CHEMISTRY
A European Journal

www.chemeurj.org

Reprint

A Journal of
ChemPubSoc Europe
ACES
Asian Chemical Editorial Society
WILEY-VCH

ChemPubSoc Europe

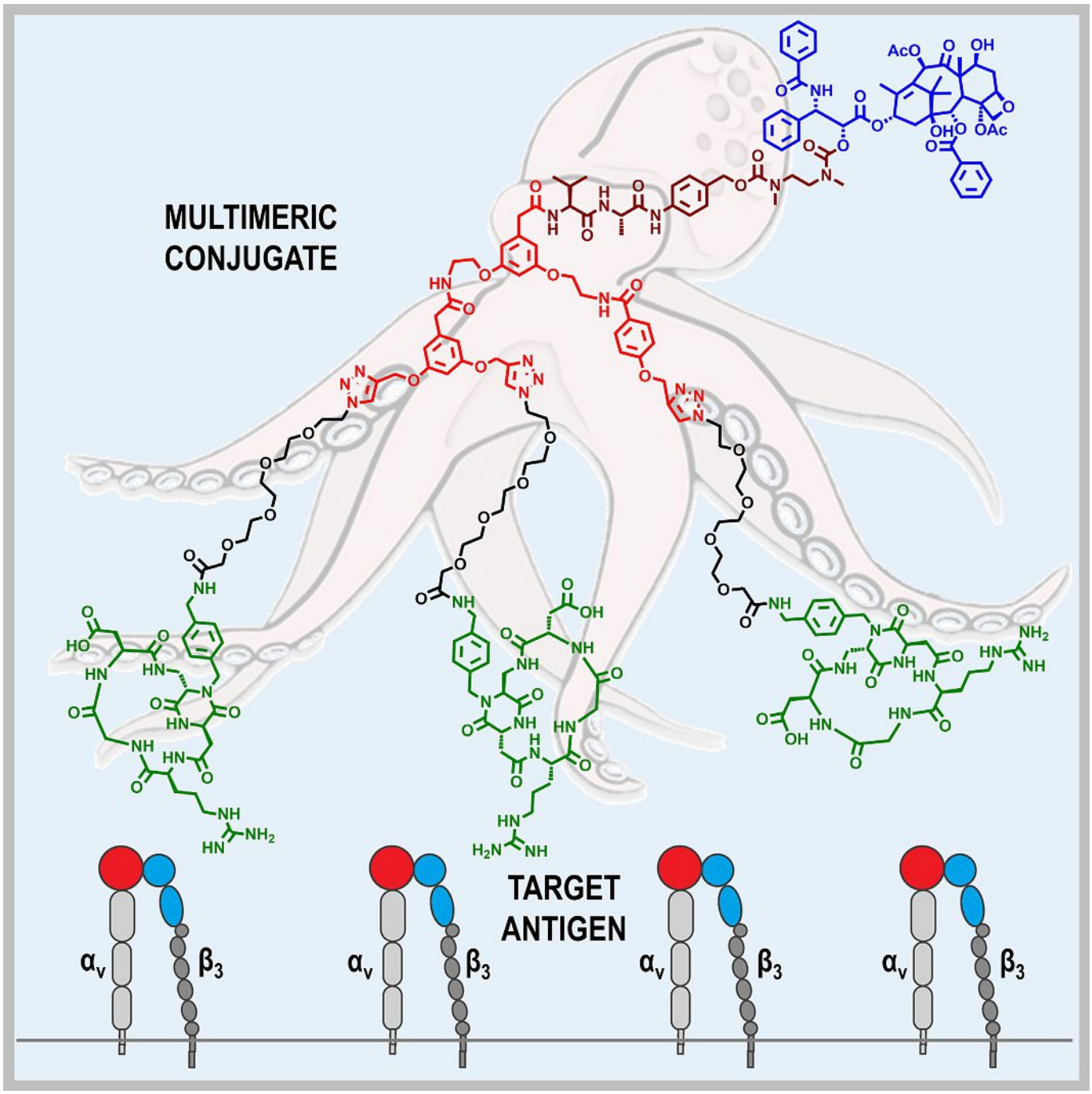
DOI: 10.1002/chem.201703093

CHEMISTRY
A European Journal
Communication

Peptidomimetics

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

André Raposo Moreira Dias,[a] Arianna Pina,[a] Alberto Dal Corso,[a] Daniela Arosio,[b] Laura Belvisi,[a, b] Luca Pignataro,*[a] Michele Caruso,[c] and Cesare Gennari*[a, b]



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]-CH_2NH_2 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 IC_{50} 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 (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 ($IC_{50} = 4.5 \pm 1.1$ nM vs. 149 ± 25 nM).[8] Later on, the functionalized ligand *cyclo*[DKP-RGD]-CH_2NH_2 (compound **2** in Figure 1), 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 decapentapeptide,[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]-CH_2NH_2 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 ($IC_{50} = 13.3 \pm 3.6$ nM in competitive binding assays with biotinylated vitronectin) and displayed fairly effective integrin targeting.[12a]

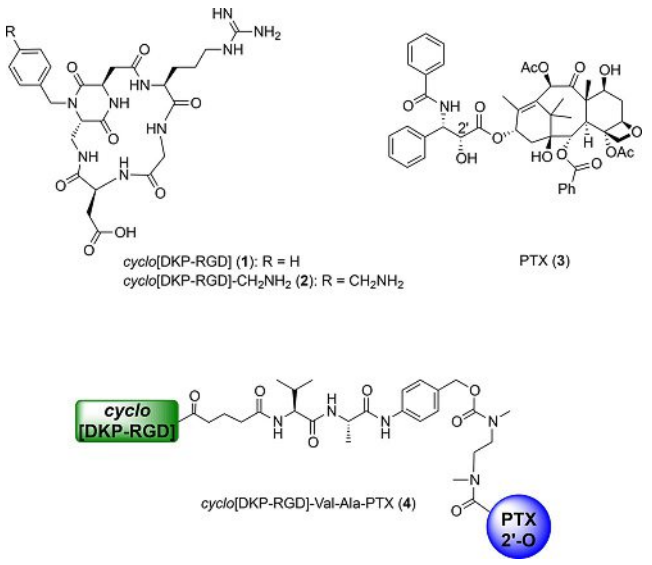


Figure 1. Molecular structures of the $\alpha_V\beta_3$ integrin ligand *cyclo*[DKP-RGD] **1**, its functionalized analogue **2**, the cytotoxic drug paclitaxel (PTX) **3**, and the SMDC *cyclo*[DKP-RGD]-Val-Ala-PTX **4**.

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-

[a] *A. Raposo Moreira Dias, A. Pina, Dr. A. Dal Corso, Prof. L. Belvisi, Dr. L. Pignataro, Prof. C. Gennari*
Università degli Studi di Milano, Dipartimento di Chimica
Via C. Golgi 19, 20133, Milan (Italy)
Fax: (+39) 02-5031-4072
E-mail: luca.pignataro@unimi.it
cesare.gennari@unimi.it

[b] *Dr. D. Arosio, Prof. L. Belvisi, Prof. C. Gennari*
CNR, Istituto di Scienze e Tecnologie Molecolari (ISTM)
Via C. Golgi, 19, 20133, Milan (Italy)

[c] *Dr. M. Caruso*
Nerviano Medical Sciences, Viale Pasteur, 10
20014 Nerviano (Italy)

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/chem.201703093.

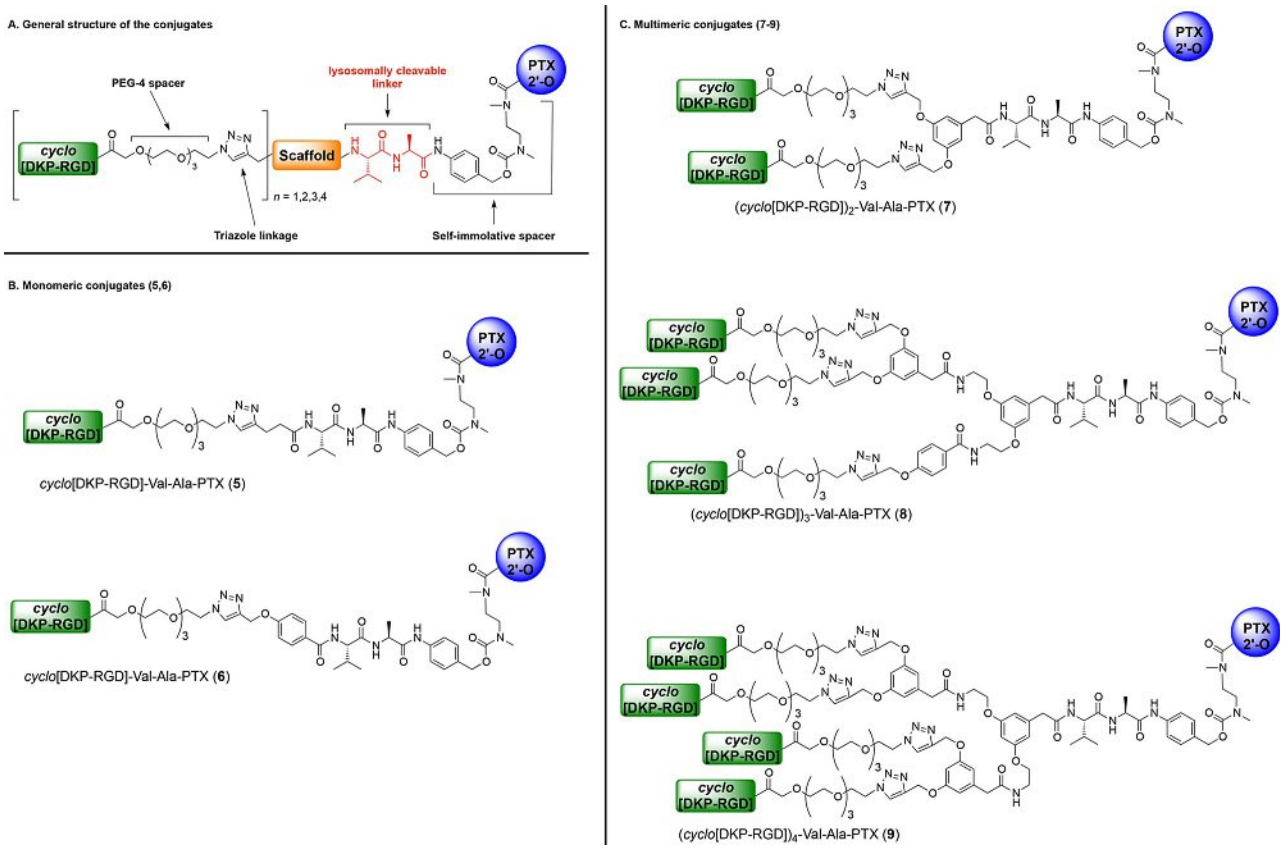


Figure 2. A) General structure of the conjugates. B) Molecular structures of monomeric conjugates (**5**, **6**). C) Molecular structures of multimeric conjugates (**7**–**9**).

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 2 A). 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 2 A).[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-

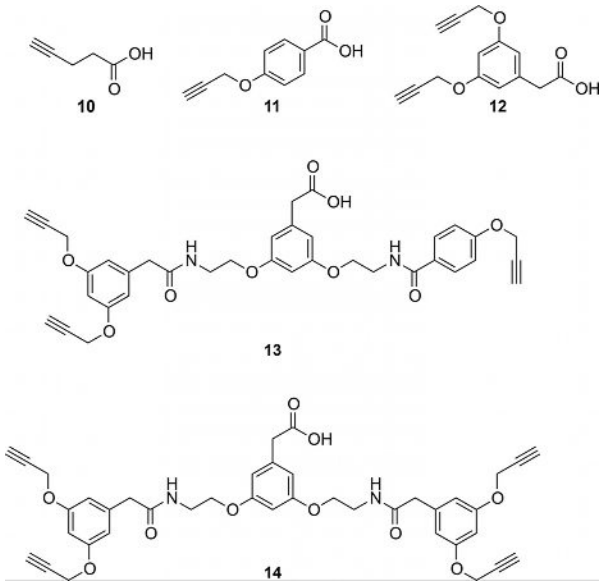
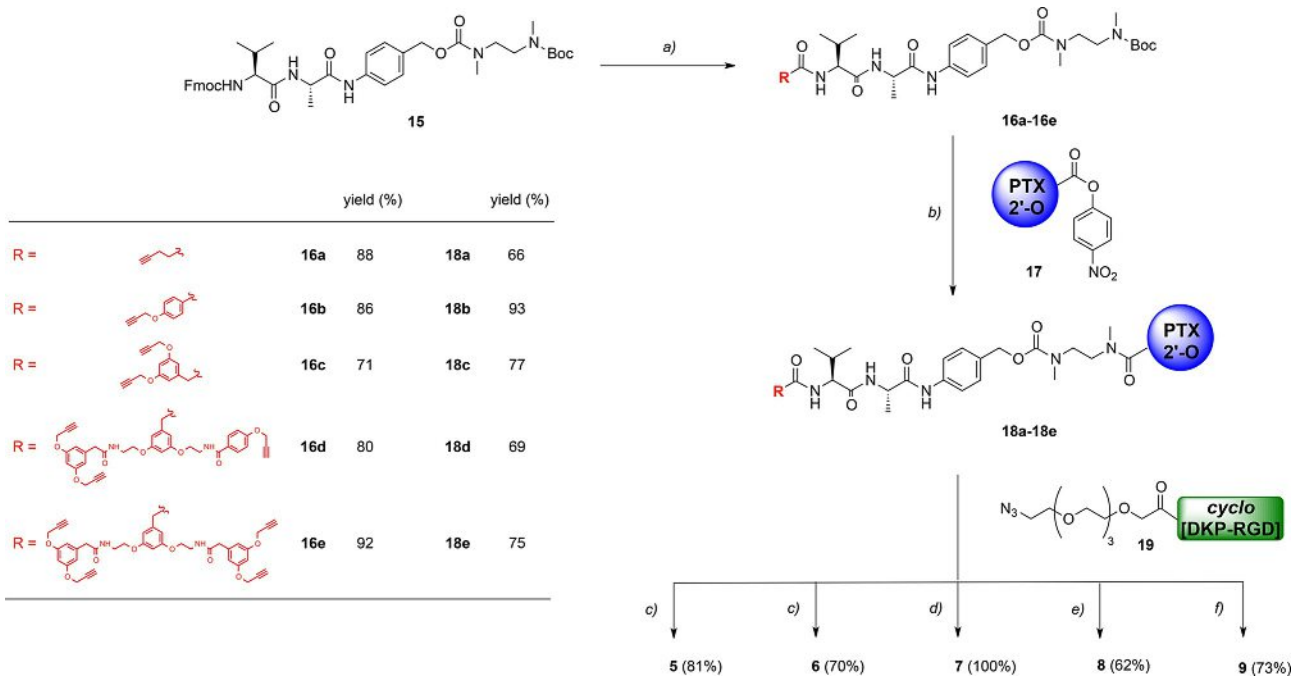


Figure 3. Mono- and polyalkyne scaffolds used for the preparation of conjugates **5**–**9**.



		yield (%)		yield (%)
R =	**16a**	88	**18a**	66
R =	**16b**	86	**18b**	93
R =	**16c**	71	**18c**	77
R =	**16d**	80	**18d**	69
R =	**16e**	92	**18e**	75

Scheme 1. Synthesis of (cyclo[DKP-RGD])$_n$-Val-Ala-PTX ($n=1$, 2, 3, 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*Pr$_2$NEt (4 equiv), DMF, RT, overnight (**16a**–**16e**); b) 1) 1:2 TFA/CH_2Cl_2, 45 min; 2) **17** (1.5 equiv), *i*Pr$_2$NEt (4 equiv), DMF, RT, overnight; c) **19** (1 equiv) **18a** or **18b** (1.5 equiv), $CuSO_4{\cdot}5H_2O$ (0.5 equiv), sodium ascorbate (0.6 equiv), 1:1 DMF/H_2O, 30 °C, overnight; d) **18c** (1 equiv), **19** (3 equiv) $CuSO_4{\cdot}5H_2O$ (1 equiv), sodium ascorbate (1.2 equiv), 1:1 DMF/H_2O, 30 °C, overnight; e) **18d** (1 equiv), **19** (3.6 equiv) $CuSO_4{\cdot}5H_2O$ (1.5 equiv), sodium ascorbate (1.8 equiv), 1:1 DMF/H_2O, 30 °C, overnight; f) **18e** (1 equiv), **19** (4.8 equiv) $CuSO_4{\cdot}5H_2O$ (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 *cyclo*[DKP-RGD]-PEG-azide **19**, prepared in two steps from *cyclo*[DKP-RGD]-CH_2NH_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, (*cyclo*[DKP-RGD])$_n$-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 conjugates at different concentrations (10^{-12} to 10^{-5} M) in the presence of biotinylated vitronectin (1 μg mL^{-1}) and measuring the concentration of bound vitronectin (Figure 4). The IC_{50} values are listed in Table 1.

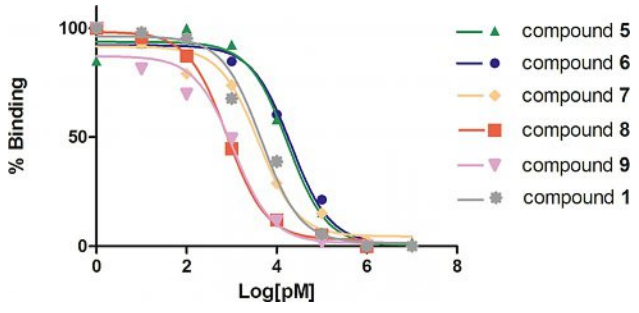


Figure 4. Inhibition of the binding of biotinylated vitronectin to $\alpha_v\beta_3$ integrin. A representative curve was selected for each compound. X-axis shows the concentration of the tested compounds **1**, **5**–**9** in logarithmic scale; Y-axis shows the percentage of inhibition of the binding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

As can be observed in Table 1, conjugates **5** (entry 1) and **6** (entry 2), featuring only one *cyclo*[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 *cyclo*[DKP-RGD]

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**	*cyclo*[DKP-RGD]-Val-Ala-PTX (aliphatic scaffold)	14.8 ± 3.9	–
2	**6**	*cyclo*[DKP-RGD]-Val-Ala-PTX (aromatic scaffold)	27.3 ± 9.8	–
3	**7**	(*cyclo*[DKP-RGD])$_2$-Val-Ala-PTX	4.0 ± 0.1	3.4
4	**8**	(*cyclo*[DKP-RGD])$_3$-Val-Ala-PTX	1.2 ± 0.5	7.6
5	**9**	(*cyclo*[DKP-RGD])$_4$-Val-Ala-PTX	1.3 ± 0.3	5.3
6	**1**	*cyclo*[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 ± 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 IC_{50} decrease can be observed (entries 1–2→3→4), to reach an IC_{50} 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] $\alpha_V\beta_3$ 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 $\alpha_V\beta_3$ (displacement of biotinylated vitronectin) show that the IC_{50} decrease with increasing number of ligand moieties, down to a plateau reached with the trimeric conjugate **8** (IC_{50} = 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]-CH_2NH_2 (**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 ^{1}H NMR and ^{13}C NMR spectra, the HPLC traces and HRMS spectra. The inhibition assays of biotinylated vitronectin binding to the $\alpha_v\beta_3$ receptor for compounds **1** and **5**–**9** are reported in the Supporting Information.

Acknowledgements

We thank the University of Milan for PhD fellowships (to A.P. and A.D.C.) and the European Commission (Marie Skłodowska-Curie ITN MAGICBULLET 642004) for a PhD fellowship (to A.R.M.D.) and financial support. We also gratefully acknowledge Ministero dell'Università e della Ricerca (PRIN 2015 project 20157WW5EH) for financial support.

Conflict of interest

The authors declare no conflict of interest.

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

[1] a) A. Barnard, D. K. Smith, *Angew. Chem. Int. Ed.* **2012**, *51*, 6572–6581; *Angew. Chem.* **2012**, *124*, 6676–6685; b) C. Fasting, C. A. Schalley, M. Weber, O. Seitz, S. Hecht, B. Koksch, J. Dernedde, C. Graf, E.-W. Knapp, R. Haag, *Angew. Chem. Int. Ed.* **2012**, *51*, 10472–10498; *Angew. Chem.* **2012**, *124*, 10622–10650; c) E. Mahon, M. Barboiu, *Org. Biomol. Chem.* **2015**, *13*, 10590–10599.

[2] a) M. Janssen, W. J. G. Oyen, L. F. A. G. Massuger, C. Frielink, I. Dijkgraaf, D. S. Edwards, M. Radjopadhye, F. H. M. Corstens, O. C. Boerman, *Cancer Biother. Radiopharm.* **2002**, *17*, 641–646; b) G. Thumshirn, U. Hersel, S. L. Goodman, H. Kessler, *Chem. Eur. J.* **2003**, *9*, 2717–2725; c) E. R. Gillies, J. M. J. Fréchet, *Drug Discovery Today* **2005**, *10*, 35–43; d) E. Garanger, D. Boturyn, J. L. Coll, M. C. Favrot, P. Dumy, *Org. Biomol. Chem.* **2006**, *4*, 1958–1965; e) S. M. Deyev, E. N. Lebedenko, *BioEssays* **2008**, *30*, 904–918; f) D. J. Welsh, D. K. Smith, *Org. Biomol. Chem.* **2011**, *9*, 4795–4801; g) D. S. Choi, H.-E. Jin, S. Y. Yoo, S.-W. Lee, *Bioconjugate Chem.* **2014**, *25*, 216–223; h) N. Krall, F. Pretto, D. Neri, *Chem. Sci.* **2014**, *5*, 3640–3644; i) A. Bianchi, D. Arosio, P. Perego, M. De Cesare, N. Carenini, N. Zaffaroni, M. De Matteo, L. Manzoni, *Org. Biomol. Chem.* **2015**, *13*, 7530–7541.

[3] a) D. Boturyn, J. L. Coll, E. Garanger, M. C. Favrot, P. Dumy, *J. Am. Chem. Soc.* **2004**, *126*, 5730–5739; b) J. Shi, L. Wang, Y.-S. Kim, S. Zhai, Z. Liu, X. Chen, S. Liu, *J. Med. Chem.* **2008**, *51*, 7980–7990; c) L. Sancey, E. Garanger, S. Foillard, G. Schoehn, A. Hurbin, C. Albiges-Rizo, D. Boturyn, C. Souchier, A. Grichine, P. Dumy, J. L. Coll, *Mol. Ther.* **2009**, *17*, 837–843; d) L. Wang, J. Shi, Y.-S. Kim, S. Zhai, B. Jia, H. Zhao, Z. Liu, F. Wang, X. Chen, S. Liu, *Mol. Pharm.* **2009**, *6*, 231–245; e) S. Liu, *Bioconjugate Chem.* **2015**, *26*, 1413–1438; f) V. Lopez-Rodriguez, C. Galindo-Sarco, F. O. García-Pérez, G. Ferro-Flores, O. Arrieta, M. A. Ávila-Rodríguez, *J. Nucl. Med.* **2016**, *57*, 404–409; g) C. Zhai, G. M. Franssen, M. Petrik, P. Laverman, D. Summer, C. Rangger, R. Haubner, H. Haas, C. Decristoforo, *Mol. Imaging Biol.* **2016**, *18*, 758–767; h) C. Imberti, S. Y. A. Terry, C. Cullinane, F. Clarke, G. H. Cornish, N. K. Ramakrishnan, P. Roselt, A. P. Cope, R. J. Hicks, P. J. Blower, M. T. Ma, *Bioconjugate Chem.* **2017**, *28*, 481–495.

[4] a) B. C. Lee, B. S. Moon, J. S. Kim, J. H. Jung, H. S. Park, J. A. Katzenellenbogen, S. E. Kim, *RSC Adv.* **2013**, *3*, 782–792; b) M. H. Lee, J. L. Sessler, J. S. Kim, *Acc. Chem. Res.* **2015**, *48*, 2935–2946; c) Z.-H. Jin, T. Furukawa, M. Degardin, A. Sugyo, A. B. Tsuji, T. Yamasaki, K. Kawamura, Y. Fujibayashi, M.-R. Zhang, D. Boturyn, P. Dumy, T. Saga, *Mol. Cancer Ther.* **2016**, *15*, 2076–2085.

[5] a) R. Mahato, W. Tai, K. Cheng, *Adv. Drug Delivery Rev.* **2011**, *63*, 659–670; b) T. Lammers, F. Kiessling, W. E. Hennink, G. Storm, *J. Controlled Release* **2012**, *161*, 175–187; c) N. Krall, J. Scheuermann, D. Neri, *Angew. Chem. Int. Ed.* **2013**, *52*, 1384–1402; *Angew. Chem.* **2013**, *125*, 1424–1443; d) G. Casi, D. Neri, *J. Med. Chem.* **2015**, *58*, 8751–8761; e) P. T. Wong, S. K. Choi, *Chem. Rev.* **2015**, *115*, 3388–3432.

[6] For the use of RGD peptides and peptidomimetics as carriers of nanoparticles, imaging agents, and anticancer drugs, see: a) F. Danhier, A. Le Breton, V. Préat, *Mol. Pharm.* **2012**, *9*, 2961–2973; b) D. Arosio, C. Casagrande, L. Manzoni, *Curr. Med. Chem.* **2012**, *19*, 3128–3151; c) F. C. Gaertner, H. Kessler, H.-J. Wester, M. Schwaiger, A. J. Beer, *Eur. J. Nucl. Med. Mol. Imaging* **2012**, *39*, 126–138; d) N. Anwar, A. Rix, W. Lederle, A. J. C. Kuehne, *Chem. Commun.* **2015**, *51*, 9358–9361; e) A. Dal Corso, L. Pignataro, L. Belvisi, C. Gennari, *Curr. Top. Med. Chem.* **2016**, *16*, 314–329; f) D. Arosio, C. Casagrande, *Adv. Drug Delivery Rev.* **2016**, *97*, 111–143; g) D. Arosio, L. Manzoni, C. Corno, P. Perego, *Recent Pat. Anti-Cancer Drug Discovery* **2017**, *12*, 148–168.

[7] a) M. Schottelius, B. Laufer, H. Kessler, H.-J. Wester, *Acc. Chem. Res.* **2009**, *42*, 969–980; b) M. Sutherland, A. Gordon, S. D. Shnyder, L. H. Patterson, H. M. Sheldrake, *Cancers* **2012**, *4*, 1106–1145.

[8] M. Marchini, M. Mingozzi, R. Colombo, I. Guzzetti, L. Belvisi, F. Vasile, D. Potenza, U. Piarulli, D. Arosio, C. Gennari, *Chem. Eur. J.* **2012**, *18*, 6195–6207.

[9] R. Colombo, M. Mingozzi, L. Belvisi, D. Arosio, U. Piarulli, N. Carenini, P. Perego, N. Zaffaroni, M. De Cesare, V. Castiglioni, E. Scanziani, C. Gennari, *J. Med. Chem.* **2012**, *55*, 10460–10474.

[10] M. Mingozzi, L. Manzoni, D. Arosio, A. Dal Corso, M. Manzotti, F. Innamorati, L. Pignataro, D. Lecis, D. Delia, P. Seneci, C. Gennari, *Org. Biomol. Chem.* **2014**, *12*, 3288–3302.

[11] S. Zanella, M. Mingozzi, A. Dal Corso, R. Fanelli, D. Arosio, M. Cosentino, L. Schembri, F. Marino, M. De Zotti, F. Formaggio, L. Pignataro, L. Belvisi, U. Piarulli, C. Gennari, *ChemistryOpen* **2015**, *4*, 633–641.

[12] a) A. Dal Corso, M. Caruso, L. Belvisi, D. Arosio, U. Piarulli, C. Albanese, F. Gasparri, A. Marsiglio, F. Sola, S. Troiani, B. Valsasina, L. Pignataro, D. Donati, C. Gennari, *Chem. Eur. J.* **2015**, *21*, 6921 – 6929; b) S. Zanella, S. Angerani, A. Pina, P. López Rivas, C. Giannini, S. Panzeri, D. Arosio, M. Caruso, F. Gasparri, I. Fraietta, C. Albanese, A. Marsiglio, L. Pignataro, L. Belvisi, U. Piarulli, C. Gennari, *Chem. Eur. J.* **2017**, *23*, 7910 – 7914.

[13] For multivalent RGD conjugates targeting integrin $\alpha_v\beta_3$, see refs. [2a,b,d,f,g,i] and [3].

[14] M. Nahrwold, C. Weiß, T. Bogner, F. Mertink, J. Conradi, B. Sammet, R. Palmisano, S. Royo Gracia, T. Preusse, N. Sewald, *J. Med. Chem.* **2013**, *56*, 1853 – 1864.

[15] a) *1,3-Dipolar Cycloadditions Chemistry*, (Ed.: R. Huisgen), Wiley, Hoboken, **1984**, Vol. 1, pp. 1 – 176; b) R. Huisgen, *Pure Appl. Chem.* **1989**, *61*, 613 – 628; c) H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004 – 2021; *Angew. Chem.* **2001**, *113*, 2056 – 2075; d) H. C. Kolb, K. B. Sharpless, *Drug Discovery Today* **2003**, *8*, 1128 – 1137.

[16] S. Ordanini, N. Varga, V. Porkolab, M. Thepaut, L. Belvisi, A. Bertaglia, A. Palmioli, A. Berzi, D. Trabattoni, M. Clerici, F. Fieschi, A. Bernardi, *Chem. Commun.* **2015**, *51*, 3816 – 3819.

[17] A. Dal Pozzo, E. Esposito, M. Ni, L. Muzi, C. Pisano, F. Bucci, L. Vesci, M. Castorina, S. Penco, *Bioconjugate Chem.* **2010**, *21*, 1956 – 1967.

[18] J. I. Gavrilyuk, U. Wuellner, S. Salahuddin, R. K. Goswami, S. C. Sinha, C. F. Barbas, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3716 – 3720.

[19] For the extracellular cleavage of non-internalizing conjugates, see: a) S. Cazzamalli, A. Dal Corso, D. Neri, *Mol. Cancer Ther.* **2016**, *15*, 2926 – 2935; b) S. Cazzamalli, A. Dal Corso, D. Neri, *J. Controlled Release* **2017**, *246*, 39 – 45; c) A. Dal Corso, S. Cazzamalli, R. Gebleux, M. Mattarella, D. Neri, *Bioconjugate Chem.* **2017**, *28*, 1826 – 1833.

[20] a) J. L. Crisp, E. N. Savariar, H. L. Glasgow, L. G. Ellies, M. A. Whitney, R. Y. Tsien, *Mol. Cancer Ther.* **2014**, *13*, 1514 – 1525; b) Z. H. Peng, J. Kopeček, *J. Am. Chem. Soc.* **2015**, *137*, 6726 – 6729.

[21] a) K. Yamaguchi, S. Shimada, S. Tashima, M. Ogawa, *Oncol. Rep.* **2000**, *7*, 1017 – 1021; b) K. Lapis, J. Timar, *Semin. Cancer Biol.* **2002**, *12*, 209 – 217; c) M. D. Albers, J. D. Baumgarten, H. G. D. Lerchen, A. D. Schoop (Bayer HealthCare AG), EP 1372732 A1, **2002**.

[22] A true multivalent effect is observed if Rp/*n* is higher than 1, see: D. Alvarez-Dorta, D. T. King, T. Legigan, D. Ide, I. Adachi, D. Deniaud, J. Désiré, A. Kato, D. Vocadlo, S. G. Gouin, Y. Blériot, *Chem. Eur. J.* **2017**, *23*, 9022 – 9025.

Manuscript received: July 5, 2017
Accepted manuscript online: August 17, 2017
Version of record online: September 6, 2017

