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Multimerization of an Apoptogenic TRAIL-Mimicking Peptide by Using Adamantane-Based Dendrons**

Giuseppe Lamanna,*[a] Cristian R. Smulski,[a, c] Neila Chekkat,[a, d] Karine Estieu-Gionnet,[b] Gilles Guichard,[b] Sylvie Fournel,[a, d] and Alberto Bianco*[a]

Abstract: We have developed a straightforward strategy to multimerize an apoptogenic peptide that mimics the natural tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by using adamantane-based dendrons as multivalent scaffolds. The selective binding affinity of the ligands to TRAIL receptor 2 (TR2) was studied by surface plasmon resonance, thus demonstrating that the trimeric and hexameric forms of the peptide exert an increased affinity of about 1500- and 20000-fold, respectively, relative to the monomer. Moreover, only the trimeric and hexameric ligands were able to induce cell death in TR2 expressing cells (BJAB), thus confirming that a multivalent form of the peptide is necessary to trigger a substantial TR2-dependent apoptotic response in vitro. These results provide interesting insight into the multivalency effect on biological ligand/receptor interactions for future therapeutic applications.

Keywords: adamantane · click chemistry · dendrimers · multivalency · peptides · TRAIL

Introduction

Polyvalent interactions that occur in biological systems are characterized by the simultaneous binding of multiple ligands to multiple receptors.[1] These interactions can induce (agonist mechanism) or inhibit (antagonist mechanism) a biological response differently to monovalent complexes.[2] To understand receptor signaling by multivalent interactions, new strategies have emerged from synthetic organic chemistry and chemical biology by the design of multivalent ligands.[3] The latter are composed of a central scaffold bearing multiple copies of a recognition element, such as a carbohydrate, peptide, small molecule, or any moiety that binds to a receptor.[4] The size, shape, flexibility, and valency of the central scaffold play important roles in the biological activity and the action mechanism of the recognition element.[5] Different scaffolds from various classes of structure, such as proteins, liposomes, dendrimers, polymers, and solid supports, have been designed.[6] Because of their nanoscale size and monodispersity, dendrimers and dendrons (wedge-shaped dendrimer sections) have appeared as ideal scaffolds for the design of multivalent ligands.[4,7]

We have recently reported the synthesis of the first- and the second-generation (G1 and G2, respectively) adamantane based-dendrons displaying the anti-inflammatory ibuprofen moiety at their periphery in a trimeric or nonameric arrangement, respectively.[8] The multivalency effect on the drug activity was studied, demonstrating that the multivalent ibuprofen/dendron conjugates exert an enhanced anti-inflammatory activity in vitro relative to free ibuprofen. Moreover, in the proposed dendritic structures, the adamantane building blocks with their rigid tetrahedral geometry, provided a three-dimensional tripodal arrangement of the attached entities. This tripodal arrangement is an ideal recognition motif for the cell surface, as several important receptors form complexes with threefold geometry.[9] On the basis of these elements, we decided to explore the adamantane-based dendrons as scaffolds to study multivalent ligand/receptor interactions.[10] We chose an apoptogenic cyclic peptide, termed **M1**, as a ligand, which was developed at Affymax,[11] which specifically recognizes human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 2 (TR2) and once oligomerized mimics the function of its natural ligand TRAIL.[12]

TRAIL is a type II transmembrane protein that, like other tumor necrosis factor (TNF) superfamily members,

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[**] TRAIL = tumor necrosis factor-related apoptosis-inducing ligand.

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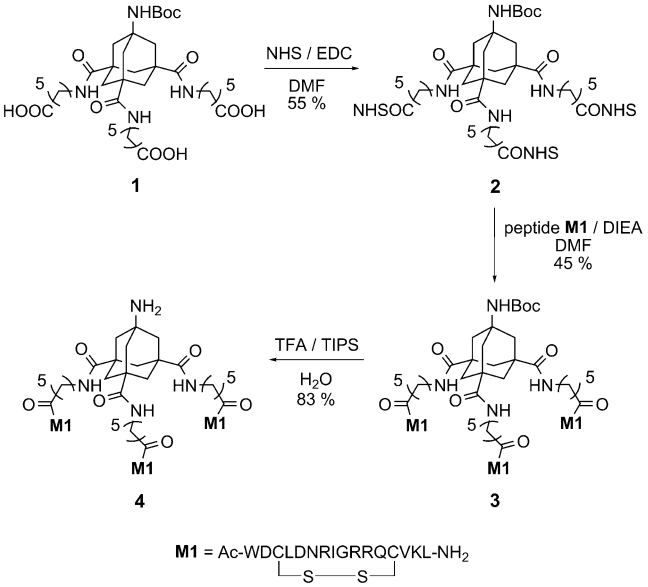
forms homotrimers. Each ligand subunit binds a receptor molecule that induces an oligomerization process, which is generally accepted to generate the minimal active unit that triggers an effective signal.[13] TRAIL receptors belong to the TNF receptor subgroup that contains an intracellular "death domain" and triggers apoptosis. There are five different TRAIL receptors: two fully functional receptors (i.e., TR1 and TR2), two membrane-bound receptors that cannot transmit an apoptotic signal (i.e., TR3 and TR4), and a soluble receptor called osteoprotegerin (OPG).[14]

Trimerization or multimerization of TR1 and TR2 results in the formation of a death-inducing signaling complex (DISC), thus leading to caspase activation and cell death by apoptosis. TRAIL is a ligand with enormous potential in cancer therapy because of its unique characteristic to induce apoptosis in tumor cells through the endogenous molecule while sparing normal cells.[15] Moreover, it is well established that death-receptor oligomerization is central for efficient formation of DISC, and structural studies provide novel insight into this process.[16] Indeed, it has been shown that strategies that increase receptor oligomerization amplify TRAIL-induced apoptosis.[17] Therefore, in the present study, we have analyzed the multimerization effect of the **M1** peptide ligand on TR2 binding by using different generations of adamantane-based dendrons as multivalent scaffolds.

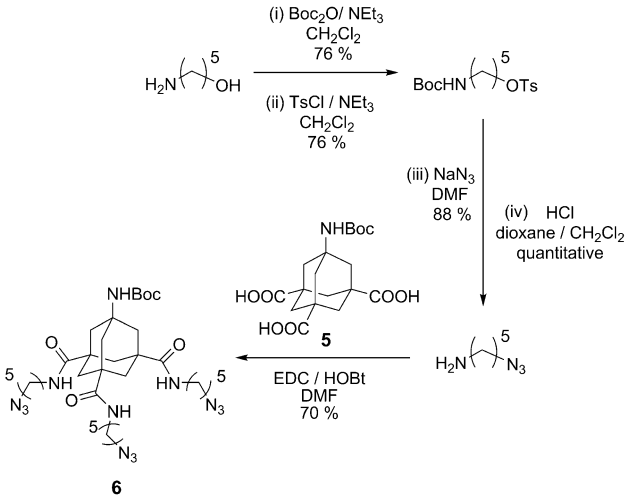


Scheme 1. The multimerization of **M1** peptide by using an amidation approach: synthesis of trimer **4**. Boc = *tert*-butoxycarbonyl, DIEA = *N*-diisopropylethylamine, EDC × HCl = *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, NHS = *N*-hydroxysuccinimidyl, TFA = trifluoroacetic acid, TIPS = triisopropylsilane.

Results and Discussion

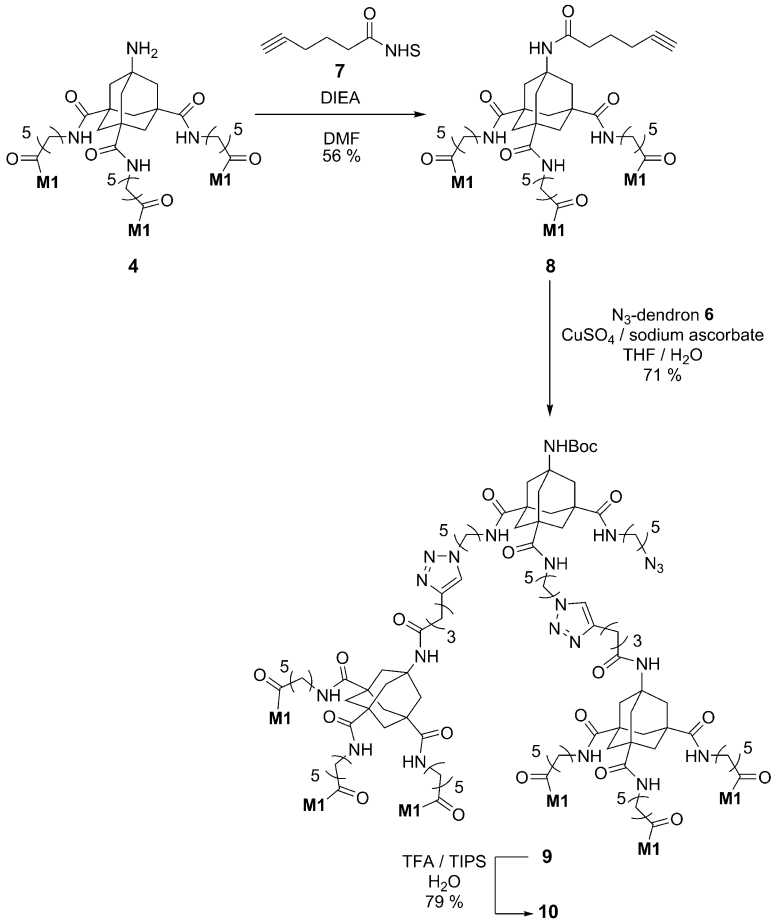
We have previously described the synthesis of the G1 adamantane-based dendron **1** with three carboxylic acid groups at its periphery.[18] We decided to use this molecule as a scaffold for the construction of the trivalent form of the **M1** peptide ligand. We initially activated the acid functions of **1** by forming the corresponding NHS esters in the presence of EDC × HCl, and then we coupled the peptide **M1** through amidation with its lysine residue. HPLC purification on a C_{18} column afforded the trimeric ligand **3** in an overall yield of 25% (Scheme 1). The Boc group at the focal point of **3** was subsequently cleaved with TFA in the presence of a TIPS scavenger to obtain the necessary building block **4** for the synthesis of the G2 dendron bearing **M1** in a nonameric arrangement. For this purpose, we decided to explore a synthetic approach based on the click chemistry between azides and alkynes.

In our first experiments, we prepared the G1 dendron **6** functionalized at its periphery with azide groups (Scheme 2). By starting from the commercially available 5-amino-1-pentanol, we generated the desired azide-terminated chain following a four-step sequence: 1) the introduction of a Boc-protecting group on the amine using Boc_2O and triethylamine;[19] 2) the activation of the hydroxy function by tosylation with TsCl;[20] 3) the conversion of the tosylate into an azide group by treatment with NaN_3; and 4) Boc-deprotection with a solution of HCl in dioxane. The resulting azide-derivatized chain was finally introduced in the 3-, 5-, 7-carboxylate positions of adamantane **5** by means of amidation with EDC × HCl/HOBt to afford the G1 azide dendron **6** in good yield after isolation by column chromatography on silica gel (Scheme 2). Tetrasubstituted adamantane **5** was synthesized, in turn, according to our protocols in six steps from 1-bromoadamantane as an inexpensive starting material.[18]



Scheme 2. Synthesis of the first-generation azide dendron **6**. Boc_2O = di-*tert*-butyl dicarbonate, HOBt = 1-hydroxybenzotriazole, TsCl = *para*-toluenesulfonyl chloride.

At this point, for the development of the nonameric ligand, we needed a second adamantane-based dendron to bring an alkyne moiety at the focal point and bearing the



Scheme 3. Synthesis of hexamer **10** by using the click-chemistry approach.

M1 peptide at its periphery. For this aim, we linked the NHS ester **7**, which terminates with an alkyne function, to the free amine of **4**, thus obtaining intermediate **8** under basic conditions (Scheme 3). This latter derivative was involved in a Huisgen cycloaddition with the azide dendron **6** by using a copper(I) catalyst in the presence of sodium ascorbate. Purification by HPLC on a C_{18} column afforded only hexameric ligand **9**. We did not observe the formation of the nonamer molecule probably because of steric hindrance. Thus, the third azide group of hexamer **9** is likely inaccessible to alkyne **8**. In the last synthetic step, the deprotection of the Boc group of **9** with TFA took place to afford the corresponding hexamer **10** with the free amine at the focal point.

Once we obtained multivalent ligands **4** and **10**, we studied their binding affinity to TR2 receptor by surface plasmon resonance (SPR) relative to monomer **M1**. Recombinant human TR2 and the mouse homologue of TR2 were immobilized on the sensor chip and the peptide-based ligands were flushed onto this chip at different concentrations. The recombinant human RANK protein, a related protein from the TNF receptor family, was used as a control.

All the compounds displayed specific binding to human TR2, and no detectable interaction was observed with mouse TR2 (see Figure 1 and Figure S1 in the Supporting Information). We also carried out SPR studies with the peptide-free dendron **1** to demonstrate that the ligand/receptor interaction is not influenced by the adamantane-based building block. Unspecific binding of **1** to the TR2 protein was not recorded (see Figure S2 in the Supporting Information). SPR experiments with the monovalent peptide **M1** provided an equilibrium dissociation constant (or "binding constant"; K_D) of 1.29×10^{-7} M, calculated from the dissociation kinetic rate ($k_{off}=3.07\times10^{-3}\,s^{-1}$) divided by the association kinetic rate ($k_{on}=2.39\times10^{4}\,M^{-1}s^{-1}$; Table 1). The ligand in its trivalent form **4** substantially improved the binding affinity to human TR2 by approximately 1500-fold ($K_D=9.10\times10^{-11}$ M). Interestingly, this effect was mainly due to a change on the dissociation rate ($k_{off}=1.04\times10^{-6}\,s^{-1}$) as the k_{on} value for trivalent ligand **4** ($k_{on}=1.15\times10^{4}\,M^{-1}s^{-1}$) is almost the same than the k_{on} value for monomer **M1**.

Table 1. Kinetic parameters of the **M1** ligand in its monovalent, trivalent, or hexavalent forms.

Analyte	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]	K_D [M]
monomer **M1**	$(2.39\pm1.91)\times10^{4}$	$(3.07\pm0.02)\times10^{-3}$	$(1.29\pm0.04)\times10^{-7}$
trimer **4**	$(1.15\pm0.22)\times10^{4}$	$(1.04\pm0.33)\times10^{-6}$	$(9.10\pm1.92)\times10^{-11}$
hexamer **10**	$(8.01\pm0.23)\times10^{4}$	$(5.74\pm0.54)\times10^{-7}$	$(7.16\pm1.35)\times10^{-12}$

Moreover, hexamer **10** exerted a 14-fold increased affinity to human TR2 ($K_D=7.16\times10^{-12}$ M) relative to trimer **4**. Reconstitution of the sensor chip surface after injection at high concentrations of **10** was only effective by using 50 mM HCl solution probably due to high stability of the formed ligand/receptor complexes (Table 1). Here again, the higher binding affinity of **10** was determined by a strong decrease in the k_{off} value ($k_{off}=5.74\times10^{-7}\,s^{-1}$), while keeping an association rate ($k_{on}=8.01\times10^{4}\,M^{-1}s^{-1}$) similar to **4** and **M1**. The latter results can be interpreted as an improved stability of the complex ligand/receptor due to the multimerization of peptide **M1** that provides a multivalent interaction with TR2.

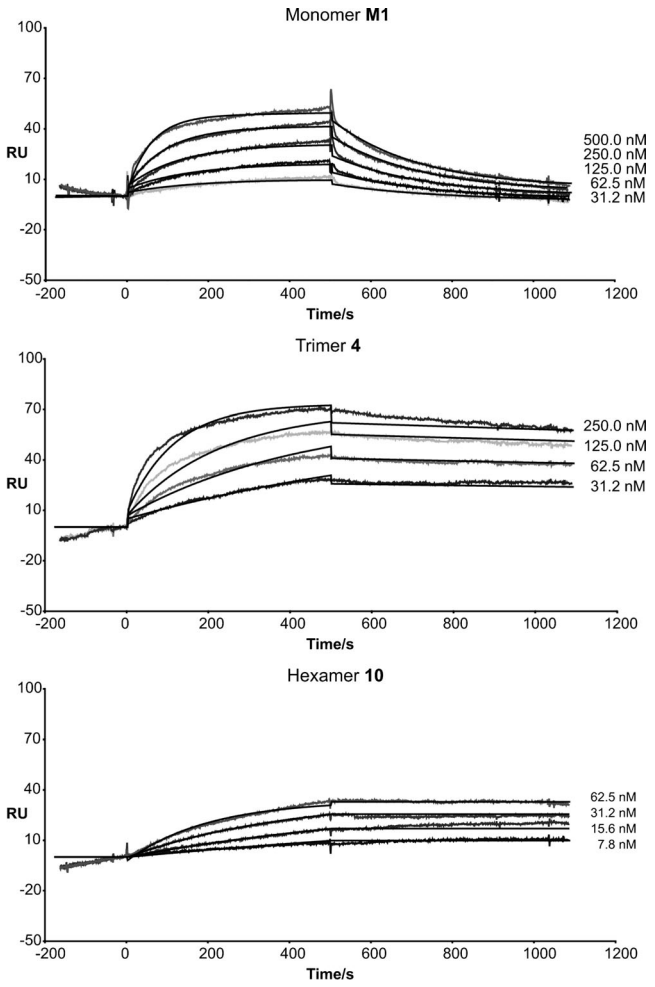


Figure 1. Sensorgrams of monomer **M1**, trimer **4**, and hexamer **10** on human TR2. The fittings correspond to the Langmuir binding model.

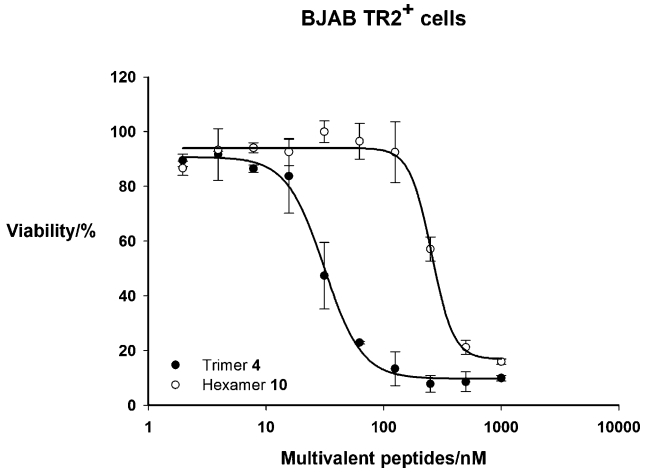


Figure 2. Cell-viability assay (MTS) in which BJAB TR2$^+$ cells were treated with increasing concentrations of trivalent **4** or hexavalent **10** peptides for 16 h. The IC_{50} values were obtained by a sigmoidal, logistic, four-parameter fitting and correspond to (31.23 ± 1.59) and (255.64 ± 13.90) nM for the trivalent and hexavalent peptides, respectively.

In addition, we can suggest that the applied strategy for ligand multimerization by using adamantane-based dendrons does not alter complex formation but contributes to increased stability during the dissociation phase.

Encouraged by these positive results, we decided to analyze the efficiency and specificity of trimer **4** and hexamer **10** to induce cell death in a TR2-dependent manner by using an in vitro model of human Burkitt lymphoma (BJAB) that either does or does not express (BJAB TR2$^+$ or BJAB TR2$^-$ cells, respectively) TRAIL receptor 2.[21] The BJAB TR2$^+$ and BJAB TR2$^-$ cells were incubated with increasing concentrations of either trivalent **4** or hexavalent **10** ligand and cell viability was analyzed after 16 h of incubation. Whereas a significant death of BJAB TR2$^+$ cells was observed upon treatment with both compounds, no cell death was seen in BJAB TR2$^-$ cells, thus indicating high TR2 selectivity of the multivalent peptides (Figure 2) and no toxic effect at the assessed concentrations (see Figure S3 in the Supporting Information).

As it has been previously shown, the monomer **M1** has no effect on BJAB TR2$^+$ cell death, thus confirming that a multivalent form of the peptide is necessary to trigger a substantial TR2-dependent apoptotic response in vitro.[12a] Unexpectedly, hexavalent ligand **10** displayed a decreased activity relative to trimer **4**. Indeed, the IC_{50} value of **10** was approximately 255 nM relative to the trivalent ligand **4**, which displayed an IC_{50} value of approximately 31 nM (Figure 2). This seemingly discordant result, when compared with SPR data, can be due to unfavorable interactions between the six **M1** peptides of **10** and TR2 on the cell surface for reasons of steric hindrance, thus avoiding the formation of a functional signaling complex. The TNF superfamily ligands are type-II transmembrane proteins that can be active in a membrane-bound form or as soluble cytokines after proteolytic shedding.[9a] The C-terminal, extracellular domain of TRAIL forms a homotrimer and mediates receptor binding and oliogomerization.[22] This ligand-induced oligomerization is generally accepted to trigger the signal cascade, but increasing the oligomerization level improves the effectiveness of the signal.[23] This effect can be related to the differential activity of membrane-bound ligands relative to their soluble products. Our goal was to set up the basis for mimicking a highly oligomeric ligand by using a G2 dendron that presented the TRAIL receptor 2 agonistic peptide **M1** on its periphery. However, contrary to what it was expected from SPR data, the hexameric peptide **10** displayed a decreased cellular activity relative to the trimeric peptide **4**. This behavior can be a consequence of a biological incompatible three-dimensional arrangement of both trimers present on the hexameric molecule, thus avoiding the formation of a functional signaling complex. Unfortunately information is not available about the three-dimensional distribution of highly multimeric ligands within the TNF superfamily members. Further synthetic studies are needed to find the optimal length of the spacer arms between the peptide and adamantane core to obtain effective apoptotic signaling, even with multivalent ligands higher than trivalent forms.

Conclusion

Our studies have demonstrated that adamantane-based dendrons are promising scaffolds because they provide a three-dimensional tripodal arrangement for the multimerization of peptides such as the TRAIL-mimicking ligand **M1**. Indeed, we carried out a straightforward synthesis of trimer **4** and hexamer **10**. These multivalent ligands proved to specifically bind the human TR2, showing an increased affinity (higher avidity) relative to the monomer **M1** in SPR assays. Moreover, both the trimeric and hexameric ligands could induce selective cell death in TR2 expressing cells (BJAB). These results together with the fact that the monomeric **M1** peptide could not induce cell death[12a] confirm that a multivalent form of the peptide is necessary to trigger a substantial TR2-dependent apoptotic response in vitro. Unexpectedly, hexamer **10** displayed a decreased biological activity relative to the trimeric ligand **4**. This outcome is probably due to unfavorable interactions between the six **M1** peptides of **10** and TR2 on the cell surface for reasons of steric hindrance. To overcome this limitation, in silico models are needed to predict the most favorable distance between the ligands and the adamantane core for the formation of a functional signaling ligand/receptor complex. The insertion of a longer spacer between the adamantane units might also render the functional groups more accessible, thus generating higher multimeric structures.

Experimental Section

General: All the starting materials, chemicals, and anhydrous solvents were obtained from commercial suppliers and used without purification. Peptide **M1** was purchased from PolyPeptide. ^{1}H and ^{13}C NMR spectra were recorded using a Brucker 300, 400, or 500 MHz spectrometer; the protons of the residual solvent were used to reference the chemical shift in ppm. Coupling constants J are reported in Hertz (Hz), and the splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). IR spectra were measured on a Perkin–Elmer Spectrum One ATR-FTIR spectrometer. ESI mass spectra were recorded on ThermoFisher Finnigan LCQ Advantage Max instrument. Elemental analyses were performed at the Micro-Analytical Facility Core of the University of Strasbourg. All the peptide-based compounds were purified by preparative HPLC on a Beckman instrument equipped with a System Gold 166 NMP detector with a Macherey–Nagel Nucleodur 100–16 C_{18} column (gradient: 5–65% B in 20 min; eluent: A = H_2O + 0.1% TFA, eluent B = MeCN + 0.08% TFA; λ = 220 nm). The following compounds were synthesized according to reported procedures: 5-*O*-tosyl-1-*tert*-butoxycarbonylaminopentane,[19,20] 1,3,5-tri(5-carboxy-pentylcarbamoyl)-7-*tert*-butoxycarbonylaminoadamantane (**1**), and 1,3,5-tricarboxy-7-*tert*-butoxycarbonylaminoadamantane (**5**).[18]

5-Azido-1-*tert*-butoxycarbonylaminopentane: Sodium azide (0.54 g, 8.39 mmol) was added to a solution of 5-*O*-tosyl-1-*tert*-butoxycarbonylaminopentane (1.00 g, 2.79 mmol) in dry DMF (20 mL). The resulting mixture was stirred at 70 °C for 24 h in an atmosphere of argon. After the addition of CH_2Cl_2 (150 mL), the organic phase was washed with ice-cold deionized water (3 × 150 mL), dried over Na_2SO_4, and filtered. After removal of the solvent, the crude product was purified by column chromatography on silica gel with CH_2Cl_2 as the eluent to give the product as a colorless oil (0.56 g, 2.45 mmol, 88%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 4.78 (bs, 1H), 3.17 (t, J = 6.7 Hz, 2H), 2.96–3.06 (m, 2H), 1.33 (s, 9H), 1.25–1.55 ppm (m, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz): D = 155.82, 78.77, 51.08, 29.44, 28.30, 28.18, 23.71 ppm; FTIR (neat) $\tilde{\nu}$ = 3362, 2972, 2931, 2861, 2091, 1689, 1515, 1244, 1163 cm^{-1}; MS (ESI): m/z: 229.2 $[M+H]^+$; elemental analysis calcd for $C_{10}H_{20}N_4O_2$: C 52.61, H 8.83, N 24.54; found: C 52.92, H 8.38, N 24.59.

5-Azidopentylamine: A solution of HCl in dioxane (4 M, 6.5 mL, 26.28 mmol) was added to a solution of 5-azido-1-*tert*-butoxycarbonylaminopentane (1.00 g, 4.38 mmol) in CH_2Cl_2 (8 mL). The resulting mixture was stirred at room temperature for 1 h. Evaporation of the solvent in vacuo afforded the product as a beige solid (0.56 g, 4.38 mmol, quant.). ^{1}H NMR ($CDCl_3$, 400 MHz): D = 8.27 (bs, 2H), 3.30 (t, J = 6.4 Hz, 2H), 2.93–3.08 (m, 2H), 1.77–1.87 (m, 2H), 1.60–1.67 (m, 2H), 1.47–1.55 ppm (m, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz): D = 50.94, 39.74, 28.14, 26.92, 23.62 ppm; FTIR (neat) $\tilde{\nu}$ = 2931, 2866, 2091, 1605, 1509, 1282, 1254 cm^{-1}; MS (ESI): m/z: 129.3 $[M+H]^+$; elemental analysis calcd for $C_5H_{12}N_4$: C 46.85, H 9.44, N 43.71; found: C 47.00, H 9.46, N 43.57.

NHS triester 2: EDC × HCl (0.120 g, 0.63 mmol) and *N*-hydroxysuccinimide (0.066 g, 0.57 mmol) were added to a solution of **1** (0.130 g, 0.18 mmol) in dry DMF (3 mL). The resulting mixture was stirred overnight at room temperature. After the addition of AcOEt (60 mL), the organic phase was washed with saturated aqueous NH_4Cl solution (1 × 60 mL) and water (1 × 60 mL), dried over Na_2SO_4, and filtered. Evaporation of the solvent in vacuo yielded the desired product **2** as a beige solid (0.101 g, 0.10 mmol, 55%). ^{1}H NMR (CD_3OD, 300 MHz): D = 3.17–3.24 (m, 6H), 2.84 (s, 12H), 2.64 (t, J = 7.2 Hz, 6H), 1.98 (s, 6H), 1.82–1.91 (m, 6H), 1.69–1.79 (m, 6H), 1.49–1.58 (m, 6H), 1.42 (s, 9H), 1.39–1.47 ppm (m, 6H); ^{13}C NMR (CD_3OD, 75 MHz): D = 178.03, 177.95, 170.22, 156.33, 79.78, 52.98, 44.57, 42.57, 42.52, 40.32, 31.54, 29.81, 28.88, 26.89, 26.57, 25.40 ppm; FTIR (neat) $\tilde{\nu}$ = 3352, 2937, 2866, 1810, 1780, 1729, 1633, 1522, 1201, 1161, 1065 cm^{-1}; MS (ESI): m/z: 1037.2 $[M+Na]^+$, 1015.0 $[M+H]^+$; elemental analysis calcd for $C_{48}H_{67}N_7O_{17}$: C 56.85, H 6.66, N 9.67; found: C 57.01, H 6.69, N 9.23.

Trimer 4: Peptide **M1** (0.065 g, 0.0323 mmol) and DIEA (50 μL) were added to a solution of NHS triester **2** (0.008 g, 0.0079 mmol) in dry DMF (3 mL). The resulting mixture was stirred at room temperature for 48 h. After removal of the solvent, the crude product was purified by preparative HPLC (t_r = 13.8 min) to obtain trimer **3** as a white solid (0.024 g, 0.0036 mmol, 45%). MS (ESI): m/z: 6710.7 $[M+H]^+$.

The obtained trimer **3** (0.019 g, 0.0028 mmol) was dissolved in TFA/H_2O/TIPS (1.5 mL, 44:5:1), and the resulting mixture was stirred at room temperature for 3 h. Purification by preparative HPLC (t_r = 12.9 min) afforded the desired compound **4** as a white solid (0.018 g, 0.0027 mmol, 83%). MS (ESI): m/z: 6609.9 $[M+H]^+$.

1,3,5-Tri(5-azido-pentylcarbamoyl)-7-*tert*-butoxycarbonylaminoadamantane (6): EDC × HCl (0.348 g, 1.82 mmol) and HOBt (0.246 g, 1.82 mmol) were added to a solution of **5** (0.100 g, 0.26 mmol) in dry DMF (3 mL). The resulting mixture was stirred at room temperature for 30 min. A solution of 5-azidopentylamine (0.200 g, 1.56 mmol) and DIEA (0.70 mL, 3.90 mmol) in dry DMF (2 mL) was added to the reaction mixture, which was stirred at 65 °C for 72 h. After the addition of AcOEt (80 mL), the organic phase was washed with saturated aqueous NH_4Cl solution (2 × 80 mL), saturated aqueous $NaHCO_3$ solution (2 × 80 mL), and brine (1 × 80 mL), dried over Na_2SO_4, and filtered. The solvent was removed in vacuo and the crude product was purified by column chromatography on silica gel with AcOEt as the eluent to yield the G1 azide dendron **6** as an orange oil (0.128 g, 0.18 mmol, 70%). ^{1}H NMR ($CDCl_3$, 300 MHz): D = 5.92 (t, J = 5.7 Hz, 3H), 4.77 (s, 1H), 3.15–3.27 (m, 12H), 1.98 (s, 6H), 1.78–1.90 (m, 6H), 1.44–1.61 (m, 12H), 1.37 (s, 9H), 1.29–1.37 ppm (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): D = 174.9, 154.1, 79.3, 51.8, 51.0, 43.1, 41.6, 39.21, 39.2, 28.9, 28.3, 28.28, 23.8 ppm; FTIR (neat) $\tilde{\nu}$ = 3326, 2926, 2855, 2086, 1701, 1630, 1519, 1244, 1158 cm^{-1}; MS (ESI): m/z: 714.8 $[M+H]^+$; elemental analysis calcd for $C_{33}H_{55}N_{13}O_5$: C 55.52, H 7.77, N 25.51; found: C 55.11, H 7.90, N 25.03.

5-Hexynoic NHS ester 7: EDC × HCl (0.256 g, 1.33 mmol) and *N*-hydroxysuccinimide (0.123 g, 1.07 mmol) were added to a solution of commercial 5-hexynoic acid (0.100 g, 0.89 mmol) in dry DMF (2 mL). The resulting mixture was stirred at room temperature for 24 h. After the addition of AcOEt (60 mL), the organic phase was washed with saturated aqueous NH_4Cl solution (1 × 60 mL) and water (1 × 60 mL), dried over Na_2SO_4,

and filtered. Evaporation of the solvent in vacuo afforded the desired product **7** as a yellow oil (0.164 g, 0.78 mmol, 88%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.78 (s, 4H), 2.72 (t, J = 7.4 Hz, 2H), 2.30 (dt, J = 2.8 and 6.8 Hz, 2H), 1.99 (t, J = 2.8 Hz, 1H), 1.87–1.95 ppm (m, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 169.07, 167.99, 82.29, 69.67, 29.43, 25.36, 23.13, 17.31 ppm; FTIR (neat) $\tilde{\nu}$ = 3281, 2942, 2105, 1810, 1780, 1727, 1201, 1064 cm^{-1}; MS (ESI): m/z: 210.1 $[M+H]^+$; elemental analysis calcd for $C_{10}H_{11}NO_4$: C 57.41, H 5.30, N 6.70; found: C 57.54, H 5.97, N 6.44.

Alkyne trimer 8: Amine **4** (0.016 g, 0.0024 mmol) and DIEA (30 μL) were added to a solution of **7** (0.001 g, 0.0048 mmol) in dry DMF (2 mL). The resulting mixture was stirred at 45 °C for 48 h. After removal of the solvent, the crude product was purified by preparative HPLC (t_r = 13.6 min) to obtain the alkyne **8** as a white solid (0.009 g, 0.0013 mmol, 56%). MS (ESI): m/z: 6703.3 $[M+H]^+$.

Hexamer 10: Compound **6** (357 μg, 0.5 μmol), sodium ascorbate (99 μg, 0.5 μmol), and copper(II) sulfate pentahydrate (75 μg, 0.3 μmol) were added to a solution of alkyne trimer **8** (12 mg, 1.8 μmol) in THF/H_2O (5 mL, 1:1) in an argon atmosphere,. The reaction mixture was stirred at room temperature for 48 h in an argon atmosphere. After lyophilization, the crude product was purified by preparative HPLC (t_r = 15.5 min) to obtain hexamer **9** as a white solid (5 mg, 0.35 μmol, 71%). MS (ESI): m/z: 14123.7 $[M+H]^+$.

The obtained hexamer **9** (5 mg, 0.3 μmol) was dissolved in TFA/H_2O/TIPS (0.5 mL, 44:5:1), and the resulting mixture was stirred at room temperature for 2 h. Purification by preparative HPLC (t_r = 14.1 min) afforded the desired compound **10** as a white solid (4 mg, 0.3 μmol, 79%). MS (ESI): m/z: 14023.0 $[M+H]^+$.

SPR analysis: The Biacore 3000 system, sensor chip CM5, surfactant P20, amine-coupling kit containing NHS, EDC×HCl, and ethanolamine were from Biacore (Uppsala, Sweden). Biosensor assays were performed with HBS-EP buffer as a running buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM sodium chloride, 3 mM ethylenediaminetetraacetic acid (EDTA), and 0.005% v/v surfactant P20; pH 7.4). The low carboxylated dextran matrix (B1) was activated with a mixture of 0.2 M EDC×HCl and 0.05 M NHS (35 μL) at 5 μL min^{-1}. Human RANK, human TR2, and mouse TR2-Fc fusion proteins were immobilized with the standard Biacore protocol at a density of 0.05 pmol mm^{-2}. Soluble peptides were diluted in the running buffer and injected (250 μL), followed by a dissociation phase (500 μL) at a flow rate of 30 μL min^{-1}. The sensor chip surface was regenerated after each experiment by injecting HCl (20 μL, 25 mM (or 50 mM for the hexamer **10**)). The kinetic parameters were calculated using Biaeval 4.1 software. Analysis was performed by using the simple Langmuir binding model. Specific binding profiles were obtained after subtracting the response signal from the control RANK channel and from a blank-buffer injection. The fitting to each model was judged by the chi square χ2 value and the randomness of residue distribution compared to the theoretical model. The use of others binding models did not give better fits.

Cell-viability assay: BJAB cells were grown in RPMI1640 medium (Lonza, Basel Switzerland) supplemented with 10% fetal calf serum. The cells were seeded in 96-well plates at 5×10^5 cells well^{-1} and treated with the indicated concentrations of multivalent peptides for 16 h. Stock peptides were prepared at a concentration of 1 mM in water, and all the dilutions were performed in the cell-culture medium. The CellTiter 96 aqueous non-radioactive cell proliferation assay (MTS; Promega, WI, USA) was used to estimate the percentage of viable cells by following the manufacturer's instructions. Nontreated cells were used to normalize the 100% value.

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Peptide Design

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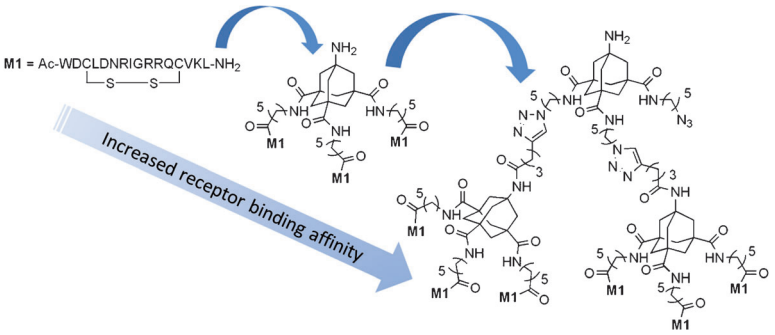
Multimerization of an Apoptogenic TRAIL-Mimicking Peptide by Using Adamantane-Based Dendrons



TRAIL-blazers: Multimerization of an apoptogenic peptide that mimics the natural tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by using adamantane-based dendrons remarkably increased the selective binding affinity to TRAIL receptor 2 (TR2; see scheme). Only multimeric forms of the ligands could induce cell death in TR2-expressing cells, thus triggering a substantial TR2-dependent apoptotic response in vitro. This multivalency effect is important in biological ligand/receptor interactions.

