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†‡


Received 2nd November 2006, Accepted 2nd January 2007
First published as an Advance Article on the web 29th January 2007
DOI: 10.1039/b615940k

This report describes the design and synthesis of a series of αvβ3 integrin-directed monomeric, dimeric and tetrameric cyclo[Arg-Gly-Asp-D-Phe-Lys] dendrimers using “click chemistry”. It was found that the unproctected N-ε-azido derivative of cyclo[Arg-Gly-Asp-D-Phe-Lys] underwent a highly chemoselective conjugation to amino acid-based dendrimers bearing terminal alkyne using a microwave-assisted Cu(1)-catalyzed 1,3-dipolar cycloaddition. The αvβ3 binding characteristics of the dendrimers were determined in vitro and in vivo αvβ3 targeting properties were assessed in nude mice with subcutaneously growing human SK-RC-52 tumors. The multivalent RGD-dendrimers were found to have enhanced affinity toward the αvβ3 integrin receptor as compared to the monomeric derivative as determined in an in vitro binding assay. In case of the DOTA-conjugated 110In-labeled RGD-dendrimers, it was found that the radiolabeled multimeric dendrimers showed specifically enhanced uptake in αvβ3 integrin expressing tumors in vivo. These studies showed that the tetrameric RGD-dendrimer had better tumor targeting properties than its dimeric and monomeric congeners.

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 αvβ3, which binds to the Arg-Gly-Asp (RGD) tripeptide motif containing ligands, plays a pivotal role in tumor angiogenesis and metastasis. Integrin expressed on endothelial cells modulate cell migration and survival during angiogenesis, while integrin expressed on carcinoma cells potentiate metastasis by facilitating invasion and movement across blood vessels. The αvβ3 integrin is expressed on activated endothelial cells during tumor induced angiogenesis, whereas it is absent on quiescent endothelial cells and normal tissues. In addition, αvβ3 is expressed on various tumor cell types (e.g. breast, ovarian, and prostate cancers). Evidence exists that inhibition of αvβ3 integrin function prevents tumor growth and induces tumor regression by antagonizing angiogenesis. Several peptidic and pepotidomimetic αvβ3 antagonists have been synthesized. Among these, the cyclo[Arg-Gly-Asp-D-Phe-Val] (c[RGDfVK]), as developed by Kessler and coworkers, is one of the most active and selective antagonists for the αvβ3 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-azo 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 imaging properties, several studies explored the multivalency effect by using dimeric and tetrameric RGD peptides with affinity toward the αvβ3 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 commercially available amino acid- or peptide-based dendrimers, 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 commercially available 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 thiol–disulfide exchange, by native chemical ligation, or via a chemoselective oxime hydrozone 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 alkyne and an azide to yield 1,4-disubstituted 1,2,3-triazoles, was reinvestigated independently by Meldal et al. and Sharpless et al. They found that an alkyne 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’, 17 has led to a plethora of applications in the literature. Recently, we synthesized multivalent dendrimeric peptides (up to octa- and hexadecavalent systems) respectively triazole-linked glycodendrimers via a microwave-assisted 1,3-dipolar cycloaddition between azido peptides respectively glycosyl azides and dendrimeric alkyne as an alternative approach to functionalize dendrimers.

Here we describe the synthesis of monomeric, dimeric and tetrameric [RGD(K)] dendrimers via a microwave-assisted 1,3-dipolar cycloaddition of dendrimeric alkyne with the N-ε-azido derivative of cyclo[Arg-Gly-Asp-d-Phe-Lys] and their subsequent evaluation as αβ-integrin antagonists. Additionally, the RGD dendrimers were conjugated with a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecanoic acid (DOTA) moiety. These analogs were radiolabeled with 111In to evaluate the in vitro receptor binding characteristics and 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 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 propargyl bromide in the presence of K2CO3 as a base and were obtained in 95 and 81% yield. Since these two compounds were also used as synths in further syntheses, the resulting methyl esters 2 and 3 were treated with Tesser’s base to yield acids 4 and 5 in nearly quantitative yield. After treatment of the previously described 6 with TFA to remove both Boc-functionalities, the resulting bisamidine 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 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 amides 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(OBu)) to give the DOTA-conjugated mono-, di- and tetravalent dendrimers.

Scheme 1 Synthesis of the mono-, di- and tetravalent dendrimeric alkyne 2, 3 and 7.
Scheme 2 Synthesis of the DOTA-conjugated dendrimeric alkynes 13, 14 and 16.

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–tBu SPPS (solid phase peptide synthesis) based on the protocol of Liu et al. 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 macro lactamization 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-amino of the lysine residue was selectively converted into the azide moiety by a diazotransfer. At pH 10, the ε-amino 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 αβ₃ integrin antagonists as shown in Scheme 4. Our first experiments were based on the literature procedure 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. 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 °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 Cu²⁺ ions. Chelated copper(ii) will result in a lower efficiency of the Cu(tii)–Cu(ti) 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 ¹¹¹In, ⁹⁹Y or ¹⁷⁷Lu. 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 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 NH₄OAc buffer of pH 6.0 and 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. Reversed phase-HPLC analysis showed a single peak for each of the three ¹¹¹In-labeled compounds with an elution time of 25.9 min, 29.5 min and 29.4 min for the ¹¹¹In-labeled monovalent 23, divalent 24, and tetravalent 25, RGD peptide dendrimers respectively.

Solid phase α,β₃ binding assay

The affinity of the DOTA-conjugated RGD dendrimers 23, 24, and 25 for the α,β₃ integrin was determined in a competitive binding assay. The results of these analyses are shown in Fig. 1. Binding of the ¹¹¹In-labeled dimeric peptide, ¹¹¹In-DOTA-Glu-(c[RGDfK])₂, to α,β₃ was competed by unlabeled 23, 24, and 25 in a concentration dependent manner. The IC₅₀ 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 α,β₃ compared to the dendrimers containing one (23) or two (24) c[RGDfK] units. Multimerization of c[RGDfK] resulted in enhanced affinity for α,β₃, as was evidenced by a decrease of the IC₅₀ concentration.

Biodistribution studies

In athymic mice with subcutaneously (s.c.) growing SK-RC-52 renal cell carcinoma, the tumor uptake of the ¹¹¹In-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 ¹¹¹In-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 $\alpha_\beta$ receptors in vivo, resulted in a significantly reduced tumor uptake of each of the three compounds: 23: $0.46 \pm 0.04$%ID/g (2 h p.i.), $0.36 \pm 0.31$%ID/g (24 h p.i.), 24: $0.76 \pm 0.09$%ID/g (2 h p.i.), not determined (24 h p.i.) and 24: $1.56 \pm 0.02$%ID/g (2 h p.i.), $1.19 \pm 0.03$%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 $\alpha_\beta$, 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.\(^{3a}\)

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 IC\(_{50}\) concentration of the tetrameric RGD dendrimer 25 was lower compared to those of the monomeric and dimeric RGD dendrimers.\(^{25}\)

In conclusion, a series of $\alpha_\beta$, 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(1)-catalyzed 1,3-dipolar cycloaddition. The $\alpha_\beta$ binding characteristics and $\alpha_\beta$ targeting properties of the dendrimers were determined both in vitro and in vivo. In the case of the DOTA-conjugated $^{111}$In-labeled RGD-dendrimers, it was found that the radiolabeled multimeric dendrimers showed specifically enhanced uptake in $\alpha_\beta$, 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 Proosphere C4 or C8 and Adsorbosphere XL C18 columns ($250 \times 4.6$ mm, particle size: $300 \AA$, particle size: $5 \mu$m) or on a Merck LiChroCART CN column ($250 \times 4.6$ mm, pore size $100 \AA$, particle size: $5 \mu$m) at a flow rate of $1.0 \text{mL min}^{-1}$ using a linear gradient of buffer B (0–100% in 25 min) in buffer A (buffer A: 0.1% TFA in H$_2$O, buffer B: 0.1% TFA in CH$_3$CN–H$_2$O 95 : 5 v/v). Preparative HPLC runs were performed on an Alltech Proosphere C4 or C8 column ($250 \times 22$ mm, pore size $300 \AA$, particle size: 10 $\mu$m), and semi-prep HPLC runs were performed on an Alltech Adsorbosphere XL C18 column ($250 \times 10$ mm, particle size: 30 $\mu$m) or on a Merck LiChroCART CN column ($250 \times 10$ mm, pore size $100 \AA$, particle size: 10 $\mu$m) at a flow rate of $10.0 \text{mL min}^{-1}$ (semi-prep HPLC: $4.0 \text{mL min}^{-1}$) using a linear gradient of buffer B (0–100% in 50 min) in buffer A (buffer A: 0.1% TFA in H$_2$O, buffer B: 0.1% TFA in CH$_3$CN–H$_2$O 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 Finnigan LCQ Deca XP MAX LC/MS equipped with a Shimadzu 10A VP analytical HPLC system. The samples were dissolved in 10% formic acid in CH$_3$CN–H$_2$O 1 : 1 v/v and analyzed using a Phenomenex Gemini C18 column ($150 \times 4.6$ mm, particle size: $3 \mu$m, pore size: $110 \AA$) at a flow rate of $1.0 \text{mL min}^{-1}$ using a linear gradient of 100% buffer A (0.1% TFA in H$_2$O–CH$_3$CN 95 : 5 v/v) to 100% buffer B (0.1% TFA in CH$_3$CN–H$_2$O 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-cyano-4-hydroxycinnamic acid or sinapinic acid as matrices.\(^{1}H\) NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm ($\delta$) relative to TMS.\(^{1}C\) NMR spectra were recorded on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to CDCl$_3$ (77.0 ppm). The \(^{13}C\) NMR spectra were recorded using the attached proton test (APT) sequence.\(^{1}H\) NMR spectra in H$_2$O–D$_2$O 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-propanesulphonic 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...
HPLC-grade acetonitrile were purchased from Biosolve. 2-(4,7,10-yl) acetic acid (DOTA(Otert-hexafluoroisopropanol (HFIP), N30 min in buffer A (buffer A: 25 mM NH4OAc, buffer B: CH3CN) Dendrimers 23 Radiolabeling of the RGD dendrimers sodium ascorbate were obtained from Acros Organics. Triiso-degassed and subsequently heated at 100 °C for 15 min. The 2-chlorotriyl chloride resin (Hecheng Science & Technology Company) was used in all solid phase syntheses. The coupling reagents 2-(1H-benzotrizol-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphate (HTBU) and benzotrizol-1-lyoxy-tris-(dimethylenino)phosphonium hexa- fluorophosphate (BOP) were obtained from Biosolve. N-Hydroxy-benzotrizole (HOBt) was from Advanced ChemTech and N9-fluorenemethyloxycarbonyl (Fmoc) amino acids were obtained from MultiSynTech. The side-chain protecting groups were removed as tert-butyl for aspartic acid, tert-butyloxy carbonyl (Boc) for lysine and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for arginine. Peptide-grade (Boc) for lysine and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for arginine. Peptide-grade tert-butanol (BuOH), dichloromethane, N,N-dimethylformamide (DMF), 1,1,3,3,3-hexafluoriosopropanol (HFIP), tert-butyl methylether (MTBE), N-methylpyrrolidone (NMP), and trifluoroacetic acid (TFA) and HPLC-grade acetonitrile were purchased from Biosolve. 2-(4,7,10-Tris(tert-butyl-2-oxoethyl)-1,4,7,10-tetraacyclododecan-1-yl) acetic acid (DOTA(OBu)) was purchased from Macrocylls. Piperidine, N,N-diisopropylethylamine (DIPEA), CuSO4 and sodium ascorbate were obtained from Acros Organics. Tripropylsilane (TIS) and HPLC-grade TFA were obtained from Merck. Trifluoroacetic acid and propargyl bromide 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 radiolabeled 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 a 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 αβ integrin was determined using a solid-phase competitive binding assay.

**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 αβ-mediated, a separate group of mice was co-injected with an excess (50 μg) of non-radiolabeled DOTA-Glu-(cRGDfK)2 to saturate all the αβ 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-ynyloxy)benzamido)ethylcarbamate (11). Acid 4 (774 mg, 4.40 mmol) and amine 10 (704 mg, 4.40 mmol) were dissolved in CH3CN (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 KHSO₄ (3 × 20 mL), H₂O (3 × 20 mL), 5% NaHCO₃ (3 × 20 mL) and brine (3 × 20 mL), dried (Na₂SO₄) to dryness. The residue was purified by column chromatography (eluents: EtOAc–hexane 1 : 1 v/v and EtOAc–hexane 8 : 2 v/v as eluents). Mp: 128–134 °C; Rᵣ (EtOAc–hexane 7 : 3 v/v): 0.31; ¹H NMR (CDCl₃): δ: 1.42 (s, 9H, (CH₃)₃ Boc), 2.55 (s, 2H, CH₃), 3.37 (m, 2H, ~NH–CH₂–CH₂–), 3.52 (m, 2H, ~CH₂–CH₂–NH–), 4.68 (s, 4H, ~O–CH₂), 5.30 (m, 1H, NH urethane), 6.72 (s, 1H, aromatic H4), 7.06 (s, 2H, aromatic 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, 118.9, 129.4, 135.6, 157.3, 157.6, 157.6; MS analysis: calcd for C₁₇H₂₀N₂O₄ 318.16, found ES-MS 319.27 [M + H]⁺, 341.33 [M + Na]⁺; Elemental analysis: calcd for C₁₇H₂₀N₂O₄ 64.13, H 6.97, N 8.80 found C 63.81, H 6.81, N 8.63%.

tert-Butyl-2-(3,5-bis(prop-2-ynyloxy)benzamido)ethylcarbamate (12). This compound was synthesized using acid 5 (506 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; Rᵣ (EtOAc–hexane 7 : 3 v/v): 0.31; ¹H NMR (CDCl₃): δ: 1.42 (s, 9H, (CH₃)₃ Boc), 2.55 (s, 2H, CH₃), 3.37 (m, 2H, ~NH–CH₂–CH₂–), 3.52 (m, 2H, ~CH₂–CH₂–NH–), 4.68 (s, 4H, ~O–CH₂), 5.30 (m, 1H, NH urethane), 6.72 (s, 1H, aromatic H4), 7.06 (s, 2H, aromatic H2/H6), 7.44 (m, 1H, NH amide); ¹³C NMR (125 MHz, CDCl₃): δ: 28.3, 40.0, 41.7, 56.0, 75.9, 78.0, 79.8, 105.5, 106.6, 136.4, 157.3, 158.6, 167.2; MS analysis: calcd for C₂₀H₂₄N₂O₅ 372.17, found ES-MS 373.24 [M + H]⁺, 395.27 [M + Na]⁺; Elemental analysis: calcd for C₂₀H₂₄N₂O₅ 64.50, H 6.50, N 7.05%.

tert-Butyl-2,2′-[10-(2-oxo-2-(3-prop-2-ynyloxy)benzamido)ethylamino]ethyl-1,4,7,10-tetraazaacyclodecane-1,4,7,10-tetraacetate (13). To a solution of compound 11 (100 mg, 0.31 mmol) in CH₂Cl₂ (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, evaporation and the residue was coevaporated with CH₂Cl₂ to remove any residual TFA. The obtained solid was used without further purification. Then, the TFA-salt was dissolved in CH₂Cl₂ (10 mL) and 2-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazaacyclodecan-1-yl)ace tic acid (DOTA(OBu)₂): 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 KHSO₄ (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). Rᵣ (CH₂Cl₂–MeOH 9 : 1 v/v): 0.49; Mp: 180–181 °C; Rᵣ (CH₂Cl₂–MeOH 9 : 1 v/v): 0.30; Rᵣ (19.70 min (C8); ¹H NMR (CDCl₃): δ: 1.45 (s, 27H, (CH₃)₃ Boc), 2.04–4.50 (broad s, 36H, CH₃, DOTA (24H)/~NH–CH₂–CH₂–NH–(4H)~O–CH₂–CH₂–NH–(4H)~), 2.55 (s, 4H, CH₂), 4.72 (m, ~O–CH₂–NH–), 6.70 (m, 1H, aromatic H4), 6.70 (m, 2H, aromatic H2/H6), 7.18–7.33 (m, 6H, aromatic H2/H6), 7.80 (m, 2H, NH), 8.75 (m, 2H, NH); ¹³C NMR (CDCl₃): δ: 27.9, 28.0, 38.8, 39.3, 39.7, 55.7, 55.9, 56.2, 66.5, 75.8, 76.0, 87.3, 82.0, 106.0, 106.3, 106.5, 106.7, 136.3, 136.8, 159.4, 166.7, 166.9, 171.5, 172.3; MS analysis: calcd for C₁₇₇H₁₇₇N₈O₁₆ 2160.63, found ES-MS 2161.75 [M + H]⁺; Elemental analysis: calcd for C₁₇₇H₁₇₇N₈O₁₆·H₂O 59.19, H 6.67, N 8.24 found C 59.60, H 6.82, N 7.71%.

-n-Azido cyclo(Arg-Gly-Asp-p-Ph-Lys)(19). Cyclic peptide 18 (200 mg, 0.33 mmol) was dissolved in tert-BuOH–H₂O (5 mL; 1 : 1 v/v) and the pH was adjusted to 10 by the addition of
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 Na₂N₃ (975 mg, 15 mmol, 4.5 equiv) in CH₂Cl₂–H₂O (13 mL; 10 : 3 v/v/v). The obtained two-phase reaction mixture was stirred at room temperature. Subsequently, the reaction mixture was concentrated for 4 h at room temperature. Subsequently, the reaction mixture was stirred for 4 h at room temperature. Then, the solvents were removed by evaporation and the residue was mixed with tert-BuOH–H₂O and subsequently lyophilized to yield 202 mg (97%) crude reaction product. Pure azido peptide 19 was obtained in 21% yield (44 mg) after purification by HPLC (C8). R₉ (CHCl₃–MeOH–AcOH 90 : 20 : 3 v/v/v): 0.25; R₉ (16.1 min (C4)); R₉ (17.33 min (CN)); FTIR (KBr) υ: 2100 cm⁻¹; ¹H NMR (500 MHz, H₂O–D₂O 1 : 9 v/v, pH 4): Arg, δ: 1.43 (m, 2H, γCH₂), 1.65/1.86 (double m, 2H, βCH₂), 3.18 (m, 2H, CH₂), 4.36 (m, 1H, CH), 7.20 (t (J 5.8 Hz), 1H, δNH), 8.04 (d (J 8.7 Hz), 1H, αNH); Gly, δ: 3.49 (dd (J 4.5 Hz, J 14.8 Hz), 1H, CH), 4.21 (dd (J 7.7 Hz, J 14.8 Hz), 1H, αCH), 1H, αCH), 8.33/8.36 (dd (J 9.7 Hz, J 7.4 Hz), 1H, αNH); Asp, δ: 2.63/2.66 (dd (J 6.7 Hz, J 16.4 Hz), 1H, βCH₂), 7.29/8.23 (dd (J 7.7 Hz, J 16.4 Hz), 1H, βCH₂), 4.73 (m, 1H, CH), 8.12 (d (J 8.8 Hz, 1H, δNH); p-Phe, δ: 2.93/2.98 (dd (J 10.3 Hz, J 13.2 Hz), 1H, βCH₂), 3.07/3.10 (dd (J 5.9 Hz, J 13.2 Hz), 1H, αCH), 1H, βCH₂), 4.45 (m, 1H, CH), 7.25 (d (J 7.3 Hz, 2H, arom H), 7.33–7.38 (3H, arom H), 8.42 (d (J 5.9 Hz, 1H, αNH); azido Lys, δ: 0.95 (m, 2H, γCH₂), 1.46/1.65 (double m, 2H, βCH₂), 1.49 (m, 2H, CH₂), 3.24 (t (J 7.1 Hz), 2H, CH₂), 3.85 (m, 1H, CH), 8.44 (d (J 5.6 Hz), 1H, αNH); C NMR (H₂O–D₂O 9 : 1 v/v, 293 K, 12.1 min (C18); MS analysis: found for C₈₅H₁₂₀N₂₈O₂₄, 1918.067 (M+Na), found MALDI-TOF 1918.431 [M + H]⁺.

**DOTA-conjugated divalent cyclo[RGDiK] peptide dendrimer (24).** Alkyne 14 (4.9 mg, 5.9 µmol) and azido peptide 19 (11 mg, 14.8 µmol, 1.3 equiv) were dissolved in DMF–2,6-lutidine (1 mL, 7 : 3 v/v) and to this solution the following reagents were subsequently added: CuOAc (1.8 mg, 14.7 µmol, 1.5 equiv), Na-ascorbate (5.9 mg, 29.8 µmol, 1.5 equiv) and DIPEA (9.8 µL, 71 µmol, 1.2 equiv). The obtained reaction mixture was heated by microwave irradiation to 100 °C for 3 × 5 min. Then, the solvents were removed under reduced pressure and the residue was dissolved in tert-BuOH–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-BuOH–H₂O 1 : 1 v/v and lyophilized. The obtained fluffy solid was purified by semi-prep HPLC (C18) to obtain compound 24 in 11% yield (1.3 mg). R₉ (12.1 min (C18); MS analysis: calculated for C₉₁H₁₃₀N₃₉O₃ₙ, 1918.067 (M+Na), found MALDI-TOF 1918.431 [M + H]⁺.

**DOTA-conjugated tetravalent cyclo[RGDiK] peptide dendrimer (25).** Alkyne 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 × 5 min. Then, the solvents were removed under reduced pressure and the residue was dissolved in tert-BuOH–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-BuOH–H₂O 1 : 1 v/v, lyophilized and purified by semi-prep HPLC (C18) to give compound 25 in 36% yield (3.9 mg). R₉ (11.6 min (C18); MS analysis: calculated for C₉₅H₁₄₀N₃₉O₃ₙ, 3611.873 (M+Na), found MALDI-TOF 3612.646 [M + H]⁺.

**Acknowledgements**

We thank Dr Hans Ippel for measuring the 500 MHz ¹H NMR spectra of compound 19.

**References**