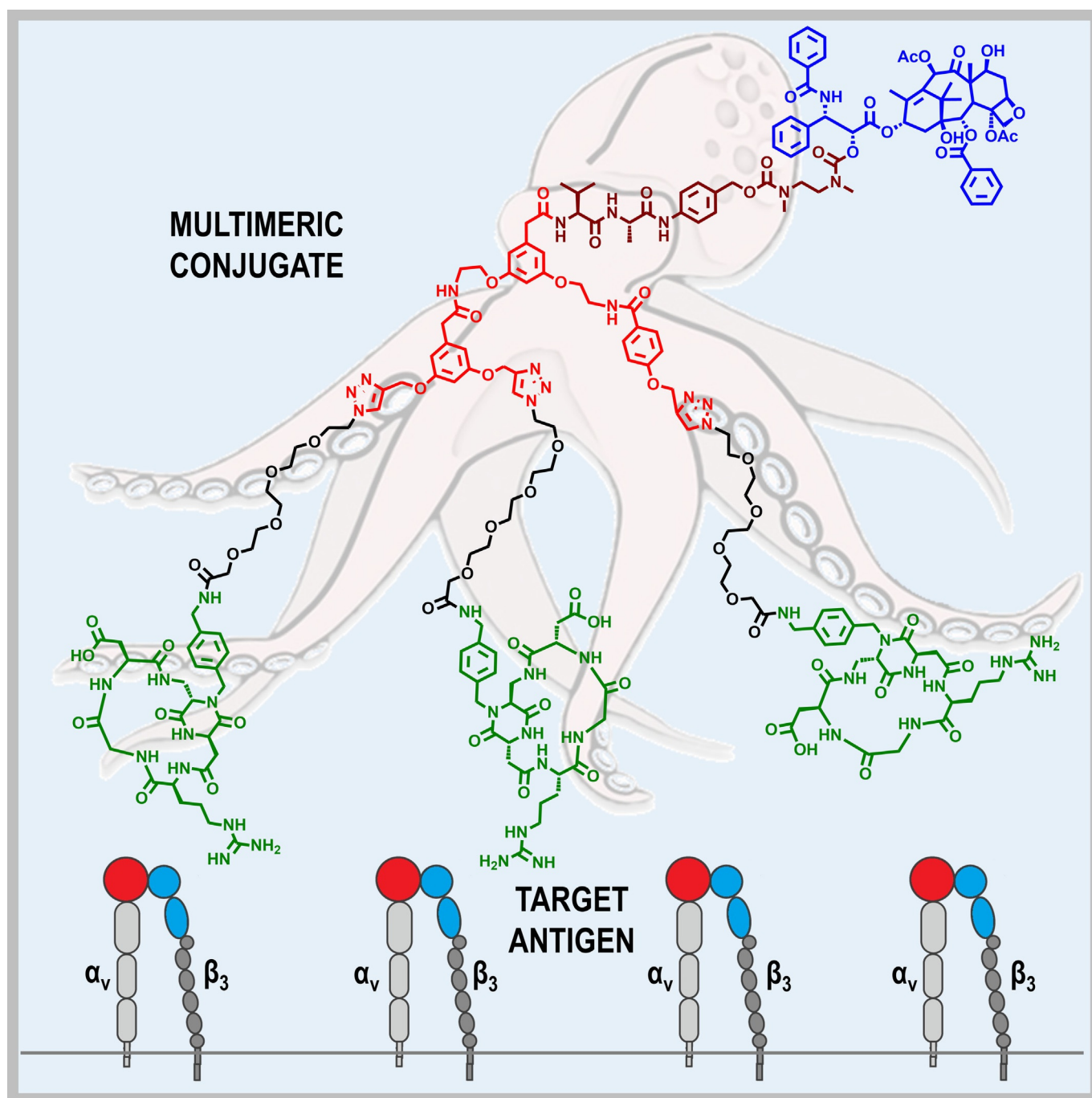


Peptidomimetics

Multivalency Increases the Binding Strength of RGD Peptidomimetic-Paclitaxel Conjugates to Integrin $\alpha_v\beta_3$

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Abstract: This work reports the synthesis of three multimeric RGD peptidomimetic-paclitaxel conjugates featuring a number of $\alpha_v\beta_3$ integrin ligands ranging from 2 to 4. These constructs were assembled by conjugation of the integrin $\alpha_v\beta_3$ ligand *cyclo*[DKP-RGD]-CH₂NH₂ with paclitaxel via a 2'-carbamate with a self-immolative spacer, the lysosomally cleavable Val-Ala dipeptide linker, a multimeric scaffold, a triazole linkage, and finally a PEG spacer. Two monomeric conjugates were also synthesized as reference compounds. Remarkably, the new multimeric conjugates showed a binding affinity for the purified integrin $\alpha_v\beta_3$ receptor that increased with the number of integrin ligands (reaching a minimum IC₅₀ value of 1.2 nM for the trimeric), thus demonstrating that multivalency is an effective strategy to strengthen the ligand–target interactions.

Nature makes widespread use of multivalency to create strong yet reversible interactions. In multivalent interactions, several covalently linked ligands bind to clustered receptors, with multiple simultaneous molecular recognition interactions. As a result, bond reinforcement occurs and strong overall binding is achieved even when the individual interactions are weak.^[1] In the last decade, multimeric ligands of cancer-overexpressed receptors have been exploited for different kinds of tumor targeting, such as drug-targeting,^[2] imaging,^[3] and the use of 'theranostic' compounds.^[4] In this context, multivalency can be envisaged as a way to improve the tumor-targeting performance of small molecule–drug conjugates (SMDCs), with the final goal of approaching the efficiency of the antibody–drug conjugates (ADCs).^[5] Indeed, SMDCs possessing multivalent ligands are expected to display enhanced affinity and selectivity for the corresponding tumor receptors, thus promoting more effectively drug accumulation at the diseased tissue.

In recent years, much research effort has been devoted to the development of SMDCs targeting integrin $\alpha_v\beta_3$,^[6] a transmembrane heterodimeric receptor that is overexpressed on

the cell surface of various tumor types (e.g., melanoma, glioblastoma, ovarian, prostatic, and breast cancer).^[7] We entered this research field reporting a low-nanomolar $\alpha_v\beta_3$ integrin ligand (compound **1** in Figure 1) featuring the Arg-Gly-Asp

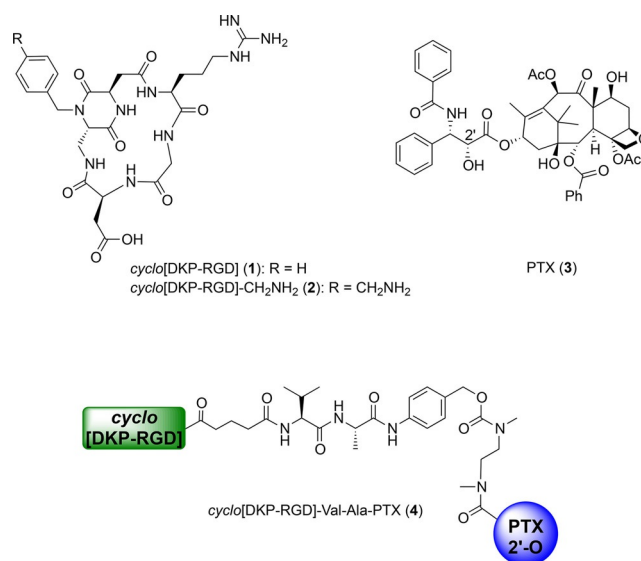


Figure 1. Molecular structures of the $\alpha_v\beta_3$ integrin ligand *cyclo*[DKP-RGD] **1**, its functionalized analogue **2**, the cytotoxic drug paclitaxel (PTX) **3**, and the SMDC *cyclo*[DKP-RGD]-Val-Ala-PTX **4**.

(RGD) sequence (i.e., the binding epitope of the endogenous ligand for this integrin) connected to a *trans*-diketopiperazine (DKP) scaffold.^[8] Remarkably, ligand **1** was found to be 33 times more selective for integrin $\alpha_v\beta_3$ with respect to integrin $\alpha_v\beta_5$ in competitive binding assays with biotinylated vitronectin (IC₅₀ = 4.5 ± 1.1 nM vs. 149 ± 25 nM).^[8] Later on, the functionalized ligand *cyclo*[DKP-RGD]-CH₂NH₂ (compound **2** in Figure 1),^[9] featuring a primary amino group, was prepared.^[9] The latter compound was conjugated to different payloads, such as the anticancer drug paclitaxel (PTX, compound **3** in Figure 1),^[9] a pro-apoptotic SMAC (second mitochondria-derived activator of caspases) mimetic compound^[10] and an anti-angiogenic VEGFR-targeting decapeptide,^[11] by means of ester and amide linkages. As a further step, to achieve selective release of PTX in the cancer cell environment, we synthesized conjugates of the *cyclo*[DKP-RGD]-CH₂NH₂ ligand **2** with paclitaxel (**3**) via a 2'-carbamate with a self-immolative spacer and the lysosomally cleavable linkers (Val-Ala and Phe-Lys dipeptide sequences).^[12] Notably, despite its remarkable size, the *cyclo*[DKP-RGD]-Val-Ala-PTX conjugate **4** (Figure 1) retained a very good affinity for the $\alpha_v\beta_3$ integrin receptor (IC₅₀ = 13.3 ± 3.6 nM in competitive binding assays with biotinylated vitronectin) and displayed fairly effective integrin targeting.^[12a]

Herein, we report our initial efforts to exploit multivalency for increasing the binding affinity of RGD ligands to integrin $\alpha_v\beta_3$.^[13] Thus, we set to synthesize a series of compounds (Figure 2) in which PTX is conjugated to one (compounds **5** and **6**), two (compound **7**), three (compound **8**), and four *cyclo*[DKP-RGD] ligands (compound **9**), respectively. In this con-

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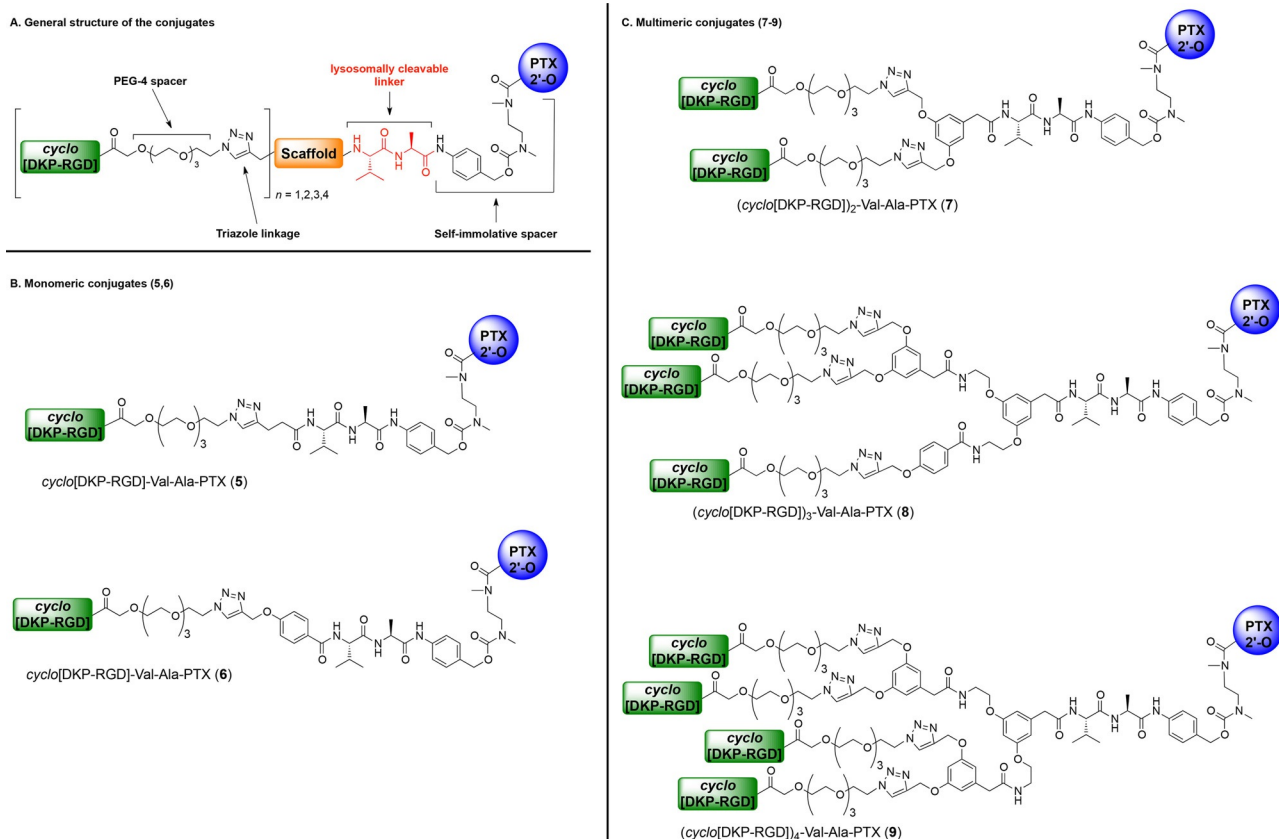


Figure 2. A) General structure of the conjugates. B) Molecular structures of monomeric conjugates (5,6). C) Molecular structures of multimeric conjugates (7–9).

text, the new conjugates were designed to release PTX intracellularly^[14] by means of a self-immolative spacer (PABC-*N,N'*-dimethylethylenediamine) and a lysosomally cleavable dipeptide linker (Val-Ala),^[12] which connects PTX to a multivalent scaffold (Figure 2A). The latter, in turn, is linked to the *cyclo*[DKP-RGD] ligand(s) via triazole group(s) deriving from copper-catalyzed azide-alkyne cycloaddition (CuAAC “click” reaction).^[15] To connect the *cyclo*[DKP-RGD] ligands to the scaffolds, tetraethylene glycol (PEG-4) spacers were employed in order to make the conjugates more water-soluble and flexible, which is reported to facilitate the binding to the receptor (Figure 2A).^[16] The choice of short-sized PEG spacers was made with the aim of minimizing the formation of bulky loops that can interfere with binding.^[17] With the exception of commercially available 4-pentynoic acid (**10**) and of the previously reported acid **11**,^[18] the alkyne scaffolds used for the synthesis of conjugates **5–9** (Figure 3) are new compounds, whose synthesis and characterization are described in the Supporting Information. The synthesis of conjugates **5–9** was carried out according to a common synthetic strategy, shown in Scheme 1. The bis-protected compound **15**, featuring the Val-Ala linker connected to the *para*-aminobenzyl carbamate (PABC)-*N,N'*-dimethylethylenediamine self-immolative spacer, was prepared according to a methodology reported by our group.^[12a] Compound **15** was Fmoc-deprotected and the resulting crude free amine was coupled to scaffolds **10–14**, affording the corresponding amides

16a–e in good yields (71–92%). Compounds **16a–e** were treated with trifluoroacetic acid for Boc removal and then reacted with 2'-(4-nitrophenoxycarbonyl)paclitaxel **17**,^[12a] affording carbamates **18a–e** again in satisfying yields (66–93%). Fi-

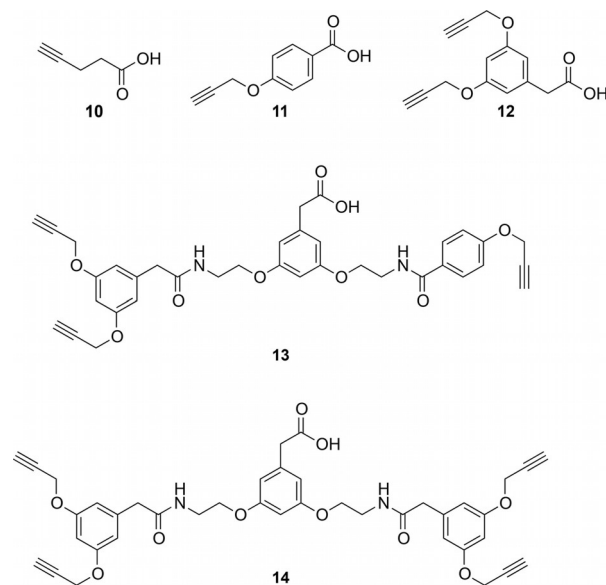
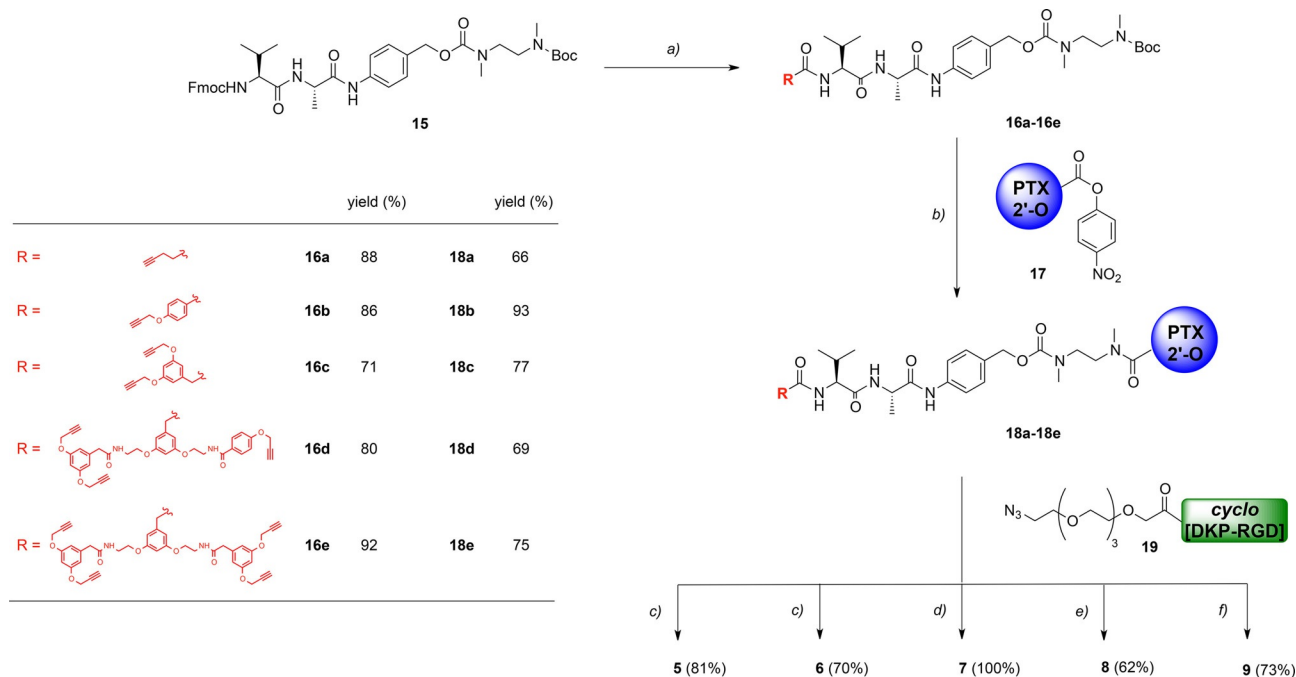


Figure 3. Mono- and polyalkyne scaffolds used for the preparation of conjugates **5–9**.



Scheme 1. Synthesis of $(\text{cyclo}[\text{DKP-RGD}])_n\text{-Val-Ala-PTX}$ ($n = 1, 2, 3, \text{ or } 4$) conjugates **5–9**. Reagents and conditions: a) 1) piperidine (5 equiv), DMF, RT, 2 h; 2) acids **10–14** (1.5 equiv), HATU (1.7 equiv), HOAt (1.7 equiv), $i\text{Pr}_2\text{NEt}$ (4 equiv), DMF, RT, overnight (**16a–16e**); b) 1) 1:2 TFA/ CH_2Cl_2 , 45 min; 2) **17** (1.5 equiv), $i\text{Pr}_2\text{NEt}$ (4 equiv), DMF, RT, overnight; c) **19** (1 equiv) **18a** or **18b** (1.5 equiv), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.5 equiv), sodium ascorbate (0.6 equiv), 1:1 DMF/ H_2O , 30 °C, overnight; d) **18c** (1 equiv), **19** (3 equiv) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1 equiv), sodium ascorbate (1.2 equiv), 1:1 DMF/ H_2O , 30 °C, overnight; e) **18d** (1 equiv), **19** (3.6 equiv) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.5 equiv), sodium ascorbate (1.8 equiv), 1:1 DMF/ H_2O , 30 °C, overnight; f) **18e** (1 equiv), **19** (4.8 equiv) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (2 equiv), sodium ascorbate (2.4 equiv), 1:1 DMF/ H_2O , 30 °C, overnight.

nally, alkynes **18a–b** and polyalkynes **18c–e** were subjected to CuAAC reaction with $\text{cyclo}[\text{DKP-RGD}]\text{-PEG-azide}$ **19**, prepared in two steps from $\text{cyclo}[\text{DKP-RGD}]\text{-CH}_2\text{NH}_2$ (**2**) as described in the Supporting Information. This reaction gave the target compounds **5–9** in good to excellent yields (62%–quantitative).

To assess the effect of ligand multipresentation on conjugates' binding properties, $(\text{cyclo}[\text{DKP-RGD}])_n\text{-Val-Ala-PTX}$ ($n = 1–4$) conjugates **5–9** were examined in vitro for their ability to inhibit biotinylated vitronectin binding to the purified $\alpha_v\beta_3$ receptor and were compared to the unconjugated ligand **1**. The screening assays were performed by incubating the immobilized integrin receptors with solutions of the RGD-PTX con-

jugates at different concentrations (10^{-12} to 10^{-5} M) in the presence of biotinylated vitronectin ($1 \mu\text{g mL}^{-1}$) and measuring the concentration of bound vitronectin (Figure 4). The IC_{50} values are listed in Table 1.

As can be observed in Table 1, conjugates **5** (entry 1) and **6** (entry 2), featuring only one $\text{cyclo}[\text{DKP-RGD}]$ ligand moiety, displayed slightly reduced binding ability (3-fold and 6-fold increase of IC_{50} , respectively) compared to the free ligand **1** (entry 6). To our delight, when the number of $\text{cyclo}[\text{DKP-RGD}]$

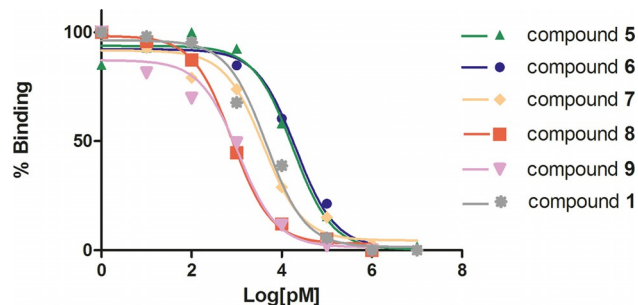


Figure 4. Inhibition of the binding of biotinylated vitronectin to $\alpha_v\beta_3$ integrin. A representative curve was selected for each compound. X-axis shows the concentration of the tested compounds **1, 5–9** in logarithmic scale; Y-axis shows the percentage of inhibition of the binding of biotinylated vitronectin in the presence of the tested compounds. Experimental data were fitted with the software, as described in the Supporting Information.

Table 1. Inhibition of biotinylated vitronectin binding to the $\alpha_v\beta_3$ receptor.				
Entry	Cpd	Structure	$\alpha_v\beta_3$ IC_{50} [nM] ^[a]	Rp/ n ^[b]
1	5	$\text{cyclo}[\text{DKP-RGD}]\text{-Val-Ala-PTX}$ (aliphatic scaffold)	14.8 ± 3.9	–
2	6	$\text{cyclo}[\text{DKP-RGD}]\text{-Val-Ala-PTX}$ (aromatic scaffold)	27.3 ± 9.8	–
3	7	$(\text{cyclo}[\text{DKP-RGD}])_2\text{-Val-Ala-PTX}$	4.0 ± 0.1	3.4
4	8	$(\text{cyclo}[\text{DKP-RGD}])_3\text{-Val-Ala-PTX}$	1.2 ± 0.5	7.6
5	9	$(\text{cyclo}[\text{DKP-RGD}])_4\text{-Val-Ala-PTX}$	1.3 ± 0.3	5.3
6	1	$\text{cyclo}[\text{DKP-RGD}]$	4.5 ± 0.1	–

[a] IC_{50} values were calculated as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding, as estimated by GraphPad Prism software. All values are the arithmetic mean \pm the standard deviation (SD) of triplicate determinations. [b] The relative potency Rp is obtained by dividing the IC_{50} of the monovalent reference **6** by the IC_{50} of each multivalent conjugate. Rp/ n values were calculated by dividing Rp of the multivalent conjugates by the valency (n) of each conjugate.^[22]

ligand moieties in the conjugates increases from 1 to 3, a clear trend of IC₅₀ decrease can be observed (entries 1–2→3→4), to reach an IC₅₀ lower than that of the free ligand **1** (entry 4 vs. entry 6). However, with the trimeric conjugate **8** a plateau is reached (entry 4, Rp/n=7.6), and no further improvement is obtained when an additional *cyclo*[DKP-RGD] ligand is present (conjugate **9**, entry 5, Rp/n=5.3). These data demonstrate that multiple presentation of the integrin ligand leads to a significant improvement of the binding affinity,^[13] although this effect seems to be partially balanced by the increasing steric bulk.

In conclusion, five new conjugates (**5–9**), featuring a number of *cyclo*[DKP-RGD] α_vβ₃ integrin ligands ranging from 1 to 4 have been synthesized using a straightforward modular approach. Binding tests carried out with the purified receptor of integrin α_vβ₃ (displacement of biotinylated vitronectin) show that the IC₅₀ decrease with increasing number of ligand moieties, down to a plateau reached with the trimeric conjugate **8** (IC₅₀=1.2 nM, Rp/n=7.6). These results demonstrate that multivalency is a valuable tool to enhance the integrin targeting performance of this kind of conjugates, and may represent a possible way to improve the in vivo tumor-targeting properties of RGD conjugates, which are often suboptimal.^[3b,d,h,6e] Moreover, it should be noted that the new ligands are also suitable for conjugation to different kinds of 'smart' linkers such as those amenable to extracellular cleavage^[19] (for example, by matrix metalloproteinases^[20] or elastases^[21]).

Experimental Section

Cyclo[DKP-RGD]-CH₂NH₂ (**2**),^[9] Fmoc-Val-Ala-N-[4-[[[(N-(Boc)-N,N'-dimethylethylenediamine)carbonyl]oxy]methyl]phenyl] (**15**)^[12] and 2'-(4-nitrophenoxycarbonyl)paclitaxel (**17**),^[12] were prepared according to literature procedures, and their analytical data were in agreement with those already published. The synthetic procedures for the preparation of compounds **5–9** and **11–14** are reported in the Supporting Information, along with the ¹H NMR and ¹³C NMR spectra, the HPLC traces and HRMS spectra. The inhibition assays of biotinylated vitronectin binding to the α_vβ₃ receptor for compounds **1** and **5–9** are reported in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antitumor agents • click chemistry • integrins • multivalency • peptidomimetics

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