64Cu-Labeled CB-TE2A and diamsar-conjugated RGD peptide analogs for targeting angiogenesis: comparison of their biological activity

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Abstract

Objectives: The αvβ3 integrin is a cell adhesion molecule known to be involved in stages of angiogenesis and metastasis. In this study, the chelators CB-TE2A and diamsar were conjugated to cyclic RGDyK and RGDfD and the biological properties of 64Cu-labeled peptides were compared.

Methods: CB-TE2A-c(RGDyK) and diamsar-c(RGDfD) were labeled with 64Cu in 0.1 M NH4OAc (pH=8) at 95°C and 25°C, respectively. PET and biodistribution studies were carried out on M21 (αvβ3-positive) and M21L (αv-negative) melanoma-bearing mice. Binding affinity of the Cu-chelator–RGD peptides to αvβ3 integrins was determined by a competitive binding affinity assay.

Results: Biological studies showed higher concentration of 64Cu-CB-TE2A-c(RGDyK) in M21 tumor compared to M21L tumor at 1 and 4 h pi. Tumor concentration of 64Cu-diamsar-c(RGDfD) was lower than that of 64Cu-CB-TE2A-c(RGDyK). The difference is not due to differing binding affinities, since similar values were obtained for the agents. Compared to 64Cu-diamsar-c(RGDfD), there is more rapid liver and blood clearance of 64Cu-CB-TE2A-c(RGDyK), resulting in a lower liver and blood concentration at 24 h pi. Both 64Cu-labeled RGD peptides show similar binding affinities to αvβ3. The differences in their biodistribution properties are likely related to different linkers, charges and lipophilicities. The M21 tumor is clearly visualized with 64Cu-CB-TE2A-c(RGDyK) by microPET imaging. Administration of c(RGDyK) as a block significantly reduced the tumor concentration; however, the radioactivity background was also decreased by the blocking dose.

Conclusions: Both 64Cu-CB-TE2A-c(RGDyK) and 64Cu-diamsar-c(RGDfD) are potential candidates for imaging tumor angiogenesis. For diamsar-c(RGDfD), a linker may be needed between the Cu-chelator moiety and the RGD peptide to achieve optimal in vivo tumor concentration and clearance from nontarget organs.

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1. Introduction

Angiogenesis, the formation and differentiation of blood vessels, is required for both tumor growth and metastasis [1–3]. The angiogenic process depends on vascular endothelial cell migration and invasion, which are regulated by cell adhesion receptors. Integrins are a family of proteins that facilitate cellular adhesion and migration to extracellular matrix proteins found in intercellular spaces and basement membranes, and regulate cellular entry and withdrawal from the cell cycles [4–7]. Alpha v beta 3
(αvβ3) integrin, one of the most extensively studied integrins, is highly expressed on activated endothelial and tumor cells during angiogenesis [8]. In contrast, expression of αvβ3 is weak in resting endothelial cells and most normal organ systems [4,9]. Therefore, αvβ3 integrin is a promising target for imaging tumor angiogenesis and for antiangiogenic therapy [10,11].

αvβ3 binds to the arginine-glycine-aspartate (RGD) amino acid sequence of extracellular matrix proteins such as vitronectin, fibrinogen or fibronectin [12,13]. In the past decade, various radiolabeled RGD peptide antagonists of αvβ3, have been developed as radiopharmaceuticals for single photon emission computed tomography and positron emission tomography (PET) imaging (see Refs. [14–16]).

64Cu-Labeled RGD peptides are of particular interest. 64Cu (t1/2=12.7 h; 38% β−, Eβmax=573 keV; 19% β+, Eβmax=656 keV; 43% EC) is an attractive radionuclide for both PET imaging and targeted radiotherapy of cancer because of its decay characteristics [17,18], and because it can be produced at high specific activity on a small biomedical cyclotron [19].

RGD peptides can be radiolabeled with 64Cu using a covalently bound bifunctional chelator, 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) have historically been used as bifunctional chelators for copper radionuclides [20–23]. We showed that 64Cu-DOTA and 64Cu-TETA complexes are moderately unstable in vivo due to the release of uncoordinated 64Cu by decomposition in the blood or transchelation in the liver, causing high uptake in nontarget tissues [24,25]. Several new macrocyclic copper-chelating agents have been investigated for improving the in vivo stability of the 64Cu complex. Novel bicyclic tetraazamacrocyclu cross-bridged cyclam chelators have recently been developed for 64Cu [26] that have shown significantly increased metal-chelate stability in vivo and, as a consequence, a reduced nontarget tissue accumulation [24,27]. 64Cu-CB-TE2A-Tyr3-Octreotate (where CB-TE2A is 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2] hexadecane) (Fig. 1) has demonstrated improved blood, liver and kidney clearance compared with the analogous 64Cu-TETA agent [27]. 64Cu-CB-TE2A-ReCCMSH(Arg11) also showed greatly improved liver and blood clearance as well as higher tumor-to-nontarget tissue ratios compared with 64Cu-DOTA-ReCCMSH(Arg11) [28].

A new class of cage-type hexaazamacrobicyclic sarcophagine (Sar) chelators and derivatives has also been developed as stable Cu chelates [29–33]. A recently developed bifunctional version of the Sar chelator — SarAr — has been conjugated to antibodies and the resulting SarAr immunoconjugates have been radiolabeled with 64Cu. 64Cu-Labeled SarAr conjugated anti-GD2 monoclonal antibody (mAb) 14.G2a and its chimeric derivative, ch14.18, were shown to have high specific activity, antigen binding and in vivo target specificity to neuroblastoma and melanoma, with minimal uptake in normal tissues [34]. SarAr has also been conjugated to the well-characterized B72.3 antibody and its fragments, and 64Cu-SarAr immunoconjugates maintain their specificity for the target and are stable in vivo [35].

Both cross-bridged and Sar chelate systems have shown significant metal-chelate stability and, consequently, low nontarget tissue accumulation. The objective of this research was to compare the in vivo and in vitro biological activities of the cross-bridged and Sar chelator using RGD peptide analogs as targeting molecules.

Fig. 1. (A) Structure of CB-TE2A-c(RGDyK) and (B) structure of diamsar-c(RGDfD).
2. Materials and methods

2.1. General

All chemicals unless otherwise stated were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Water was distilled and then deionized (18 MΩ·cm²) by passing through a Milli-Q water filtration system (Millipore Corp., Milford, MA, USA). 64Cu was produced on a CS-15 biomedical cyclotron at Washington University School of Medicine according to published procedure [19]. Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA, USA). Radioactivity was accomplished using a BIOSCAN AR2000 Imaging Scanner (Washington, DC). Analytical reversed-phase high-performance liquid chromatography (HPLC) was performed on a 600E chromatography system (Waters) with a 996 photodiode array detector (Waters) and an Ortec Model 661 radioactivity detector (EG&G Instruments, Oak Ridge, TN, USA). Electrospray mass spectrometry was accomplished using a Micromass ZQ (Waters).

2.2. Synthesis of CB-TE2A-c(RGDyK)

The cyclic peptide (Fig. 1A) was prepared in three steps consisting of solid-phase peptide synthesis, intramolecular cyclization in solution, and conjugation of peptide with CB-TE2A, as described previously [36]. The crude product was purified by HPLC and identified by electrospray mass spectrometry. The observed m/z for [MH]+ and [MH2]2+ in electrospray mass spectrometry was 444.37 and 472.77, respectively. Unconjugated c(RGDyK) for the integrin-binding assays and blocking studies in mice was purchased from CS Bio (Menlo Park, CA, USA).

2.3. Synthesis of diamsar-c(RGDfD)

Diamsar was synthesized following literature procedures [29,32]. Briefly, cobalt(III) complex of the diamine derivate of the Sar compound (diamsar) was prepared by template synthesis based on tris(ethane-1,2-diamine)cobalt (III). Co(III)–diamsar complex was reduced to its Co(II) form to enable the removal of the diamsar ligand. The reaction reduction was performed in hot aqueous solution containing excess cyanide ion.

Diamsar-c(RGDfD) (Fig. 1B) was synthesized by a similar procedure as that of CB-TE2A-c(RGDyK). Briefly, the orthogonally protected linear peptide [H-Asp(Obut)-DPhe-Asp(ODmab)-Arg(Pbf)-Gly-OH] was prepared from a H-Gly-2-chlorotrityl resin and cleaved with 1% TFA in dichloromethane. The cyclization was realized in the presence of PyBOP, N-hydroxybenzotriazole (HOBT) and diisopropylcarbodiimide in N,N-dimethylformamide/dichloromethane. The ODmab group was deprotected with 2% hydrazine in aqueous acetonitrile. Diamsar was conjugated to the carboxylic acid group of aspartic acid of the peptide in the presence of diisopropylcarbodiimide and HOBT in anhydrous N,N-dimethylformamide. All side-chain–protecting groups were removed with 95% aqueous TFA solution, and the crude product was purified by HPLC and identified by electrospray mass spectrometry. The observed m/z for [MH]+ in electrospray mass spectrometry was 444.3.

2.4. 64Cu Radiolabeling

CB-TE2A-c(RGDyK) was radiolabeled with 64Cu according to the method reported previously for radiolabeling of CB-TE2A conjugates [27,28,36]. Briefly, 64CuCl2 was added to CB-TE2A-c(RGDyK) in 0.1 M NH4OAc (pH 8) and heated at 95°C for 1 h. Radiochemical purity was >95% as confirmed by both radio-HPLC (99% A to 70% A in 15 min, 1 ml/min; A: 0.1% TFA in H2O, B: 0.1% TFA in acetonitrile; C-18 monomeric column [Vydac], 3 mm, 4.6×100 mm) and radio-TLC (Whatman MKC18F reversed-phase plates with 10% ammonium acetate/methanol (30:70) as the mobile phase).

Diamsar-c(RGDfD) was labeled with 64Cu under milder reaction conditions. 64CuCl2 was added to diamsar-c(RGDfD) solution in 0.1 M NH4OAc (pH 8), and the reaction mixture was incubated at 25°C for 1 h. Radio-HPLC and radio-TLC (conditions same as above) assessment showed that the radiochemical purity was >95%.

2.5. In vitro binding assay

The binding affinity of Cu-CB-TE2A-c(RGDyK) and Cu-diamsar-c(RGDfD) for αβ3 integrin was estimated using previously reported methods [37]. Briefly, vitronectin (Chemicon, Temecula, CA) (630 μg/ml) was biotinylated with N-hydroxysuccinimide biotin (1.27 μg/ml; 2 h at room temperature) prior to dialysis into PBS, pH 7.4. Integrin αβ3 (EMD Bioscience, San Diego, CA, USA) [1 μg/ml in 20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 1 mM MnCl2 (coating buffer)] was layered onto 96-well plates (Nunc Immuno Plate with MaxiSorp) (1 h at 4°C). Plates were then blocked (1 h at 4°C) with BSA (3% in coating buffer). After washing twice with binding buffer (0.1% BSA in coating buffer), biotinylated vitronectin (14 nM) and serially diluted peptides were allowed to bind to the integrins (3 h at 37°C). Following washing (three times in binding buffer), bound biotinylated vitronectin was detected by binding ExtrAvidin-Alkaline Phosphatase (Sigma) (1/35,000 dilution, 1 h at RT) using p-Nitrophenyl Phosphate Liquid Substrate System (Sigma) as the chromogen. Assays were performed in triplicate. Nonlinear regression was used to fit binding curves and calculate IC50 values (GraphPad Prism 4.0, San Diego, CA, USA).

2.6. Biodistribution studies

All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals established by Washington University’s Animal Studies Committee. Biodistribution studies were carried out on 24- to 29-g male Nu/Nu mice (Charles River
Laboratories, Wilmington, MA, USA) that had been implanted with cultured M21 and M21L human melanoma cells. Due to the lack of the αv subunit, M21L cells have different growth characteristics compared to M21 cells. To obtained comparable tumor xenografts, 5 × 10^6 M21 and 2 × 10^6 M21L cells were injected subcutaneously into the left and right thigh of the mice, respectively [38]. Tumors were allowed to grow for 21 days, at which time the animals received ∼10 μCi of 64Cu-CB-TE2A-(cRGDyK) or 64Cu-diamsar-c(RGDfD) in 100 μl of saline via lateral tail vein injection. For each peptide, four groups were examined at four time points [n=5 per group at 1, 2, 4 and 24 h postinjection (pi)]. In all studies following euthanasia, tissues and organs of interest were removed and weighed, and the radioactivity was measured in a γ-counter. The percent doses per gram (%ID/g) were then calculated by comparison to known standards.

### 2.7. Small animal PET studies

Whole-body small animal PET imaging was performed on a microPET Focus scanner (Concorde Microsystems, Knoxville, TN, USA) [39]. Imaging studies were carried out on Nu/Nu mice bearing 21-day M21 and M21L human melanoma tumors. The mice were injected via the tail vein on 64Cu-labeled peptides. For the purpose of direct comparison to known standards.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>24 h</th>
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</thead>
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<tr>
<td>Blood</td>
<td>0.85±0.46</td>
<td>0.27±0.13</td>
<td>0.13±0.11</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>2.38±0.86</td>
<td>1.64±0.40</td>
<td>1.06±0.05</td>
<td>0.41±0.07</td>
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<tr>
<td>Liver</td>
<td>3.86±1.98</td>
<td>2.53±0.89</td>
<td>1.92±0.29</td>
<td>0.84±0.17</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.17±0.60</td>
<td>1.90±0.78</td>
<td>1.58±0.24</td>
<td>1.66±1.15</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.96±1.20</td>
<td>5.18±1.67</td>
<td>3.96±0.53</td>
<td>2.10±0.86</td>
</tr>
<tr>
<td>Muscle</td>
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<td>0.37±0.09</td>
<td>0.47±0.18</td>
<td>0.15±0.04</td>
</tr>
<tr>
<td>Heart</td>
<td>1.22±0.48</td>
<td>0.66±0.30</td>
<td>0.34±0.04</td>
<td>0.23±0.07</td>
</tr>
<tr>
<td>Bone</td>
<td>3.80±1.15</td>
<td>0.76±0.17</td>
<td>0.50±0.08</td>
<td>0.59±0.48</td>
</tr>
<tr>
<td>M21</td>
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<td>1.68±0.58</td>
<td>1.66±0.80</td>
<td>0.70±0.17</td>
</tr>
<tr>
<td>M21L</td>
<td>1.76±0.43</td>
<td>1.40±0.54</td>
<td>0.84±0.41</td>
<td>0.80±0.35</td>
</tr>
<tr>
<td>Tumor (M21)/blood</td>
<td>4.51±2.89</td>
<td>6.46±1.30</td>
<td>18.34±5.10</td>
<td>26.31±3.24</td>
</tr>
</tbody>
</table>

### 2.8. Statistical methods

All of the data are presented as mean±S.D. For statistical classification, a Student’s t test was performed using GraphPad PRISM (San Diego, CA, USA). Differences at the 95% confidence level (P<0.05) were considered significant.

### 3. Results

#### 3.1. Synthesis of CB-TE2A-c(RGDyK) and diamsar-c(RGDfD)

The carboxylic acid arm of the cross-bridged CB-TE2A was conjugated to the ε-amine of the lysine group of cyclic pentapeptide RGDyK, while the amine group of diamsar was coupled to the carboxylic acid of the aspartic acid group of cyclic RGDfD. The conjugated peptides were prepared in three steps. First, the linear peptides were synthesized by solid-phase peptide synthesis using Fmoc chemistry, followed by intramolecular cyclization to form cyclic peptides c(RGDyK) and c(RGDfD), and conjugation of the peptides with chelates CB-TE2A and diamsar. The conjugated peptides were purified by RP-HPLC and identified by positive ion electrospray mass spectrometry.

#### 3.2. Radiolabeling of CB-TE2A-c(RGDyK) and diamsar-c(RGDfD) with 64Cu

CB-TE2A-c(RGDyK) was successfully labeled with 64Cu in 0.1 M ammonium acetate buffer (pH=8) at 95°C for 1 h. 64Cu labeling of diamsar-c(RGDfD) was performed in the same reaction buffer and same reaction time but at lower temperature (25°C). Without a purification procedure, we were able to achieve radiochemical purities of >95% for both radiolabeled peptides. For the purpose of direct comparison of their biological properties, the specific activities of the two 64Cu-labeled peptides were controlled to be similar (∼1 mCi/μg corresponding to ∼1000 mCi/μmol).

#### 3.3. In vitro competitive binding assay

The nonradioactive Cu complexes of the chelator-conjugated RGD peptides were synthesized using similar
procedures as those for the $^{64}$Cu radiolabeling. Their binding affinity to $\alpha_v\beta_3$ integrin was determined by a competitive binding affinity assay using biotinylated vitronectin and target ligands. IC$_{50}$ values (the concentration of peptide/antagonist required to inhibit 50% of vitronectin binding to integrin) of the two compounds are very similar, indicating comparable binding affinity to $\alpha_v\beta_3$ integrin (Table 1).

3.4. Biodistribution studies

Biodistribution studies for $^{64}$Cu-CB-TE2A-c(RGDyK) and $^{64}$Cu-diamsar-c(RGDfD) were carried out in nude mice using the $\alpha_v\beta_3$-positive xenotransplanted human melanoma M21 model [37,40]. M21L cells have low $\alpha_v$ expression and were therefore selected as the negative control [40,41]. M21 and M21L cells were implanted into the left and right thigh of the mice, respectively. Table 2 presents the biodistribution data at 1, 2, 4 and 24 h after injection of 10 $\mu$Ci of $^{64}$Cu-diamsar-c(RGDfD) and $^{64}$Cu-CB-TE2A-c(RGDyK).

Biodistribution data showed higher concentration in M21 tumor compared to M21L tumor for $^{64}$Cu-CB-TE2A-c(RGDyK) at 1 and 4 h pi [e.g., at 1 h pi, 2.99±0.90 (M21) vs. 1.76±0.43 %ID/g (M21L), $P≈0.02$; at 4 h pi, 1.66±0.8 (M21) vs. 0.84±0.41 %ID/g (M21L), $P<0.04$]. For $^{64}$Cu-diamsar-c(RGDfD), the M21 tumor concentration was slightly higher than M21L; however, statistical analysis showed that M21 and M21L concentrations were not significantly different [$P=NS$ (not significant)] at 1, 2 and 4 h pi. Only at 24 h pi is there a significant difference in the uptake between the two cell lines ($P≈0.03$) (Table 2). The concentration of $^{64}$Cu-CB-TE2A-c(RGDyK) in M21 tumor was higher than that of $^{64}$Cu-diamsar-c(RGDfD) at all time points (e.g., at 1 h pi, $^{64}$Cu-CB-TE2A-c(RGDyK): 2.99±0.90 vs. $^{64}$Cu-diamsar-c(RGDfD): 1.51±0.53 %ID/g, $P<0.01$; at 4 h pi, $^{64}$Cu-CB-TE2A-c(RGDyK): 1.66±0.80 vs. $^{64}$Cu-diamsar-c(RGDfD): 0.52±0.29 %ID/g, $P<0.02$) (Table 2 and Fig. 2).

Table 2 and Fig. 2 indicate that the radioactivity in the liver and blood was higher for $^{64}$Cu-CB-TE2A-c(RGDyK) than for $^{64}$Cu-diamsar-c(RGDfD) at 1 h pi [liver uptake: $^{64}$Cu-CB-TE2A-c(RGDyK): 3.86±1.98 vs. $^{64}$Cu-diamsar-c(RGDfD): 2.33±0.76 %ID/g, $P<0.05$; blood uptake: $^{64}$Cu-CB-TE2A-c(RGDyK): 3.86±1.98 vs. $^{64}$Cu-diamsar-c(RGDfD): 3.86±1.98 %ID/g, $P<0.05$].
(RGDyK): 0.85±0.46 vs. 64Cu-diamsar-c(RGDfD): 0.60±0.31 %ID/g, P=NS). However, compared to 64Cu-diamsar-c (RGDfD), there is more rapid liver and blood clearance of 64Cu-CB-TE2A-c(RGDyK), resulting in a lower liver and blood concentration at 24 h pi. For example, the liver uptake of 64Cu-CB-TE2A-c(RGDyK) at 24 h pi had fallen to ~20% of the 1-h uptake (1 h pi: 3.86±1.98 vs. 24 h pi: 0.84±0.17 %ID/g, P<0.02), while for 64Cu-diamsar-c(RGDfD), the radioactivity in liver did not change significantly from 1 to 24 h pi (1 h pi: 2.33±0.76 vs. 24 h pi: 2.24±0.41 %ID/g, P=NS). The 64Cu-CB-TE2A-c(RGDyK) activity in the blood was reduced by 98% from 1 to 24 h pi compared with an 86% reduction for 64Cu-diamsar-c(RGDfD) activity over the same period [64Cu-CB-TE2A-c(RGDyK): 0.85±0.46 (1 h pi) vs. 0.02±0.01 %ID/g (24 h pi), P<0.01; 64Cu-diamsar-c(RGDfD): 0.60±0.31 (1 h pi) vs. 0.08±0.01 %ID/g (24 h pi), P<0.02]. Because of the higher tumor concentration and faster blood clearance, the tumor (M21)-to-blood ratio of 64Cu-CB-TE2A-c(RGDyK) is higher than that of 64Cu-diamsar-c(RGDfD) at later time points. Table 2 and Fig. 2 show that the tumor/blood ratio of 64Cu-CB-TE2A-c(RGDyK) is similar to 64Cu-diamsar-c(RGDfD) at 1 and 2 h pi but is significantly higher than 64Cu-diamsar-c(RGDfD) at 4 and 24 h pi [at 4 h pi, 64Cu-CB-TE2A-c(RGDyK): 18.34±5.10 vs. 64Cu-diamsar-c (RGDfD): 9.50±1.51 %ID/g, P<0.05; at 24 h pi, 64Cu-CB-TE2A-c(RGDyK): 26.31±3.24 vs. 64Cu-diamsar-c(RGDfD): 6.31±1.04 %ID/g, P<0.05].

3.5. Small animal PET studies

Because of its higher tumor concentration, 64Cu-CB-TE2A-c(RGDyK) was chosen for a small animal PET imaging study. Small animal PET images were obtained with nude mice bearing 21-day M21 and M21L human melanoma tumors. Fig. 3 shows the projection images of the mice at 1 h after injection of 150 μCi of 64Cu-CB-TE2A-c (RGDyK). The image of the control mice on the right side demonstrated that the M21 tumor was clearly visualized, with much lower activity accumulation was found in M21L tumor, which is consistent with the biodistribution data. The mouse on the left side was preinjected with 15 mg of c (RGDyK) per kilogram of body weight as a block. The results show that the block dramatically reduced the radioactivity accumulation in the tumor by such an extent that very little activity can be seen in the tumor. However, Fig. 3 shows that treatment with c(RGDyK) also reduced uptake in other tissues such as the kidney, resulting in lower radioactivity concentrations in both tumor and the background for the c (RGDyK)-treated mouse.

4. Discussion

The procedures for the conjugation of both chelating groups with the RGD peptides were similar. Although CB-TE2A possesses two carboxylic acid functions, previous studies demonstrated the selective conjugation of partially protected c(RGDyK) with only one of the acid groups [27,28,36]. Unlike CB-TE2A, however, diamsar contains eight amino acid groups that can potentially react with the peptide. To prepare diamsar-c(RGDfD), we used orthogonally protected RGD peptide, c[R(Pbf)GD(OBut)fD] and reaction conditions that favor conjugation of only one of the diamsar’s primary amines with the unprotected aspartic acid group of the peptide. HPLC purification gave a major peak that was identified by both ES-MS and analytical HPLC as the mono-conjugate. The synergistic effects of mild reaction conditions and higher reactivity of the primary relative to the secondary amines because of steric factors further support the structure depicted in Fig. 1.

To facilitate 64Cu labeling, antibodies or peptides have been historically conjugated to one of the bifunctional chelators, DOTA or TETA. Biodistribution and metabolism studies of 64Cu-labeled DOTA/TETA monoclonal antibody or peptide conjugates have demonstrated significant dissociation of the 64Cu from the bifunctional chelator resulting in transchelation to liver superoxide dismutase and other proteins [24,27,42–44]. 64Cu-labeled DOTA/TETA conjugates often show poor blood and liver clearance, high uptake in some nontarget organs and increased background radioactivity levels, resulting in reduced imaging sensitivity, poor image quality and radiation toxicity [45,46].

A series of copper(II) cross-bridged cyclam complexes have been synthesized and characterized, and have demonstrated improved kinetic stability compared with TETA and DOTA complexes [26,47]. The biodistribution of four 64Cu-labeled cross-bridged cyclam complexes has been evaluated, and it was found that 64Cu-CB-TE2A had the most improved...
blood, liver and kidney clearance [25]. Another type of Cu chelator has been developed based on hexa-aza macrobicyclic sarcophagine (Sar) cage [29,31,48]. The cage-type ligands, designed with the additional linking strand incorporating two nitrogen atoms, form a three-dimensional “cage” around the Cu$^{2+}$ ion which leads to an increased thermodynamic and kinetic stability [49,50]. In this study, we intended to compare the biological properties of the cross-bridged and Sar chelators using RGD peptide analogs as targeting molecules.

One of the selective $\alpha_v\beta_3$ antagonists is a cyclic pentapeptide c(RGDfV) with IC$_{50}$ values in the lower nanomolar range [51]. Additional studies showed that, besides the essential RGD sequence, a hydrophobic amino acid in Position 4 increases the affinity, whereas the amino acid in Position 5 has no effect on the affinity [52]. We selected two cyclic RGD peptides, c(RGDyK) and c(RGDfD), both of which have hydrophobic amino acids (tyrosine or phenylalanine, respectively) in Position 4. Lysine group in Position 5 in c(RGDyK) offers an ε-amino group which can be coupled to the carboxylic acid group of the chelate CB-TE2A. For c(RGDfD), the aspartic acid group was chosen for Position 5 to facilitate the conjugation with the amine group of the diamsar chelator. The chelator-conjugated peptides were synthesized by similar procedures. The linear peptides were first prepared by Fmoc solid-phase peptide synthesis technique, followed by intramolecular cyclization and finally conjugation of the peptides with the chelators.

CB-TE2A-c(RGDyK) and diamsar-c(RGDfD) were radiolabeled with $^{64}$Cu in the same basic reaction buffer (0.1 M NH$_4$OAc, pH 8). Radiolabeling of CB-TE2A-c(RGDyK) was performed at high temperature (95°C), while milder temperature (25°C) was required for the radiolabeling of diamsar-c(RGDfD). It was reported that cage-type chelate Sar and its derivative, SarAr, can be labeled with $^{64}$Cu over a pH range of 4–9 [30,31], and SarAr immunoconjugates were radiolabeled with $^{64}$Cu at pH 5 [34,35]. Both SarAr and SarAr-conjugated antibodies could be rapidly radiolabeled (<10 min) at room temperature [30,31,34,35]. However, in our study, by monitoring the labeling reaction with radio-TLC, we found that the $^{64}$Cu labeling of diamsar-c(RGDfD) was not completed within 30 min, and 1 h is the optimal reaction time to ensure the highest radiolabeling yield. The $^{64}$Cu labeling of diamsar-c(RGDfD) at pH 6 and 8 showed similar reaction rates. However, a complete investigation of the labeling conditions, such as other pH values, different buffers and molar ratios of diamsar-c(RGDfD) to $^{64}$Cu, was not performed. Therefore, the labeling conditions may not be optimized. Moreover, the chelator used in previously reported SarAr-conjugated antibody B72.3 [35] contains a benzyl amine linker, while in diamsar-c(RGDfD) the Sar chelating moiety is closer to the RGD peptide. Also, the peptide c(RGDfD) was attached to the chelator diamsar by a short aspartic acid linker. The short distance between diamsar and c(RGDfD) may impact the kinetics of radiolabeling.

For in vivo evaluation, we chose the human xenograft M21 and M21L melanoma model. The M21 cells express $\alpha_v\beta_3$ [37,40], while the M21L cell line is a stable variant cell line of M21 failing to transcribe the $\alpha_v$ gene and therefore serves as a negative control [41]. At 1 and 4 h postinjection, the M21 tumor concentration is higher than M21L concentration for $^{64}$Cu-CB-TE2A-c(RGDyK). $^{99m}$Tc-DKCK-c(RGDfK) was studied previously using the same M21/M21L tumor model, and it was reported that the M21 tumor accumulation decreases from 1.91±0.24 %ID/g at 30 min pi to 1.10±0.22 %ID/g at 4 h pi In contrast, the M21L tumor uptake ranges from 0.74±0.06 %ID/g at 30 min pi to 0.31±0.09 %ID/g at 4 h pi [38]. The magnitude of the tumor uptake values of $^{64}$Cu-labeled RGD peptides in this study as shown in Table 2 is in the same range as the M21 and M21L tumor uptakes of $^{99m}$Tc-DKCK-c(RGDfK).

Small animal PET imaging also showed higher radioactivity concentration in the M21 tumor compared to M21L tumor for $^{64}$Cu-CB-TE2A-c(RGDyK), which is consistent with the biodistribution data. The blocking study showed that adding excess RGD peptide c(RGDyK) significantly decreased the tumor concentration, as well as reduced the uptake in other organs such as kidney. Similar multiorgan blocking has been reported for a tetrameric RGD peptide $^{64}$Cu-DOTA-E[c(RGDfK)]$_2$ in the U87MG tumor model [53] and also for $^{64}$Cu-CB-TE2A-c(RGDyK) in the parathyroid hormone mouse model [36]. This is not unexpected, as it is well known that RGD peptides do show low-level affinity for other integrins [54]. Also, nontarget organs express a low amount of integrin $\alpha_v\beta_3$ by Western blot analysis [53].

The biodistribution study demonstrated that, compared to $^{64}$Cu-diamsar-c(RGDfD), $^{64}$Cu-CB-TE2A-c(RGDyK) showed higher tumor uptake, faster liver and blood clearance, and higher tumor-to-blood ratio at 4 and 24 h pi, indicating superior properties of $^{64}$Cu-CB-TE2A-c(RGDyK). The in vitro binding assay showed similar $\alpha_v\beta_3$ binding affinity for $^{64}$Cu-CB-TE2A-c(RGDyK) and $^{64}$Cu-diamsar-c(RGDfD), which proved that the binding affinity does not contribute to the differences of their biodistribution properties. The chelator conjugation seems to have minimal impact on the integrin binding affinity of the RGD peptides. Similar results were reported previously for DOTA-conjugated dimeric and tetrameric RGD peptides E[c(RGDfK)]$_2$ and E[c(RGDfK)]$_4$ [53].

The differences in the biodistribution data of the two $^{64}$Cu-labeled RGD peptides are likely related to their molecular structures. Cu-CB-TE2A-c(RGDyK) has a longer lysine linker, while Cu-diamsar-c(RGDfD) has a shorter aspartic acid linker. Moreover, different charges and lipophilicities may also cause their pharmacokinetic differences. The ligand donors for $^{64}$Cu-diamsar-c(RGDfD) are amines which make the labeled peptide more positively charged compared to $^{64}$Cu-CB-TE2A-c(RGDyK) whose ligand donors are with a combination of amines, amide and carboxylic acid moieties. $^{64}$Cu-CB-
TE2A-c(RGDyK) is more hydrophilic than $^{64}$Cu-diamasar-c (RGDfD) with tyrosine in Position 4 instead of phenylalanine. A future direction is to modify the structure of the Sar-RGD peptide in order to directly compare CB-TE2A and Sar-conjugated RGD peptides. For example, a successful synthesis of the carboxylic acid derivatives of the Sar ligand would allow coupling of the acid group with the $\varepsilon$-amine of lysine to isolate diamasar-c(RGDyK). Alternatively, utilizing the SarAr bifunctional chelator that has been evaluated for radiolabeling $^{64}$Cu-labeled mAbs [27] may provide more optimal results.

5. Conclusions

We successfully synthesized and purified CB-TE2A-c (RGDyK) and diamasar-c(RGDfD), and radiolabeled the peptides with $^{64}$Cu. The tumor concentration of $^{64}$Cu-CB-TE2A-c(RGDyK) in the $\alpha_v\beta_3$-positive M21 tumor is higher than that in the negative control M21L tumor at 1 and 4 h postinjection. The $\alpha_v\beta_3$ binding affinities of the two Cu-chelator-RGD peptides are similar. Compared to $^{64}$Cu-diamasar-c(RGDfD), $^{64}$Cu-CB-TE2A-c(RGDyK) is a superior compound for $\alpha_v\beta_3$ integrin targeting with higher tumor uptake, faster liver and blood clearance, and a higher tumor/blood ratio. For diamasar-c(RGDfD), a suitable linker may be needed between the Cu-chelate moiety and the cyclic RGD peptide to achieve optimal in vivo stability and tumor uptake.

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