Synthesis and Evaluation of a Dimeric RGD Peptide as a Preliminary Study for Radiotheranostics with Radiohalogens

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Abstract: We recently developed $^{125}$I- and $^{211}$At-labeled monomer RGD peptides using a novel radiolabeling method. Both labeled peptides showed high accumulation in the tumor and exhibited similar biodistribution, demonstrating their usefulness for radiotheranostics. This study applied the labeling method to a dimer RGD peptide with the aim of gaining higher accumulation in tumor tissues based on improved affinity with $\alpha_v\beta_3$ integrin. We synthesized an iodine-introduced dimer RGD peptide, E[c(RGDfK)] (6), and an $^{125/131}$I-labeled dimer RGD peptide, E[c(RGDfK)][$^{125/131}$I][c(RGDf(4-I)K)] ([125/131][6], and evaluated them as a preliminary step to the synthesis of an $^{211}$At-labeled dimer RGD peptide. The affinity of 6 for $\alpha_v\beta_3$ integrin was higher than that of a monomer RGD peptide. In the biodistribution experiment at 4 h postinjection, the accumulation of [125I]6 (4.12 ± 0.42% ID/g) in the tumor was significantly increased compared with that of $^{125}$I-labeled monomer RGD peptide (2.93 ± 0.08% ID/g). Moreover, the accumulation of [125I]6 in the tumor was greatly inhibited by co-injection of an excess RGD peptide. However, a single injection of [131I]6 (11.1 MBq) did not inhibit tumor growth in tumor-bearing mice. We expect that the labeling method for targeted alpha therapy with $^{211}$At using a dimer RGD peptide could prove useful in future clinical applications.

Keywords: RGD peptide; integrin; tumor; alpha-particle

1. Introduction

“Theranostics” is a term that combines “therapeutics” and “diagnostics” and refers to the use of specific agents or techniques that combine diagnosis and targeted therapy [1]. Theranostics has recently gathered considerable attention in oncology as being a safe and effective method for providing personalized medical treatment using a tailored combination of medications to diagnose and subsequently target tumors [2]. In nuclear medicine, cancer is confirmed via molecular imaging with positron emission tomography (PET) or conventional nuclear medicine imaging, including planar or single-photon emission computed tomography (SPECT) imaging, and subsequent therapy involves the delivery of targeted radionuclide therapy with $\alpha$-particle or $\beta$-particle emitter radionuclides. Supposing the diagnostic and therapeutic radiopharmaceuticals show similar biodistribution, absorbed doses in the tumor and each normal tissue during therapy can be calculated based on the quantitative imaging at the time of diagnosis. As the therapeutic effects and side effects of delivered radionuclides are predictable, we can more accurately select the correct therapeutics for each individual patient prior to treatment. Therefore, nuclear medicine is a reasonable method for integrating theranostics. The method of using radioisotopes for theranostics is called “radiotheranostics” [3,4].
In nuclear medicine therapy, targeted alpha therapy (TAT) has gained much attention because of the excellent therapeutic effects derived from the high linear energy transfer (LET) of alpha-particles [5]. Among various alpha emitters, 211At has become more popular because the half-life (t_{1/2} = 7.2 h) of 211At could be sufficient for TAT, and 211At can be produced from natural bismuth targets via the 209Bi(α,2n)211At nuclear reaction by cyclotron [6]. Numerous promising preclinical studies with 211At have been reported in recent years [7].

We recently developed a novel 211At-labeling method for peptides using RGD peptide as a model peptide [8]. The 211At-labeled RGD peptide [211At]c[RGDf(4-At)K] and the corresponding radioiodine-labeled RGD peptide [125I]c[RGDf(4-I)K] showed high accumulation in the tumor and similar biodistribution. The results indicated that radiotheranostics combining [123I]c[RGDf(4-I)K] imaging and [211At]c[RGDf(4-At)K] therapy is possible. On the other hand, the superior biodistribution of tracers, such as higher tumor accumulation, prolonged tumor retention, and lower uptake in normal tissues, would be required for specific therapeutic effects. As one of the strategies to improve the tracer characteristics, it has been reported that the affinity of RGD peptide for α3β3 integrin is improved by the usage of multivalent peptides, such as dimeric peptides or tetrameric peptides [9].

In this study, we hypothesize that applying the labeling method to a dimeric RGD peptide leads to the development of superior probes for radiotheranostics using a combination of radioiodine and 211At. Thus, we synthesized and evaluated the [125/131I]-labeled dimer RGD peptide E[c(RGDfK)][{125/131I}c(RGDf(4-I)K)] ([125/131I]6) (Figure 1) as a preliminary step for radiotheranostics with 211At-labeled dimer RGD peptide. This is because the radiolabeling with radiohalogens such as [125/131I] and 211At can be achieved using the same tributyltin precursors, and 211At-labeled probes showed very similar biodistribution patterns in our previous studies [8,10,11]. Moreover, 125I is commercially available and has a long half-life (t_{1/2} = 59.4 d), which is appropriate for fundamental research. 131I is also commercially available and was used for radionuclide therapy to compare the therapeutic effects of 211At in the future.

![Figure 1. A chemical structure of [125/131I]6.](image)

2. Results

2.1. Preparation of [125/131I]6

Scheme 1 shows a synthetic scheme of [125/131I]6 and its precursor. After 1 and 2 were synthesized using a general Fmoc solid-phase synthesis method, 3 was synthesized by conjugating Fmoc-Glu-OAll with 1. 4 was synthesized by deprotecting the allyl group of 3, and 5 was synthesized by conjugation of 4 with 2. 6 was synthesized by deprotection of 5. The iodo group in 5 was replaced with a tributylstannyl group via a Pd-catalyzed stannylation reaction to synthesize 7. We then performed [125/131I]-labeling and deprotection of the protecting groups. The radiochemical yields in the two-step method of [125I]6 and [131I]6 were 38% and 24%, respectively. Following HPLC purification, [125I]6 and [131I]6 had radiochemical purities of over 96% and 92%, respectively. The total radiosynthesis time for [125/131I]6 was about 3 h including HPLC purification. The radiochemical yields
of $[^{125/131}I]6$ were low due to the complicated radiolabeling procedure by two steps. The identities of $[^{125/131}I]6$ were verified by comparing their retention times with 6 (Figure S1). Although the radiochemical purity of $[^{131}I]6$ was not enough even after HPLC purification, the therapeutic experiments were performed without further purification because the amount of radioactivity was prioritized.

![Scheme 1](image)

Scheme 1. Synthetic scheme of 6 and $[^{125/131}I]6$. Reagents: (a) Fmoc-Glu-OAll, TBTU, DIPEA, DMF (b) phenylsilane, DMF (c) (i) $[\text{R(Pbf)}\text{GD(OBu)}(4-\text{I})\text{K}]$ (2), TBTU, DIPEA, DMF (ii) piperidine (d) TFA, water, TIS. (e) Bis(tributyltin), tris(dibenzylideneacetone)dipalladium(0), DIPEA, methanol (f) (i) $[^{125}I]\text{NaI}$, NCS, acetic acid, acetonitrile (ii) TFA, water, TIS.

2.2. αvβ3 Integrin Binding Assay

The affinity of 6 and E[c(RGDfK)$_2$], a dimer RGD peptide without iodine, for αvβ3 integrin was determined via a competitive binding assay with U-87 MG cells. Representative displacement curves of the assay are shown in Figure 2. Binding of the radioligand $[^{125}I]\text{c[RGDy(3-I)V]}$ to αvβ3 integrin was inhibited by 6 and E[c(RGDfK)$_2$] in a concentration-dependent manner. The half-maximal inhibitory concentration (IC$_{50}$) values (nM) for 6 and E[c(RGDfK)$_2$] were 1.2 ± 0.5 and 0.8 ± 0.4 (mean ± SD for three independent experiments), respectively. The results indicate that 6 and E[c(RGDfK)$_2$] possess a specific affinity for αvβ3 integrin. Furthermore, these similar values of IC$_{50}$ of 6 and E[c(RGDfK)$_2$]...
ments), respectively. The results indicate that in vivo deiodination of \([^{125}\text{I}]6\) hardly occurred.

2.3. Determination of the Partition Coefficient

An experimental Log \(p\) value of \([^{125}\text{I}]6\) was \(-2.33 \pm 0.04\). The value was higher than that of the monomeric radioiodine-labeled RGD peptide \([^{125}\text{I}]c\text{[RGDf(4-I)K]} (-3.04 \pm 0.46\) from our previous study [8]. This result indicates that lipophilicity was increased by dimerization of the RGD peptide.

2.4. In Vitro Stability

An in vitro stability experiment of \([^{125}\text{I}]6\) in PBS(\(-\)) (pH 7.4) solution was performed. After incubation at 37 °C for 24 h, 90.8 \pm 0.7% (mean \pm SD for three samples) of its radioactivity remained intact.

2.5. Biodistribution Experiments

A comparison of the biodistribution experiments in U-87 MG tumor mice between \([^{125}\text{I}]6\) and \([^{125}\text{I}]c\text{[RGDf(4-I)K]}\) is shown in Figure 3 and Table S1. \([^{125}\text{I}]6\) was highly accumulated in the tumor based on the results of the in vitro assay. Specifically, at 4 h postinjection, the tumor accumulation of \([^{125}\text{I}]6\) (4.12 \pm 0.42% ID/g) was significantly higher than that of \([^{125}\text{I}]c\text{[RGDf(4-I)K]}\) (2.93 \pm 0.08% ID/g) [8]. The accumulation of \([^{125}\text{I}]6\) in the liver and intestines also tended to be higher than that of \([^{125}\text{I}]c\text{[RGDf(4-I)K]}\). Meanwhile, it is known that the radioactive accumulation in the thyroid gland and stomach is an index of deiodination of radioiodine labeled probes. In this study, the accumulation of radioactivity in the neck containing the thyroid glands and stomach was low (Figure 3), suggesting that in vivo deiodination of \([^{125}\text{I}]6\) hardly occurred.

An in vivo blocking study was performed to evaluate whether tumor accumulation was derived from \(\alpha_v\beta_3\) integrin specificity. The effect of c(RGDfK) on tumor uptake of \([^{125}\text{I}]6\) at 1 h postinjection is shown in Figure 4. Co-injection of an excess of c(RGDfK) drastically decreased the tumor uptake of \([^{125}\text{I}]6\), indicating that the tumor accumulation of \([^{125}\text{I}]6\) is caused by its specific binding via \(\alpha_v\beta_3\) integrin. Moreover, it also significantly reduced the accumulation of radioactivity in numerous types of normal tissues (Table S1). It is known that \(\alpha_v\beta_3\) integrin is expressed in the microvessels of normal tissues, such as the liver and lungs [12]. Thus, the result of the blocking study is reasonable for \([^{125}\text{I}]6\) as an \(\alpha_v\beta_3\) integrin-directing agent.
An in vivo blocking study was performed to evaluate whether tumor accumulation is caused by its specific binding via \( \alpha_\beta_3 \) integrin. The effect of c(RGDfK) on tumor uptake of \([^{125}\text{I}]c[\text{RGDf(4-I)K}]\)† is significant in U-87 MG tumor bearing mice (mean ± SD).† Data from reference [8]. No data of neck for \([^{125}\text{I}]c[\text{RGDf(4-I)K}]\).

**Figures:***

**Figure 3.** Biodistribution experiments. Biodistribution of radioactivity at (a) 1 h and (b) 4 h after intravenous injection of \([^{125}\text{I}]c[\text{RGDf(4-I)K}]\)† and \([^{125}\text{I}]\)6 in U-87 MG tumor bearing mice (mean ± SD).† Expressed as % injected dose. Significance was determined by unpaired Student’s t-test (*** \( p < 0.001 \)).

**Figure 4.** Blocking study. Comparison of tumor uptake (mean ± SD) of \([^{125}\text{I}]\)6 at 1 h postinjection under no-carrier-added conditions and with co-injection of an excess of c(RGDfK). Significance was determined by unpaired Student’s t-test (*** \( p < 0.001 \)).

**2.6. Radionuclide Therapy**

The tumor volume and body weight of tumor-bearing mice after treatment in the \([^{131}\text{I}]\)6 treatment and control groups are shown in Figure 5 and Figure S2, respectively. There was no significant difference in tumor volume and body weight between the treatment and control groups.
3. Discussion

We recently reported the simple one-step reaction of an 125I- and 211At-labeling method of a monomeric RGD peptide via a Pd-catalyzed stannylation reaction after deprotection [8], whereas, in this study, deprotection after the 125I/131I-labeling reaction, namely the two-step reaction, was performed because the Pd-catalyzed stannylation reaction failed after deprotection. The reason for this failure is not apparent; however, the yield of the stannylation reaction of the monomeric RGD peptide is lower (25%) than that of other stannylation reactions in previous studies (45–75%) [8,13,14]. We suppose that this difference might be derived from impeding the efficient stannylation reaction by functional groups of the amino acid residues of RGD peptides. As the number of the functional groups in E[c(RGDfK)]c[c(RGDf(4-I)K)] (6) is more than that of c[RGDF(4-I)K], it might significantly impede the stannylation reaction. Meanwhile, we expect that the reaction of the 211At-labeled dimer RGD peptide by the two-step method is possible because the 211At-labeled monomer RGD peptide was also synthesized by a similar two-step method [8]. However, the radiochemical yield of the 211At-labeled dimeric RGD peptide could prove to be too low by this method. Thus, modifying the labeling method might be necessary to improve the complication of the labeling procedure and the radiochemical yields of 125I/131I and 211At-labeled dimer RGD peptides. For this purpose, a one-step radiolabeling method using a radiolabeling precursor without protecting groups would be required. To achieve the precursor synthesis, we will explore the direct stannylation reaction of a non-protected dimer RGD peptide by improving the metal-catalyzed stannylation reaction or investigating other stannylation reactions, such as photochemical stannylation reactions [15].

The binding affinity of 6 for αvβ3 integrin (IC50: 1.2 ± 0.5 nM) was higher than that of c[RGDF(4-I)K] (IC50: 23.2 ± 17.2 nM) [11]. It was reported that IC50 values of dimeric RGD peptides in the αvβ3 integrin competitive binding assay were an order lower than those of original monomeric RGD peptides [16]. Thus, the IC50 value of dimerizing peptide 6 was consistent with those described in previous studies.

This biodistribution study found that the accumulation of a dimeric RGD peptide, 125I6, in the tumor tissue was higher than that of a monomeric RGD peptide, 125Ic[RGDF(4-I)K], as reflected in the results of the in vitro αvβ3 integrin binding assay. Therefore, 211At-labeled dimeric RGD peptide could also accumulate highly in the tumor because the similarity biodistribution of 125I- and 211At-labeled dimeric RGD peptides is expected [8]. However, the accumulation of 125I6 in the liver and intestines was higher than that of 125Ic[RGDF(4-I)K]; we suggest that the reason for this is the increased lipophilicity due to the dimerization of the RGD peptide. Notably, nearly all published studies of radiometal-labeled RGD peptides have reported that dimeric RGD peptides showed higher uptakes in the tumor and kidneys than did corresponding monomeric RGD peptides [9,16]. Although Log p values increased by dimerization in those reports, the Log p values were still much lower than those in this study. Thus, the renal excretion of the radiometal-labeled RGD peptides should not be changed. Dijkgraaf et al. proposed that the increased kidney
uptake of multimeric RGD peptides was caused by two factors [16]: (1) the expression of \( \beta_3 \) integrins on the endothelial cells of glomeruli vessels [17] and (2) the change in charge brought about by multimerization. Considering the results of this study and these previous reports, \(^{125}\text{I}\)6 could lead to higher accumulation in the kidneys by dimerization; however, the rate of hepatobiliary excretion increased due to increased lipophilicity. Thus, kidney accumulation of \(^{125}\text{I}\)6 did not change significantly compared with that of \(^{125}\text{I}\)c\([\text{RGDf(4-I)K}]\). On the other hand, to further increase the tumor uptake, the introduction of a linker should be effective because it was reported that an appropriate distance between cyclic RGD peptides is important for multivalent effects [18].

In radionuclide therapy, a single administration of \(^{131}\text{I}\)6 (11.1 MBq) did not inhibit tumor growth in tumor-bearing mice (Figure 5). In a previous study, multiple administrations of the same dose of \(^{90}\text{Y}\)-labeled RGD peptide inhibited tumor growth in tumor-bearing mice; however, a single administration of \(^{90}\text{Y}\)-labeled RGD peptide did not affect tumor growth [19]. As no significant decrease in the body weight was observed in this study (Figure S2), a higher radiation dosage or multiple administrations may be appropriate and may further inhibit tumor growth. Meanwhile, it has also been reported that conjugation of a \(^{177}\text{Lu}\)-labeled RGD peptide with Evans Blue (EB) as an albumin-binding moiety positively affected its pharmacokinetics to elevate uptake and the residence time in the tumor, and it showed higher tumor growth inhibition than the \(^{177}\text{Lu}\)-labeled RGD peptide without EB [20]. Therefore, increased tumor accumulation of \(^{131}\text{I}\)6 by structural modification such as conjugation with an albumin binder may make it possible to inhibit tumor growth.

4. Materials and Methods

4.1. Materials

\(^{125}\text{I}\)Sodium iodide (644 GBq/mg) and \(^{131}\text{I}\)Sodium iodide (185 GBq/mg) were purchased from PerkinElmer (Waltham, MA, USA). Electrospray ionization mass spectra (ESI-MS) were obtained with a JEOL JMS-T100TD (JEOL Ltd., Tokyo, Japan). Purification and identification of peptides and labeled peptides were performed using an HPLC system (LC-20AD pump, SPD-20A UV detector at a wavelength of 220 nm, and CTO-20A column oven maintained at 40 °C, Shimadzu, Kyoto, Japan). Fmoc-Lys(Boc)-OH was purchased from Merck (Darmstadt, Germany). 2-Chlorotrityl chloride resin, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-D-Tyr(Bu)-OH, Fmoc-D-Phe-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Val-OH, and Fmoc-Glu-OAll were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). 1,4,7,10-Tetraazacyclododecane-1,4,7-tris-(t-butyl acetate) (DOTA-tris) was purchased from Macrocyclus (Dallas, TX, USA). U-87 MG glioblastoma cells were purchased from DS Pharma Biomedical (Osaka, Japan). Fmoc-4-iodo-\( \text{D}-\text{phenylalanine [Fmoc-} \text{D}-\text{Phe(4-I)]} \) was synthesized according to a previous report [21]. \( \text{N,N}-\text{Diisopropylethylamine (DIPEA) was purchased from Nacalai Tesque (Kyoto, Japan).} \) 1,3-Diisopropylcarbodiimide (DIPCdi) and 1-hydroxybenzotriazole hydrate (HOBt) were purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). 2-\( (1\text{H-Benzotriazol-1-yl})-1,3,3\text{-tetracylamino} \) 4fluoro-borate (TBTU) was purchased from Chem Impex International, Inc. (Wood Dale, IL, USA). Other reagents were of reagent grade and used as received.

4.2. Synthesis of Reference Compounds and Radiolabeled Compounds

\text{E}[c(\text{RGDfK})_c(\text{RGDf(4-I)K})] (6) and \text{E}[c(\text{RGDfK})][^{125/131}\text{I}][c(\text{RGDf(4-I)K})] ([^{125/131}\text{I}]6) were synthesized according to the procedure outlined in Scheme 1.}

4.2.1. Preparation of c[R(Pbf)GD(OtBu)fK] (1) and c[R(Pbf)GD(OtBu)f(4-I)K] (2)

Cyclic[Arg(Pbf)-Gly-Asp(OtBu)-D-Phe-Lys] (1) and cyclic[Arg(Pbf)-Gly-Asp(OtBu)-D-Phe(4-I)-Lys] (2) was synthesized manually using a standard Fmoc-based solid-phase methodology according to previous reports with slight modifications [8,22]. The crude peptide of 1 and 2 were purified by reversed phase (RP)-HPLC on Cosmosil 5C18-AR-II column (20 × 250 mm; Nacalai Tesque) at a flow rate of 12 mL/min with an isocratic
mobile phase of 65% methanol in water with 0.1% trifluoroacetic acid (TFA). The solvents were removed by lyophilization to provide 1 (27.1 mg, 30%) and 2 (26.5 mg, 25%) as a white powder.

\[ \text{c[R(Pbf)GD(OtBu)fK]} \] (1): MS (ESI\(^+\)) calcd for \( C_{44}H_{65}N_9O_{10}S \) [M + H]\(^+\): \( m/z = 912.46 \)

\[ \text{c[R(Pbf)GD(OtBu)f(4-I)K]} \] (2): MS (ESI\(^+\)) calcd for \( C_{44}H_{64}IN_9O_{10}S \) [M + H]\(^+\): \( m/z = 1038.36 \)

4.2.2. Preparation of Fmoc-E\{c[R(Pbf)GD(OtBu)fK]\}OAll (3)

Fmoc-Glu(OAll)-OH (29.6 mg, 72.4 µmol) was dissolved in 1 mL of DMF, and then TBTU (11.2 mg, 65.8 µmol) and DIPEA (23.0 µL, 132 µmol) were added to the solution. After stirring at room temperature for 1 h, compound 1 (20.0 mg, 22.0 µmol) was added to the reaction mixture. After further stirring for 1 h, the reaction mixture was purified by RP-HPLC with a Cosmosil 5C\(\text{18}^{-}\)-AR-II column (20 × 250 mm) at a flow rate of 12 mL/min with an isocratic mobile phase of 85% methanol in water with 0.1% TFA for 20 min. The solvent was removed by lyophilization to yield 3 (16.9 mg, 59%) as a white powder.

\[ \text{Fmoc-E\{c[R(Pbf)GD(OtBu)fK]\}OAll} \] (3): MS (ESI\(^+\)) calcd for \( C_{67}H_{87}N_{10}O_{15}S \) [M + H]\(^+\): \( m/z = 1303.61 \); found, 1304.02.

4.2.3. Preparation of Fmoc-E\{c[R(Pbf)GD(OtBu)fK]\}OH (4)

Compound 3 (9.7 mg, 7.4 µmol) was dissolved in 1 mL of DMF, and then Pd(PPh\(_3\))\(_4\) (8.6 mg, 7.4 µmol) and phenylsilane (9.1 µL, 74 µmol) were added to the solution. After stirring at room temperature for 1 h, the reaction mixture was purified by RP-HPLC with a Cosmosil 5C\(\text{18}^{-}\)-AR-II column (20 × 250 mm) at a flow rate of 12 mL/min with a gradient mobile phase of 80% methanol in water with 0.1% TFA to 90% methanol in water with 0.1% TFA for 20 min. The solvent was removed by lyophilization to yield 4 (7.8 mg, 83%) as a white powder.

\[ \text{Fmoc-E\{c[R(Pbf)GD(OtBu)fK]\}OH} \] (4): MS (ESI\(^+\)) calcd for \( C_{64}H_{83}N_{10}O_{15}S \) [M + H]\(^+\): \( m/z = 1263.58 \); found, 1263.95.

4.2.4. Preparation of E\{c[R(Pbf)GD(OtBu)fK]\}{c[R(Pbf)GD(OtBu)f(4-I)K]} (5)

Compound 4 (7.8 mg, 6.2 µmol) was dissolved in 1 mL of DMF, and then TBTU (3.0 mg, 9.3 µmol) and DIPEA (3.3 µL, 19 µmol) were added to the solution. After stirring at room temperature for 1 h, compound 2 (9.6 mg, 9.3 µmol) was added to the reaction mixture. After 1 h stirring, solvent was removed by rotary evaporation then 1 mL of piperidine was added to the residue. After 1 h stirring at room temperature, purification was performed by RP-HPLC with a Cosmosil 5C\(\text{18}^{-}\)-AR-II column (20 × 250 mm) at a flow rate of 12 mL/min with an isocratic mobile phase of 85% methanol in water with 0.1% TFA for 20 min. The solvent was removed by lyophilization to yield 5 (6.9 mg, 54%) as a white powder.

\[ \text{E\{c[R(Pbf)GD(OtBu)fK]\}{c[R(Pbf)GD(OtBu)f(4-I)K]}} \] (5): MS (ESI\(^+\)) calcd for \( C_{93}H_{135}IN_{19}O_{22}S_2 \) [M + H]\(^+\): \( m/z = 2060.85 \); found, 2061.94.

4.2.5. Preparation of E\{c[RG(DfK)]\}{c[RG(Df(4-I)K)]} (6)

Compound 5 (3.0 mg, 1.5 µmol) was treated with a mixture of 95% TFA, 2.5% water, and 2.5% trisopropylsilane (TIS) for 2 h at room temperature. The crude peptide was purified by RP-HPLC on Cosmosil 5C\(\text{18}^{-}\)-AR-II column (10 × 250 mm) at a flow rate of 4 mL/min with a gradient mobile phase of 35% methanol in water with 0.1% TFA to 55% methanol in water with 0.1% TFA for 20 min. The solvent was removed by lyophilization to provide 6 (1.5 mg, 71%) as a white powder.

\[ \text{E\{c[RG(DfK)]\}{c[RG(Df(4-I)K)]}} \] (6): MS (ESI\(^+\)) calcd for \( C_{59}H_{82}IN_{19}O_{16} \) [M + H]\(^+\): \( m/z = 1444.56 \); found, 1444.59.
4.2.6. Preparation of E[c[R(Pbf)GD(OtBu)fK]](c[R(Pbf)GD(OtBu)f(4-SnBu3)K]) (7)

Compound 5 (500 µg, 292 nmol) was dissolved in 500 µL of methanol. Bis(tributyltin) (23.3 µL, 46.6 µmol), tris(dibenzylideneacetone)dipalladium(0) (1.54 mg, 1.68 µmol), and DIPEA (4.8 µL, 28.0 µmol) were added to the solution of 5. After stirring at 60 °C for 1 h, the reaction mixture was purified by RP-HPLC with a Cosmosil 5C18-AR-II column (10 × 250 mm) at a flow rate of 4 mL/min with a gradient mobile phase of 85% methanol in water with 0.1% TFA to 95% methanol with 0.1% TFA for 20 min. The solvent was removed by lyophilization to yield 7 as colorless oil.

E[c[R(Pbf)GD(OtBu)fK]](c[R(Pbf)GD(OtBu)f(4-SnBu3)K]) (7) MS (ESI+) calcd for C105-H163N19O22S2Sn [M + 2H]2+: m/z = 1112.53; found, 1112.98.

4.2.7. Preparation of E[c(RGDfK)][125I]c[RGDf(4-I)K]2 ([125I]6)

A small amount of 7 was dissolved in 5 µL of acetonitrile in the reaction vial. A measure of 10 µL of 1% acetic acid in acetonitrile, 3 µL of [125I]NaI (3.7 MBq) solution, and 15 µL of N-chlorosuccinimide (NCS) in acetonitrile (1 mg/mL) were added to the vial. After heating at 80 °C for 15 min, the reaction mixture was quenched with 15 µL of NaHSO3 solution (1 mg/mL). After evaporating the solvent using N2 gas, the residue was treated in 100 µL of a mixture of 95% TFA, 2.5% water, and 2.5% TIS for 90 min at room temperature, and then purified by RP-HPLC with a Cosmosil 5C18-AR-II column (4.6 × 150 mm) at a flow rate of 1 mL/min with a gradient mobile phase of 35% methanol in water with 0.1% TFA to 55% methanol in water with 0.1% TFA for 20 min. The radiochemical yield and the radiochemical purities of [125I]6 were 38% and >96%, respectively.

4.2.8. Preparation of E[c(RGDfK)][131I]c[RGDf(4-I)K]2 ([131I]6)

[131I]6 was synthesized in the same synthetic method as [125I]6, using [131I]NaI (185 MBq) instead of [125I]NaI. The radiochemical yields of [131I]6 was 24%. The radiochemical purity of [131I]6 was >92%.

4.3. αvβ3 Integrin Binding Assay

Binding affinities of synthesized peptides E[c(RGDfK)]2 and 6 for αvβ3 integrin were evaluated by competitive inhibition between the peptides and [125I]c[RGDy(3-I)V], which was prepared by Fmoc solid-phase synthesis and following 125I-labeling with chloramine-T method [23], to αvβ3 integrin according to a previously reported procedure [24]. The peptides’ half maximal inhibitory concentration (IC50) values were calculated by curve fitting with nonlinear regression using GraphPad Prism 8.4.3 (GraphPad Software Inc., San Diego, CA, USA). Each data point is the average of four determinations, and IC50 values were expressed as mean ± standard deviation (SD) from three independent experiments.

4.4. Determination of the Partition Coefficient

The partition coefficient of [125I]6 was measured as described previously [25]. The radioactivity in each phase was measured with an auto-well gamma counter. The partition coefficient was determined by calculating the ratio of 1-octanol to the buffer and was expressed as a common logarithm (log P).

4.5. In Vitro Stability

To evaluate the stability of [125I]6 in PBS(−), 10 µL of each tracer (37 kBq) was added to 90 µL of PBS(−) (pH 7.4), and the solutions were incubated at 37 °C for 24 h. After incubation, the samples were drawn, and the radioactivity was analyzed by RP-HPLC.

4.6. Biodistribution of E[c(RGDfK)][125I]c[RGDf(4-I)K]2 ([125I]6) in Tumor-Bearing Mice

Experiments with animals were conducted in strict accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University. The experimental protocols were approved by the Committee on Animal Experimentation of Kanazawa University. The animals were housed with free access to food and water at 23 °C with a
12 h alternating light/dark schedule. We used a U-87 MG cell line because it was reported that the U-87 MG highly expresses $\alpha_v\beta_3$ integrin [26]. U-87 MG cells were grown and inoculated subcutaneously into 4-week-old female BALB/c nude mice (15–19 g, Japan SLC, Inc., Hamamatsu, Japan) as previously reported [22]. Biodistribution experiments were performed approximately 14 days post-inoculation. A solution of $^{125}$I (37 kBq) was intravenously administered to groups of four mice. Mice were sacrificed at 1 and 4 h postinjection. Tissues of interest were removed and weighed. A neck containing thyroid was resected. The radioactivity counts of $^{125}$I was determined with an auto-well gamma counter (ARC-7010, Hitachi, Ltd., Tokyo, Japan) and corrected for background radiation and physical decay during counting. A window from 16 to 71 keV was used for counting $^{125}$I.

To investigate the effect of an excess amount of RGD peptide on biodistribution, U-87 MG tumor-bearing mice were intravenously administered 100 $\mu$L of a mixture solution of $^{125}$I (37 kBq) with c(RGDfK) peptide (0.2 mg/mouse). Mice ($n = 4$) were sacrificed at 1 h postinjection, and biodistribution experiments were conducted as described above.

4.7. Radionuclide Therapy

U-87 MG tumor-bearing mice ($n = 4$) as a treated group were intravenously administered 100 $\mu$L of $^{131}$I (11.1 MBq), and U-87 MG tumor-bearing mice ($n = 3$) as a control group were intravenously administered 100 $\mu$L of saline. The dose of $^{131}$I was unified with a previous therapeutic study with $^{90}$Y-DOTA-c(RGDfK) (11.1 MBq) [19]. The tumor volume and body weight of mice were monitored 3–5 times weekly. The tumor size was measured with a slide caliper, and the tumor volume was calculated using the following formula: volume = $4/3 \pi (1/2 \text{ length} \times 1/2 \text{ width} \times 1/2 \text{ height})$.

4.8. Statistical Evaluation

Animal experiments were compared using unpaired Students’ $t$-test.

5. Conclusions

In this study, we synthesized an iodine-introduced dimer RGD peptide, E[c(RGDfK)]$\{c[RGDf(4-I)K]\}$ (6), and an $^{125/131}$I-labeled dimer RGD peptide, E[c(RGDfK)]$[^{125/131}]c[-(RGDf(4-I)K)]$ ([$^{125/131}$I]6) and evaluated them as a preliminary step toward the synthesis of an $^{211}$At-labeled dimer RGD peptide. The affinity of 6 for $\alpha_v\beta_3$ integrin and the accumulation of $^{125}$I 6 in tumor tissues of U-87 MG tumor-bearing mice improved compared to monomer RGD peptides. However, a single injection of $^{131}$I 6 (11.1 MBq) did not inhibit tumor growth in tumor-bearing mice. This labeling method using the same precursor may be applicable to $^{123}$I-labeling for SPECT using E[c(RGDfK)]$[^{123}]c[RGDf(4-I)K]_2$ ([$^{123}$I]6) and $^{211}$At-labeling for TAT using an $^{211}$At-labeled dimer RGD peptide. The use of such radiolabeled peptides is expected to play a significant role in the future of clinical radiotheranostics.

Supplementary Materials: The following are available online, Figure S1: The chromatogram of (a) $^{125}$I 6 and (b) 6, Figure S2: Body weight of U-87 MG tumor-bearing mice treated with $^{131}$I 6 or with no treatment, Table S1: Biodistribution of radioactivity after intravenous injection of $^{125}$I 6 in U-87 MG tumor bearing mice.

Author Contributions: Conceptualization, K.O.; methodology, H.E., K.M. and K.O.; validation, H.E., K.M., T.F., K.S. and K.O.; formal analysis, H.E. and K.M.; investigation, H.E.; resources, K.O.; writing—original draft preparation, H.E. and K.O.; writing—review and editing, K.M. and K.O.; supervision, K.M. and K.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported in part by Grants-in-Aid for Scientific Research (21H02867) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, Mitani Foundation for Research and Development, Pancreas Research Foundation of Japan, Kanazawa University SAKIGAKE project 2020.
Institutional Review Board Statement: The animal experimental protocols were approved by the Committee on Animal Experimentation of Kanazawa University (AP-204165).

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

Sample Availability: Samples of the compounds synthesized are available from the authors.

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