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Synthesis of DOTA-conjugated multivalent cyclic-RGD peptide dendrimers via 1,3-dipolar cycloaddition and their biological evaluation: implications for tumor targeting and tumor imaging purposes†‡

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Introduction

Integrins are a class of heterodimeric transmembrane proteins, which play an important role in cell-signaling, cell-cell adhesion, apoptosis and cell-matrix interactions. Integrin αβ3, which binds to the Arg-Gly-Asp (RGD) tripeptide motif containing ligands, plays a pivotal role in tumor angiogenesis and metastasis. αβ3 Integrin expressed on endothelial cells modulate cell migration and survival during angiogenesis, whereas αβ3 integrin expressed on carcinoma cells potentiate metastasis by facilitating invasion and movement across blood vessels. The αβ3 integrin is expressed on activated endothelial cells during tumor induced angiogenesis, whereas it is absent on quiescent endothelial cells and normal tissues. In addition, αβ3 is expressed on various tumor cell types (e.g. breast, ovarian, and prostate cancers). Evidence exists that inhibition of αβ3 integrin function prevents tumor growth and induces tumor regression by antagonizing angiogenesis. Several peptidic and peptidomimetic αβ3 antagonists have been synthesized. Among these, the cyclo[Arg-Gly-Asp-D-Phe-Val] (c[RGDVal]), as developed by Kessler and coworkers, is one of the most active and selective antagonists for the αβ3 integrin.

Structure–activity relationship studies on this cyclic pentapeptide showed that the exchange of the valine by a lysine residue (Lys, K) did not significantly influence activity and selectivity. Because the ε-amino moiety of the lysine residue can be easily modified, numerous applications of c[RGDfK] have been studied for tumor targeting and imaging.

Multivalency is a well accepted approach to increase the interaction of weakly interacting individual ligands with their respective receptors. Dendrimers are macromolecules consisting of multiple perfectly branched monomers and this architecture makes them versatile constructs for the simultaneous presentation of receptor binding ligands and other biologically relevant molecules. Additionally, dendrimers might serve as promising molecular scaffolds containing a number of ligands thereby inducing an apparent increase of ligand concentration and increasing the probability of statistical rebinding. Alternatively, dendrimers may align these ligands and induce multivalency when receptor clustering occurs or is initiated after initial monovalent binding. To improve tumor targeting efficacy and to obtain better in vivo imaging properties, several studies explored the multivalency effect by using dimeric and tetrameric RGD peptides with affinity toward the αβ3 integrin. These studies clearly demonstrated the multivalency effect, since the in vivo affinity significantly increased going from monomer via dimer to tetramer. Moreover, also with respect to tumor-uptake and tumor-to-organ ratios, a similar increase was observed. These are promising results in view of the development of integrin-targeted radionuclide therapy.

To decorate the dendrimer end-groups with biologically relevant peptides as ligands, it is of crucial importance to have the disposal of efficient and chemoselective conjugation chemistry to ensure the complete attachment of the ligands to the dendrimer. In cases of completely amino acid- or peptide-based dendrimers,
this is often achieved using peptide coupling reagents, however, in most cases, the peptide ligands are attached to dendrimers by chemoselective reaction of sulphydryl groups of cysteine residues with maleimide or iodoacetamide functionalities, by thiol-disulfide exchange, by native chemical ligation \( ^{17} \) or via a chemoselective oxime \( ^{16}_{\text{a-d-l-m}} \) respectively hydrazone \( ^{18} \) ligation. However, new bioconjugation reactions with mutually reactive conjugation partners with increased efficiency and chemoselectivity which are synthetically easily accessible would be very welcome.

Recently, the well-known reaction between an alkylene and an azide to yield 1,4-disubstituted 1,2,3-triazoles, was reinvestigated independently by Meldal \( ^{19} \) and Sharpless \( ^{19} \). They found that an alkylene and an azide in the presence of Cu(i) undergo a 1,3-dipolar cycloaddition to the corresponding triazole under very mild reaction conditions with very high chemoselectivity and efficiency which make this reaction particularly suitable for bioconjugations. So far, this 1,3-dipolar cycloaddition denoted as a ‘click reaction’, \( ^{20} \) has led to a plethora of applications in the literature. \( ^{20} \) Recently, we synthesized multivalent dendrimeric peptides \( ^{20} \) (up to octa- and hexadecavalent systems) respectively triazole-linked glycodendrimers \( ^{20} \) via a microwave-assisted 1,3-dipolar cycloaddition between azido peptides respectively glycosyl azides and dendrimeric alkenes as an alternative approach to functionalize dendrimers. \( ^{20} \)

Here we describe the synthesis of monomeric, dimeric and tetrameric [RGD(1K)] dendrimers via a microwave-assisted 1,3-dipolar cycloaddition of dendrimeric alkenes with the \( ^{N-c} \)-azido derivative of cyclo[Arg-Gly-Asp-D-Phe-Lys] and their subsequent evaluation as \( \alpha_1 \beta_3 \) integrin antagonists. Additionally, the RGD dendrimers were conjugated with a 1,4,7,10-tetraazacyclododecan-\( ^{N,N',N''}\)-tetraacetic acid (DOTA) moiety. These analogs were radiolabeled with \( ^{11} \text{In} \) to evaluate the \( \text{in vitro} \) receptor binding characteristics and \( \text{in vivo} \) tumor targeting properties.

**Results and discussion**

**Synthesis**

Schemes 1 and 2 illustrate our approach for the convergent synthesis of amino acid based dendrimers \( ^{23} \) and their corresponding DOTA-conjugated derivatives. Monovalent compound 2 and divalent 3 respectively, were synthesized starting from 3-hydroxy methyl benzoate or 3,5-dihydroxy methyl benzoate and propargylbromide in the presence of \( \text{K}_2\text{CO}_3 \) as a base and were obtained in 95 and 81% yield. Since these two compounds were also used as synthons in further syntheses, the resulting methyl esters 2 and 3 were treated with Tesser’s base \( ^{24} \) to yield acids 4 and 5 in nearly quantitative yield. After treatment of the previously described 6 \( ^{24} \) with TFA to remove both Boc-functionalities, the resulting bisamine TFA salt was coupled to acid 5 in the presence of BOP–DIPEA to give the tetravalent dendrimer 7 with 75% yield. To conjugate the tetravalent dendrimer with a DOTA-moiety at a later stage of the synthesis, its methyl ester was saponified with Tesser’s base and acid 8 was obtained quantitatively.

The DOTA-moiety was connected to the dendrimer core via a short ethylene spacer. For this purpose, 1,2-diaminoethane was converted into the mono-protected Boc derivative 10 which was obtained in 56% yield. Unfortunately, although a large excess of the amine was used, the bis-protected side product was obtained in a considerable amount. Compound 10 was coupled in the presence of BOP–DIPEA to either the monovalent, divalent or tetravalent dendrimer acids 4, 5 or 8 to obtain the corresponding amides 11, 12 or 15, respectively, generally in yields higher than 90%. The Boc-protected dendrimers were treated with TFA to obtain the corresponding amines and they were treated with BOP–DIPEA in the presence of 2-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl) acetic acid (DOTA(O\text{Bu})\text{)} to give the DOTA-conjugated mono-, di- and tetravalent dendrimers 13, 14.

**Scheme 1** Synthesis of the mono-, di- and tetravalent dendrimeric alkenes 2, 3 and 7.

\[ \text{Scheme 1} \quad \text{Synthesis of the mono-, di- and tetravalent dendrimeric alkenes 2, 3 and 7.} \]
and 16 respectively. It is important to note that the solubility of the DOTA-conjugated dendrimer is an important factor that determines the yield of the coupling reaction. Compounds 13 and 14 were isolated in very high yields (>94%) but compound 16 was isolated with a modest yield of 60% due to its low solubility in solvents like EtOAc and CH₂Cl₂.

The next step in the synthesis was the preparation of the N-ε-azido cyclo(Arg-Gly-Asp-D-Phe-Lys) peptide 19 (Scheme 3). To obtain this compound, peptide resin 17 was synthesized using Fmoc-’Bu SPPS (solid phase peptide synthesis) based on the protocol of Liu et al.³⁵ It was decided to cleave the protected peptide acid from the resin by HFIP–CH₂Cl₂ instead of AcOH–TFE to avoid premature acetylation during the BOP–DIPEA-mediated macrolactamization step. Cyclic peptide 18 was obtained in 36% overall yield based on the initial resin loading of 0.64 mmol g⁻¹. Subsequently, the ε-amine of the lysine residue was selectively converted into the azide moiety by a diazotransfer.²⁷ At pH 10, the ε-amine can be deprotonated in the presence of a guanidino functionality, since the latter is a much stronger base and will not act as a nucleophile in the diazotransfer reaction. Finally, the peptide N-ε-azido cyclo(Arg-Gly-Asp-D-Phe-Lys) 19 was obtained in 21% yield after purification by HPLC and was characterized by ¹H-NMR (500 MHz) and mass spectrometry (LC-MS). Incorporation of Fmoc-Lys(N₃)-OH, to avoid the diazo transfer as the final reaction step, did not substantially improve the isolated yield.

At this stage of the synthesis, the challenge was the chemoselective coupling of the different dendrimeric alkynes (2, 3, 7, 13, 14, or 16) to the cyclic RGD azido peptide (19) to furnish the DOTA-conjugated dendrimeric cyclo-RGD peptides as V₃ integrin antagonists as shown in Scheme 4. Our first experiments were based on the literature procedure¹⁹b in which acetylene 3 was coupled to azido glycine ethyl ester (ethyl 2-azidoacetate) in the presence of CuSO₄–Na-ascorbate–Cu-wire in tert-BuOH–H₂O for 16 h at room temperature. Monitoring the reaction by TLC showed that formation of the monovalent cycloadduct proceeded rapidly, but the conversion into the divalent product was sluggish. However, a tremendous improvement was achieved by running this reaction under microwave irradiation. After 10 min at 100°C using DMF–H₂O as solvent in the presence of CuSO₄–Na-ascorbate, the divalent cycloaddition product was obtained in 96% yield. This microwave-assisted cycloaddition of dendrimeric alkynes and azido peptides was recently reported as a versatile approach to obtain multivalent dendrimeric peptides.²²a The optimized reaction conditions were used to couple the cyclic RGD azido peptide (19) to the different dendrimeric alkynes (2, 3, 7, 13, 14, or 16).

In case of alkynes 2, 3 and 7 the formation of the cycloadducts 20, 21 and 22 could be followed by TLC and LC-MS. It turned
out that the formation of 20 and 21 was complete after 10 to 20 min microwave irradiation at 100 \(^\circ\)C, whereas the formation of 22 was complete after 30 min. Although HPLC analysis of the crude cycloaddition products evidenced a complete conversion as judged by the absence of the alkyne starting material, the RGD-dendrimers 20–22 were obtained in yields varying between 14 to 57%. Then, the DOTA-conjugated alkyne dendrimers 13, 14 and 16 were subjected to the cycloaddition reaction conditions in the presence of azido peptide 19. It should be emphasized that the carboxyl functionalities of the DOTA-moiety needed to be protected by tert-butyl groups to avoid premature and irreversible sequestering of the Cu\(^{2+}\) ions. Chelated copper(II) will result in a lower efficiency of the Cu(II)–Cu(I) redox couple to generate the active Cu(I)-catalyst. More importantly, it will hamper the radiolabeling of the DOTA-moiety of compounds 23–25 with trivalent radiometals such as \(^{111}\)In, \(^{89}\)Y or \(^{177}\)Lu. As a result, after the click reaction an additional reaction step was needed in which the partially protected cycloadducts were treated with TFA, in the presence of suitable scavengers, to give the unprotected DOTA-conjugated RGD-dendrimers 23–25.

The cycloaddition reaction of the DOTA-conjugated dendrimeric alkyne 13, 14 and 16 was difficult to monitor by mass spectrometry. As was described above, reaction times of 10 to 30 min were used and the cycloaddition reaction was directly followed by a TFA-treatment without isolation of the cycloaddition intermediates. The isolated yield (13\%) of monovalent 23 was rather disappointing. Recently, optimized conditions with respect to the generation of the catalytic active Cu(i) species were published\(^{28}\) and these conditions were applied in the cycloaddition of 14 and 19. Unfortunately, an increase of the isolated yield was not observed using these modified reaction conditions. As was mentioned earlier, the cycloaddition reaction was complete according to HPLC analysis, and the low isolated yield was mainly due to the difficult purification. The DOTA-conjugated RGD-dendrimers were obtained in yields varying between 11 and 36%.

Radiolabeling of the RGD dendrimers

Dendrimers 23, 24 and 25 were radiolabeled by dissolving these compounds in an NH\(_4\)OAc buffer of pH 6.0 and 22.2–37 MBq \(^{111}\)InCl\(_3\), was added to each of the reaction mixtures. The reaction mixtures were degassed and subsequently heated at 100 \(^\circ\)C for 15 min. Reversed phase-HPLC analysis showed a single peak for each of the three \(^{111}\)In-labeled compounds with an elution time of 25.9 min, 29.5 min and 29.4 min for the \(^{111}\)In-labeled monovalent 23, divalent 24, and tetravalent 25, RGD peptide dendrimers respectively.

Solid phase $\alpha, \beta_3$ binding assay

The affinity of the DOTA-conjugated RGD dendrimers 23, 24, and 25 for the $\alpha, \beta_3$ integrin was determined in a competitive binding assay. The results of these analyses are shown in Fig. 1. Binding of the $^{111}$In-labeled dimeric peptide, $^{111}$In-DOTA-Glu-(c[RGDfK])$_2$, to $\alpha, \beta_3$ was competed by unlabeled 23, 24, and 25 in a concentration dependent manner. The IC$_{50}$ values were 212 nM for monovalent 23, 356 nM for divalent 24, and 50 nM for tetravalent 25. The dendrimer containing four c[RGDfK] units (25) showed an increased affinity for $\alpha, \beta_3$ compared to the dendrimers containing one (23) or two (24) c[RGDfK] units. Multimerization of c[RGDfK] resulted in enhanced affinity for $\alpha, \beta_3$, as was evidenced by a decrease of the IC$_{50}$ concentration.

Biodistribution studies

In athymic mice with subcutaneously (s.c.) growing SK-RC-52 renal cell carcinoma, the tumor uptake of the $^{111}$In-labeled tetrameric RGD dendrimer 25 at 2 h post-injection (p.i.; 7.27 ± 2.06%ID/g) was significantly higher ($P < 0.05$) compared to that of the $^{111}$In-labeled monomeric RGD dendrimer 23 (1.69 ± 0.41%ID/g) as shown in Fig. 2A. At 2 h p.i., the tumor uptake of tetrameric RGD dendrimer 25 was also significantly higher ($P < 0.05$) than the dimeric analog 24 (3.15 ± 0.51%ID/g). The tumor-to-blood ratios of the tetramer 25 (5.66 ± 1.74%ID/g, 34.73 ± 5.93%ID/g) were significantly higher ($P < 0.05$)—both at 2 h p.i. and at 24 h p.i.—than those of the monomer 23 (3.12 ± 1.92%ID/g, 19.65 ± 12.42%ID/g) and dimer 24 (1.70 ± 0.50%ID/g, 14.66 ± 0.25%ID/g). At 24 h post injection, the tumor uptake of the tetrameric RGD dendrimer 25 (5.83 ± 1.18%ID/g) was significantly higher compared to the dimeric RGD dendrimer 24 (2.82 ± 0.59%ID/g, $P < 0.05$) and the monomeric RGD dendrimer 23 (1.19 ± 0.31%ID/g, $P < 0.01$) which is shown in Fig. 2B. Co-injection of an excess of non-radiolabeled RGD
peptide (DOTA-Glu-(c[RGDfK])₃) to saturate all α₅β₃ receptors in vivo, resulted in a significantly reduced tumor uptake of each of the three compounds: 23: 0.46 ± 0.04%ID/g (2 h p.i.), 0.36 ± 0.31%ID/g (24 h p.i.), 24: 0.76 ± 0.09%ID/g (2 h p.i.), not determined (24 h p.i.) and 24: 1.56 ± 0.02%ID/g (2 h p.i.), 1.19 ± 0.03%ID/g (24 h p.i.), indicating that each of the three RGD dendrimers of this study showed receptor mediated uptake in the tumor. These in vivo results were in line with the in vitro binding assay. The tetrameric RGD dendrimer showed enhanced affinity for α₅β₃, as compared to the monomeric and dimeric RGD dendrimer, respectively. The results of this study correlated nicely with the results observed in a previous study in which we evaluated multimeric RGD peptides in the same animal model.19

The affinity of the dendrimers as determined in an in vitro binding assay are in agreement with the results obtained from the in vivo experiment: the IC₅₀ concentration of the tetrameric RGD dendrimer 25 was lower compared to those of the monomeric 23 and dimeric 24 analogs, resulting in a significantly higher uptake of the former in α₅β₃-expressing tumors and better tumor-to-blood ratios compared to the monomeric and dimeric RGD dendrimers. In conclusion, a series of α₅β₃ integrin-directed monomeric, dimeric and tetrameric cyclo[Arg-Gly-Asp-d-Phe-Lys] dendrimers using “click chemistry” was successfully synthesized, since the unprotected N-ε-azido derivative of cyclo[Arg-Gly-Asp-d-Phe-Lys] underwent a highly chemoselective conjugation to amino acid-based dendrimers bearing terminal alkynes using a microwave-assisted Cu(i)-catalyzed 1,3-dipolar cycloaddition. The α₅β₃ binding characteristics and α₅β₃ targeting properties of the dendrimers were determined both in vitro and in vivo. In the case of the DOTA-conjugated ¹¹¹In-labeled RGD-dendrimers, it was found that the radiolabeled multimeric dendrimers showed specifically enhanced uptake in α₅β₃ integrin expressing tumors in vivo. These studies showed that the tetrameric RGD-dendrimer had better tumor targeting properties than its dimeric and monomeric congeners.

Experimental

Instruments and methods

Peptides were synthesized on an ABI 433A automatic Peptide Synthesizer using the FastMoc solid phase peptide synthesis protocols. Microwave-assisted reactions were carried out in a Biotage microwave reactor. Analytical HPLC runs were carried out on a Shimadzu HPLC system and preparative HPLC runs were performed on a Gilson HPLC workstation. Analytical HPLC runs were performed on Alltech Prosphere C4 or C8 and Adsorbosphere XL C18 columns (250 × 4.6 mm, pore size 300 Å, particle size: 5 μm) or on a Merck LiChroCART CN column (250 × 4.6 mm, pore size 100 Å, particle size: 5 μm) at a flow rate of 1.0 mL min⁻¹ using a linear gradient of buffer B (0–100% in 25 min) in buffer A (buffer A: 0.1% TFA in H₂O, buffer B: 0.1% TFA in CH₃CN–H₂O 95:5 v/v). Preparative HPLC runs were performed on an Alltech Prosphere C4 or C8 column (250 × 22 mm, pore size 300 Å, particle size: 10 μm), and semi-prep HPLC runs were performed on an Alltech Adsorbosphere XL C18 column (250 × 10 mm, pore size 300 Å, particle size: 10 μm) or on a Merck LiChroCART CN column (250 × 10 mm, pore size 100 Å, particle size: 10 μm) at a flow rate of 10.0 mL min⁻¹ (semi-prep HPLC: 4.0 mL min⁻¹) using a linear gradient of buffer B (0–100% in 50 min) in buffer A (buffer A: 0.1% TFA in H₂O, buffer B: 0.1% TFA in CH₃CN–H₂O 95:5 v/v). Liquid chromatography electrospray ionization mass spectrometry was measured on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. LC/MS(MS) runs were performed on a Finngan LCQ Deca XP MAX LC/MS equipped with a Shimadzu 10A VP analytical HPLC system. The samples were dissolved in 10% formic acid in CH₃CN–H₂O 1:1 v/v and analyzed using a Phenomenex Gemini C18 column (150 × 4.6 mm, particle size: 3 μm, pore size: 110 Å) at a flow rate of 1.0 mL min⁻¹ using a linear gradient of 100% buffer A (0.1% TFA in H₂O–CH₃CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH₃CN–H₂O 95:5 v/v) in 50 min. MALDI-TOF analysis was performed on a Kratos Axima CFR apparatus with bradykinin(1–7) (monoisotopic [M + H]⁺ 757.399), human ACTH(18–39) (monoisotopic [M + H]⁺ 2465.198) and bovine insulin oxidized B chain (monoisotopic [M + H]⁺ 3494.651) as external references and α-cyan-4-hydroxycinnamic acid or sinapinic acid as matrices. ¹H NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS. ¹³C NMR spectra were recorded on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to CDCl₃ (77.0 ppm). The ¹³C NMR spectra were recorded using the attached proton test (APT) sequence. ¹H NMR spectra in H₂O–D₂O 9:1 v/v were recorded on a Varian Inova-500 (500 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to 3-(trimethylsilyl)-1-propanesulfonyl acid sodium salt (0.00 ppm). Peak assignments are based on DQF-COSY, TOCSY (mixing times: 20 or 60 ms) and ROESY (mixing times: 150 or 250 ms) spectra. HSQC and HMBC spectra were measured on a Varian Inova-500 spectrometer and...
chemical shifts are given in ppm (δ) relative to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (0.00 ppm). Fourier transform infrared spectra (FTIR) were measured on a Bio-Rad FTS-25 spectrophotometer. Melting points were measured on a Büchi Schmelzpunktbestimmungsapparat and are uncorrected. Elemental analyses were done by Kolbe Mikroanalytisches Labor (Mülheim/Ruhr, Germany). Rf values were determined by thin layer chromatography (TLC) on Merck precoated silica gel 60F254 plates. Spots were visualized by UV-quenching, ninhydrin or Cl2–TDM.

The 2-chlorotriyl chloride resin (Hecheng Science & Technology Company) was used in all solid phase syntheses. The coupling reagents 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) were obtained from Biosolve. N-Hydroxybenzotriazole (HOBt) was from Advanced ChemTech and N,N-dimethylformamide (DMF), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), tert-butyl methyl ether (MTBE), N-methylpyrrolidone (NMP), and trifluoroacetic acid (TFA) and HPLC-grade acetonitrile were purchased from Biosolve. 2-(4,7,10-Tris-(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid (DOTA(OBu)) was purchased from Macrocycles. Piperidine, N,N-diisopropylethylamine (DIPEA), CuSO4 and sodium ascorbate were obtained from Acros Organics. Trifluoropropylsilane (TIS) and HPLC-grade TFA were obtained from Merck. Trifluoroacetic acid and propargylbromide were purchased from Aldrich.

**Radiolabeling of the RGD dendrimers**

Dendrimers 23 (25 µg, 20 nmol), 24 (25 µg, 13 nmol), and 25 (120 µg, 33 nmol) were radio-labeled by dissolving these compounds in 500 µL 0.5 M NH4OAc buffer, pH 6.0, containing 0.6 mg mL−1 gentisic acid. Then 22.2–37 MBq 111InCl3 was added to each of the reaction mixtures. The reaction mixtures were degassed and subsequently heated at 100 °C for 15 min. The 111In-labeled dendrimers were further purified on Waters C-18 SepPak cartridge (Milford, MA). After applying the sample on the methanol-activated cartridge, the cartridge was washed with 5 mL 25 mM NH4OAc and eluted with 25% CH3CN in 25 mM NH4OAc. The radiochemical purity was determined by reversed-phase HPLC (HP 1100 series, Hewlett Packard, Palo Alto, CA, USA) using a Zorbax RX-C18 column (250 × 4.6 mm) eluted with a linear gradient of buffer B (8–20% in 25 min or 8–100% in 30 min in buffer A (buffer A: 25 mM NH4OAc, buffer B: CH3CN) at a flow rate of 1 mL min−1. The radioactivity of the eluate was monitored using an in-line radiodetector (Flo-One Beta series, Radiomatic, Meriden, CT, USA).

**Solid phase α, β, binding assay**

The affinity of the DOTA-conjugated monovalent 23, divalent 24 and tetravalent 25 RGD dendrimers for the α, β, integrin was determined using a solid-phase competitive binding assay. 111In-labeled DOTA-Glu-(c[RGDfK])2 (3 MBq µg−1) was prepared as described above and was used as the tracer in the assay. Microtiter 96-well vinyl assay plates (Corning B.V., Schiphol-Rijek, The Netherlands) were coated with 100 µL/well of a solution of purified human integrin α, β, (150 ng mL−1) in Triton X-100 Formulation (Chemicon International, Temecula, CA, USA) in coating buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 0.5 mM MgCl2 and 1 mM MnCl2) for 17 h at 4 °C. The plates were washed twice with binding buffer (0.1% bovine serum albumin (BSA) in coating buffer). The wells were blocked for 2 h with 200 µL blocking buffer (1% BSA in coating buffer). The plates were washed twice with binding buffer. Then 100 µL binding buffer containing 11.1 kBq of 111In-DOTA-Glu-(c[RGDfK])2 and appropriate dilutions of non-labeled monovalent 23, divalent 24 and tetravalent 25 RGD dendrimers in binding buffer were incubated in the wells at 37 °C for 1 h. After incubation, the plates were washed three times with binding buffer. The retained radioactivity in each well was determined in a γ-counter (1480 Wizard, Wallac, Turku, Finland). The IC50 values of the RGD dendrimers were calculated by nonlinear regression using GraphPad Prism (GraphPad Prism 4.0 Software, San Diego, CA, USA). Each data point represents the average of three individual determinations.

**Biodistribution studies**

In the right flank of 6–8 weeks old female nude BALB/c mice, 0.2 mL of a cell suspension of 8.5 × 106 cells/mL SK-RC-52 cells was injected subcutaneously (s.c.). Two weeks after inoculation of the tumor cells, mice were randomly divided into three groups. The mice were injected with 0.25–0.29 MBq of the 111In-labeled dendrimers 23, 24, or 25 via a tail vein. The mice were euthanized by CO2 asphyxiation, 2 and 24 h postinjection (p.i.) (2–5 mice/group). Blood, tumor, and the major organs and tissues were collected, weighed, and counted in a γ-counter. The percentage injected dose per gram (%ID/g) was determined for each sample. To investigate whether the uptake of each of the three RGD dendrimers is α, β-mediated, a separate group of mice was co-injected with an excess (50 µg) of non-radioabeled DOTA-Glu-(c[RGDfK])2 to saturate all the α, β, integrin receptors.

**Statistical analysis**

All mean values are given ± standard deviation (S.D.). Statistical analysis was performed using the One-way Analysis of Variance. Tukey corrections for multiple comparisons were applied. The level of significance was set at P < 0.05.

**Syntheses**

Details of the synthetic procedures for compounds 2–5, 7, 8, 10, 17, 18 and 20–22 are given in the ESI.

**tert-Butyl-2-(3-prop-2-ynylxoy)benzamido)ethylcarbamate (11)**

Acid 4 (774 mg, 4.40 mmol) and amine 10 (704 mg, 4.40 mmol) were dissolved in CH2Cl2 (25 mL) and BOP (1.95 g, 4.41 mmol) followed by DIPEA (1.77 mL, 10 mmol, 2.27 equiv) were added and the obtained reaction mixture was stirred for 16 h. Then, the solvent was removed by evaporation and the residue was redissolved in EtOAc (50 mL) and subsequently washed with H2O.
(3 × 20 mL), 1 N KHCO₃ (3 × 20 mL), H₂O (3 × 20 mL), 5% NaHCO₃ (3 × 20 mL) by evaporation to dryness. The residue was purified by column chromatography (eluents: EtOAc–hexane 1 : 1 v/v) and obtained as a white solid with 98% yield (1.38 g). Mp: 118–121 °C; Rₐ (EtOAc–hexane 1 : 1 v/v): 0.20; ¹H NMR (CDCl₃) δ: 1.42 (s, 9H, (CH₃)₃Boc), 2.53 (s, 1H, CH), 3.33 (m, 2H, ~NH–CH₂–CH₂~), 3.52 (m, 2H, ~NH–CH₂–CH₂–NH~), 4.03 (m, 4H, ~NH–CH₂–CH₂–NH~), 5.53 (m, 1H, CH urethane), 7.10–7.47 (broad, 5H, arom H/NH amide); ¹³C NMR (CDCl₃) δ: 28.3, 39.9, 41.7, 55.8, 75.7, 78.1, 79.7, 113.4, 118.3, 119.8, 129.4, 135.6, 157.3, 157.6, 157.9; MS analysis: calcd for C₃₀H₆₀N₆O₉, 532.48, found ES-MS 533.39 [M + H]+, 555.6; Elemental analysis: calcd for C₃₀H₆₀N₆O₉: C 63.81, H 6.81, N 8.63%; Found: C 63.81, H 6.81, N 8.63%.

tert-Butyl-2-(3,5-bis(prop-2-ynloxy)benzamido)ethylcarbamate (12). This compound was synthesized using acid 5 (306 mg, 2.20 mmol) and amine 10 (352 mg, 2.20 mmol) as described for 11. Compound 12 was obtained in 96% yield (760 mg) after column chromatography with EtOAc–hexane 8 : 2 v/v as eluents. Mp: 128–134 °C; Rₐ (EtOAc–hexane 7 : 3 v/v): 0.57; Rₐ (EtOAc–hexane 1 : 1 v/v): 0.50; ¹H NMR (CDCl₃) δ: 1.42 (s, 9H, (CH₃)₃Boc), 2.52 (s, 2H, CH), 3.37 (m, 2H, ~NH–CH₂–CH₂~), 3.52 (m, 2H, ~NH–CH₂–CH₂–NH~), 4.68 (s, 4H, ~O–CH₂), 5.30 (m, 1H, CH urethane), 6.72 (s, 1H, arom H), 7.06 (s, 2H, arom H2/H6), 7.44 (m, 1H, NH amide); ¹³C NMR (125 MHz, CDCl₃) δ: 28.3, 39.9, 41.7, 55.8, 75.7, 78.1, 79.7, 113.4, 118.3, 119.8, 129.4, 135.6, 157.3, 157.6, 157.9; MS analysis: calcd for C₂₇H₄₂N₂O₆, 424.30, found ES-MS 425.27 [M + H]+, 447.15; Elemental analysis: calcd for C₂₇H₄₂N₂O₆: C 63.81, H 6.81, N 8.63%; Found: C 63.81, H 6.81, N 8.63%.

tert-Butyl-2,2′-((10-(2-oxo-2-oxoethyl)-(3-prop-2-ynloxy)benzamido)-ethylamino)-ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (13). To a solution of compound 11 (100 mg, 0.31 mmol) in CHCl₃ (5 mL), TFA (5 mL) was added to remove the Boc protecting group. After 1 h of stirring at room temperature, the volatiles were removed by evaporation and the residue was coevaporated with CHCl₃ to remove any residual TFA. The obtained solid was used without further purification. Then, the TFA-salt was dissolved in CHCl₃ (10 mL) and 2-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododec-1-yl)acetic acid (DOTA(OB′u)); 177 mg, 0.31 mmol), BOP (137 mg, 0.31 mmol) followed by DIPEA (220 μL, 1.24 mmol, 4 equiv) were added and the obtained reaction mixture was stirred for 16 h at room temperature. Subsequently, the solvent was removed by evaporation and the residue was redissolved in EtOAc (50 mL) and this solution was washed with H₂O (3 × 20 mL), 1 N KHCO₃ (3 × 20 mL), H₂O (3 × 20 mL), 5% NaHCO₃ (3 × 20 mL), brine (3 × 20 mL) and dried (Na₂SO₄). Finally, the solvent was evaporated in vacuo after which 13 was obtained as a pale yellow oil with 94% yield (227 mg). Rₐ (CHCl₃–MeOH 9 : 1 v/v): 0.49; Rₐ (CH₂Cl₂–MeOH 1 : 1 v/v): 0.30; ¹H NMR (CDCl₃) δ: 1.42 (s, 27H, (CH₃)₃Boc), 2.52–3.70 (broad, 28H, 8CH₂, 2CH, DOTA (24H) ~NH–CH₂–CH₂–NH~ (4H)), 2.52 (s, 1H, CH), 4.75 (m, 2H, ~O–CH₂–CH₂~), 5.41 (s, 1H, CH, 7.08 (m, 1H, arom H), 7.25–7.32 (m, 2H, arom H), 7.15 (m, 2H, arom H/NH); ¹³C NMR (CDCl₃) δ: 27.9, 39.2, 39.7, 39.9, 39.6, 55.6, 55.8, 56.0, 75.5, 78.4, 81.8, 112.9, 118.9, 120.3, 129.6, 135.5, 157.6, 167.4, 172.0, 172.4; MS analysis: calcd for C₄₀H₆₀N₆O₉ 772.47, found ES-MS 773.90 [M + H]+.
1 N NaOH. To this solution were added: CuSO₄·5H₂O (8 mg, 0.03 mmol, 0.1 equiv) and a solution of triflic azide (587 mg, 3.3 mmol, 10 equiv) in CH₃Cl (freshly prepared from triflic anhydride (555 µL, 3.3 mmol, 10 equiv) and Na₃N (975 mg, 15 mmol, 4.5 equiv) in CH₂Cl₂·H₂O (13 mL; 10 : 3 v/v)). The obtained two-phase reaction mixture was stirred for 16 h at room temperature. The solvents were removed by evaporation and the residue was mixed with tert-ButOH·H₂O and subsequently lyophilized to yield 202 mg (97%) crude reaction mixture. After purification by HPLC (C18) and to this solution, 0.05 M Na-ascorbate (72 mg, 23 µmol) and NaN₃ (975 mg, 23 mmol, 10 equiv) were added. The mixture was placed in the microwave reactor and irradiated for 5 min at 100 °C. Then, the solvents were removed under reduced pressure and the residue was dissolved in tert-ButOH·H₂O (1 mL; 95 : 5 v/v and stirred for 4 h at room temperature. Subsequently, the reaction mixture was heated by microwave irradiation to 100 °C for 3 x 5 min. Then, the solvents were removed under reduced pressure and the residue was dissolved in tert-ButOH·H₂O (1 mL; 95 : 5 v/v and lyophilized. The obtained fluffy solid was dissolved in TFA·H₂O (1 mL; 95 : 5 v/v) and stirred for 4 h at room temperature. Subsequently, the reaction mixture was concentrated in vacuo and the residue was redissolved in tert-ButOH·H₂O (1 : 1 v/v), lyophilized and purified by semi-prep HPLC (C18) to obtain compound 24 in 11% yield (1.3 mg). R₁ 12.1 min (C18); MS analysis: calcd for C₉₅H₇₉N₁₇O₁₆, 1234.340 (M ave), found MALDI-TOF 1918.431 [M + H]^+.

**References**


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**DOTA-conjugated divalent cyclo[RGDK] peptide dendrimer (24).** Alkyn 14 (4.9 mg, 5.9 µmol) and azido peptide 19 (11 mg, 14.8 µmol, 1.3 equiv) were dissolved in DMF·2·lucidone (1 mL; 7 : 3 v/v) and to this solution the following reagents were subsequently added: CuOAc (1.8 mg, 14.7 µmol, 2.5 equiv), Na-ascorbate (5.9 mg, 29.8 µmol, 1.5 equiv) and DIPEA (9.8 µL, 7.1 µmol, 1.2 equiv). The obtained reaction mixture was heated by microwave irradiation to 100 °C for 3 x 5 min. Then, the solvents were removed under reduced pressure and the residue was dissolved in tert-ButOH·H₂O (1 : 1 v/v) and lyophilized. The obtained fluffy solid was dissolved in TFA·H₂O (1 mL; 95 : 5 v/v and stirred for 4 h at room temperature. Subsequently, the reaction mixture was concentrated in vacuo and the residue was redissolved in tert-ButOH·H₂O (1 : 1 v/v), lyophilized and purified by semi-prep HPLC (C18) to obtain compound 24 in 11% yield (1.3 mg). R₁ 12.1 min (C18); MS analysis: calcd for C₉₅H₇₉N₁₇O₁₆, 1234.340 (M ave), found MALDI-TOF 1918.431 [M + H]^+.

**DOTA-conjugated tetravalent cyclo[RGDK] peptide dendrimer (25).** Alkyn 16 (3.8 mg, 3.0 µmol) and azido peptide 19 (11 mg, 14.8 µmol, 1.2 equiv) were dissolved in DMF (500 µL) and to this solution, 0.05 M Na-ascorbate (30 µL, 1.5 µmol, 0.50 equiv) followed by 6 mM CuSO₄·5H₂O (25 µL, 0.15 µmol, 0.05 equiv) were added. The obtained reaction mixture was heated by microwave irradiation to 100 °C for 2 x 5 min. Then, the solvents were removed under reduced pressure and the residue was dissolved in tert-ButOH·H₂O (1 mL; 95 : 5 v/v and lyophilized. The obtained fluffy solid was dissolved in TFA·H₂O (1 mL; 95 : 5 v/v) and stirred for 4 h at room temperature. Subsequently, the reaction mixture was concentrated in vacuo and the residue was redissolved in tert-ButOH·H₂O (1 : 1 v/v), lyophilized and purified by semi-prep HPLC (C18) to give compound 25 in 36% yield (3.9 mg). R₁ 16.1 min (C18); MS analysis: calcd for C₉₅H₇₉N₁₇O₁₆, 3611.873 (Mₙ), found MALDI-TOF 3612.646 [M + H]^+.

**General procedure for the micro-assisted click reaction.** The alkyn (1 equiv) and the azide (1.3 equiv per arm) were dissolved in DMF·H₂O. To this solution, CuSO₄·5H₂O (0.05 equiv) and Na-ascorbate (0.50 equiv) were added. The reaction mixture was placed in a microwave reactor and irradiated during 10–30 min at 100 °C. The cycloaddition was monitored on TLC and LC-MS for completion of the reaction.

**DOTA-conjugated monovalent cyclo[RGDK] peptide dendrimer (23).** Alkyn 13 (5.5 mg, 7.1 µmol) and azido peptide 19 (6.0 mg, 8.1 µmol, 1.1 equiv) were dissolved in DMF (500 µL) and 0.05 M Na-ascorbate (72 µL, 3.6 µmol, 0.50 equiv) followed by 6 mM CuSO₄·5H₂O (60 µL, 0.36 µmol, 0.05 equiv) were added. The reaction mixture was placed in the microwave reactor and irradiated for 3 x 5 min at 100 °C. Then, the solvents were removed under reduced pressure and the residue was dissolved in tert-ButOH·H₂O : 1 v/v and lyophilized. The obtained fluffy solid was dissolved in TFA·H₂O (1 mL; 95 : 5 v/v) and stirred for 4 h at room temperature. Subsequently, the reaction mixture was concentrated in vacuo and the residue was redissolved in tert-ButOH·H₂O (1 : 1 v/v), lyophilized and purified by semi-prep HPLC (C18) to give compound 23 in 13% yield (1.1 mg). R₁ 10.3 min (C18); MS analysis: calcd for C₅₅H₇₉N₁₇O₁₆, 1234.340 (Mₙ), found MALDI-TOF 1234.807 [M + H]^+.