate. However, there was high concentration of radioactivity in normal kidneys, possibly due to the lysine-containing tetrapeptide used as the chelating sequence (*45*).

Other attempts at targeting $\alpha_v\beta_3$ receptor expression have focused on the labeling of a disulfide-bridged undecapeptide found via a phage display library. In this study, a shortened derivative of RGD-4C ((Cys^1-Cys^9-Cys^3-Cys^7)H-Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys-OH) was coupled with HYNIC and radiolabeled with ^{99m}Tc (*11*, *46*). In two murine tumor models, only marginal tumor uptake was observed, presumably due to only modest association of this conjugate for the $\alpha_v\beta_3$ receptor ($K_a = 7 \times 10^6$ M^{-1}) (*11*, *46*).

Recent studies by Psimadas and co-workers and Line and co-workers have led to some interesting developments toward development of new $[^{99m}Tc(CO)_3$-RGD]$^+$ conjugates (*47*, *48*). For example, Psimadas et al., have conjugated picolinamine-*N*,*N*-diacetic acid (PADA) and histidine to the epsilon amine of lysine for coordination to the *fac*-$[M(CO)_3]^+$ metal center. Studies show these new conjugates can be produced in very high yields (∼98%) as single radiochemical products. Furthermore, these new conjugates were stable to histidine and cysteine challenge experiments even at 24 h postincubation with exchange ligand. In vivo evaluation of the biodistribution of

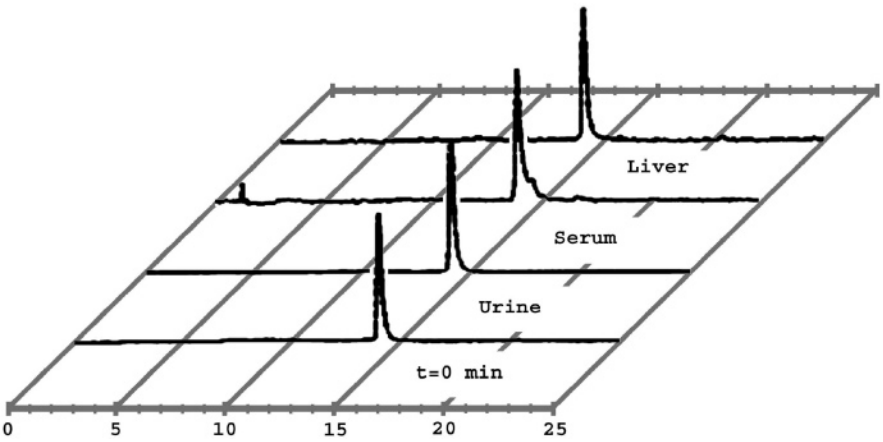


Figure 3. RP-HPLC stability profile of $[^{99m}Tc(CO)_3-$*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in murine urine, serum, and liver tissue at 1 h p.i. (t_R = 14.2 min).

Table 1. Biodistribution Studies (%ID/g(SD), $n = 5$) of $[^{99m}Tc(CO)_3-$*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ Conjugate in Normal, CF-1 Mice

tissue/organ	1 h	4 h	24 h
urine[a]	74.5(3.65)	81.9(3.40)	88.5(3.63)
bladder	2.34(1.42)	1.06(0.23)	0.24(0.33)
heart	0.56(0.09)	0.31(0.11)	0.08(0.13)
lungs	1.24(0.21)	0.59(0.12)	0.19(0.09)
liver	2.03(0.20)	0.90(0.21)	0.18(0.03)
kidney	3.11(0.32)	1.75(0.27)	0.56(0.10)
spleen	1.14(0.31)	0.45(0.06)	0.42(0.60)
stomach	0.71(0.16)	0.76(0.27)	0.18(0.07)
intestines	4.25(0.70)	6.80(1.26)	0.57(0.18)
muscle	0.34(0.05)	0.26(0.05)	0.12(0.05)
bone	1.06(0.36)	0.66(0.12)	0.23(0.07)
pancreas	0.53(0.11)	0.33(0.10)	0.04(0.01)
blood	0.23(0.06)	0.03(0.01)	0.01(0.01)

[a] Calculated as percent injected dose.

Table 2. Biodistribution Studies (%ID/g(SD), $n = 5$) of $[^{99m}Tc(CO)_3-$*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ Conjugate in M21 and M21L, Tumor-Bearing, Nude Mice

tissue/organ	1 h (M21)	4 h (M21)	1 h (M21L)
tumor	2.50(0.29)	1.62(0.44)	0.71(0.08)
heart	0.97(0.13)	0.51(0.09)	0.89(0.09)
lungs	2.12(1.15)	1.07(0.15)	2.34(0.40)
liver	8.89(0.71)	4.58(0.46)	10.5(1.32)
kidney	5.65(0.88)	3.12(0.22)	5.24(0.42)
spleen	2.08(0.27)	1.22(0.14)	2.30(0.33)
stomach	2.12(1.15)	1.05(0.01)	1.63(0.58)
intestines	10.1(1.30)	4.59(0.35)	10.2(1.57)
muscle	1.04(0.68)	0.32(0.08)	0.76(0.45)
bone	2.73(2.92)	0.57(0.13)	1.44(0.99)
pancreas	0.80(0.34)	0.34(0.04)	0.58(0.03)
blood	0.96(0.06)	0.48(0.11)	0.91(0.06)

these new conjugates indicated rapid clearance from the blood pool with excretion predominantly the hepatobiliary pathway (*47*, *48*). Studies by Line et al. have conjugated *fac*-$[M(CO)_3]^+$ metal chelator *N*-*ω*-bis(2-pyridyl-methyl)-L-lysine to an *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer with side chains terminated in doubly cyclized, integrin-specific RGD derivative KACDCRGDCFCG (RGD4C) or nonreactive RGE derivative KACDCRGECFCG (RGE4C) (*49*, *50*). In this study, they were able to produce conjugates in very high yield (≥93%) with specific activities ranging from 16.8 to 19.5 MBq/nmol ($4.5-5.3 \times 10^6$ Ci/mol). $[^{99m}Tc(CO)_3$-HPMA-RGD4C] conjugate showed very high uptake in each DU145 and PC-3 prostate tumor xenografts. For example, uptake and retention of activity at 24 h p.i. was determined to be 4.60 ± 1.80% ID/g and 5.92 ± 0.41% ID/g in DU145 and PC-3 prostate tumor models, respectively. Scintigraphic images in SCID mice bearing either DU145 or PC-3 prostate tumor xenografts demonstrated the high affinity and selectivity of these conjugates for $\alpha_v\beta_3$ integrin receptors expressed in these tumor models (*49*, *50*).

In the current study, we have developed a monomeric ^{99m}Tc-radiolabeled cyclic RGD conjugate, *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] (PZ = 3,5-Me_2-pz$(CH_2)_2$N$((CH_2)_3$COOH$)(CH_2)_2NH_2$), having high affinity for the $\alpha_v\beta_3$ integrin receptor. 3,5-Me_2-pz-$(CH_2)_2$N$((CH_2)_3$COOH$)(CH_2)_2NH_2$ coordinates technetium and rhenium metal centers in tridentate fashion through the pyrazolyl and amine nitrogen donor atoms giving the metal complexes sufficient stability for in vivo radiochemical investigations (*38*, *39*). In this study, we were able to effectively show that the new conjugate *cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)] could be radiolabeled in very high yield upon simple heating with the *fac*-$^{99m}Tc(CO)_3(H_2O)_3$-synthon. Upon simple radiolabeling of conjugate, a specific activity ~ 6×10^6Ci/mol was determined for $[^{99m}Tc(CO)_3-$*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$. This is comparable to other $[^{99m}Tc(CO)_3$-RGD] targeting vectors having very high affinity for the $\alpha_v\beta_3$ integrin receptor. Studies indicated production of a single species even at ligand concentrations of 10^{-5} M, precluding the potential for kit development for radiolabeled conjugates of this type. The new conjugate was stable in human serum for an extended period, demonstrating the ability of the nitrogen-based pyrazolyl chelator to effectively stabilize the metal center against transmetallation reactions to serum-based proteins. For example, $[^{99m}Tc(CO)_3-$*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ was ≥98% stable for periods up to 4 h postincubation as indicated by RP-HPLC.

It is essential to maximize uptake and residualization of radioactivity in human tumor tissue to optimize the diagnostic/therapeutic efficacy of the radiolabeled targeting vector. Results of internalization studies in M21 receptor-positive cells show that most of the radioactivity is not surface bound and is not lost from the cells upon incubation in pH 4.2 buffer. These studies indicate that $[^{99m}Tc(CO)_3-$*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ has high specificity for $\alpha_v\beta_3$ receptor-expressing cells. For example, there was approximately 1.0 ± 0.09% cellular uptake/mg of protein of in M21 cells at 90 min postincubation. Blocking studies indicated reduced cellular uptake/mg of protein for $[^{99m}Tc(CO)_3-$*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ at 90 min postincubation. Only 0.34 ± 0.04% cellular uptake/mg of protein was observed, further demonstrating the selectivity of conjugate for the integrin receptor. Cellular uptake/mg of protein of $[^{99m}Tc(CO)_3-$*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in M21L receptor-negative cells was only 0.37 ± 0.11%. Uptake of ^{125}I-radiolabeled RGD in each M21 and M21L human melanoma cells showed less than 0.25% cellular uptake/mg of protein comparatively.

In vivo evaluation of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ conjugate demonstrated rapid clearance from the bloodstream and excretion primarily via the renal−urinary pathway. Subsequent stability evaluation of the conjugate in human tissue showed that the conjugate was not readily metabolized in vivo, with 98% of the conjugate remaining intact upon in vivo administration. High tumor uptake and retention of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in M21 melanoma tumors was observed. For example, accumulation of radioactivity in tumor tissue was 2.50 ± 0.29 and 1.62 ± 0.44% ID/g at 1 and 4 h p.i., respectively. Studies in nude mice bearing xenografted receptor-negative, M21L melanoma tumors further demonstrates high specificity and affinity of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ for the integrin receptor. Tumor accumulation in this receptor-negative cell line was only 0.71 ± 0.08% ID/g at 1 h p.i. These results show some similarity to those studies by Haubner and co-workers in the same tumor model (12−15). Tumor-to-blood and tumor-to-muscle ratios of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ in the receptor-positive M21 cell line were ∼2.6 and 2.4 at 1 h p.i, respectively. The moderately high tumor-to-blood and tumor-to-muscle ratios are a reflection of the high binding affinity of this conjugate for the integrin receptor. These ratios are maintained even at 4 h postinjection (3.3 and 5.2, respectively) demonstrating receptor-mediated transport and subsequent intracellular trapping of this conjugate in M21, $\alpha_v\beta_3$, receptor-expressing cells. Janssen and co-workers have reported $\alpha_v\beta_3$ receptor expression in murine tissues such as the liver and spleen (*51*). In fact, they were able to effectively block uptake in muscle, lung, spleen, and liver indicating localization of their ^{111}In-RGD conjugate to be $\alpha_v\beta_3$ receptor-mediated in these tissues. In the present study, we were not able to effectively block uptake in these specific tissues and therefore cannot conclude that uptake in these organs was $\alpha_v\beta_3$ receptor-mediated.

In conclusion, these studies have demonstrated the potential utility of [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ to image angiogenesis and tumor formation in vivo. The pyrazolyl−RGD conjugate, when radiolabeled with the [^{99m}Tc(H$_2$O)$_3$(CO)$_3$]$^+$ aquaion, provided for kinetically inert complexes of very high specific activity. [^{99m}Tc(CO)$_3$−*cyclo*-[Arg-Gly-Asp-D-Tyr-Lys(PZ)]]$^+$ showed extraordinary stability in vitro and in vivo thereby retaining biological activity with high selectivity for the $\alpha_v\beta_3$ receptor.

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