Peptidomimetics

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

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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. 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 context, the new conjugates were designed to release PTX intracellularly by means of a self-immolative spacer (PABC-cyclo(DKP-RGD)) and a lysosomally cleavable linker (Val-Ala), which connects PTX to a multivalent scaffold (Figure 2A). The latter, in turn, is linked to the cyclo(DKP-RGD) (RGD) sequence (i.e., the binding epitope of the endogenous ligand for this integrin) connected to a trans-diketopiperazine (DKP) scaffold. Remarkably, ligand 1 was found to be 33 times more selective for integrin $\alpha_v\beta_3$ with respect to integrin $\alpha_v\beta_1$, in competitive binding assays with biotinylated vitronectin (IC$_{50}$ = 4.5 ± 1.1 nM vs. 149 ± 25 nM). Later on, the functionalized ligand cyclo(DKP-RGD)-CH$_2$NH$_2$ (compound 2 in Figure 1), featuring a primary amino group, was prepared. The latter compound was conjugated to different payloads, such as the anticancer drug paclitaxel (PTX, compound 3 in Figure 1), a pro-apoptotic SMAC (second mitochondria-derived activator of caspases) mimetic compound and an anti-angiogenic VEGFR-targeting decapentapeptide, 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)-Val-Ala-PTX conjugate (Figure 1) retained a very good affinity for the $\alpha_v\beta_3$ integrin receptor (IC$_{50}$ = 13.3 ± 3.6 nM in competitive binding assays with biotinylated vitronectin) and displayed fairly effective integrin targeting.

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.
ligand(s) via triazole group(s) deriving from copper-catalyzed azide-alkyne cycloaddition (CuAAC “click” reaction).\textsuperscript{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).\textsuperscript{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.\textsuperscript{17} With the exception of commercially available 4-pentyenoic acid (10) and of the previously reported acid 11,\textsuperscript{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'-dimethylethylene-diamine self-immolative spacer, was prepared according to a methodology reported by our group.\textsuperscript{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,\textsuperscript{12a} affording carbamates 18a–e again in satisfying yields (66–93\%). Finally, alkynes 18a–b and polyalkynes 18c–e were subjected to CuAAC reaction with cyclo[DKP-RGD]-PEG-azole 19, prepared in two steps from cyclo[DKP-RGD]-CH$_2$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, (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 $\mu$g/mL\textsuperscript{19}) and measuring the concentration of bound vitronectin (Figure 4). The IC\textsubscript{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 cyclo[DKP-RGD] ligand moiety, displayed slightly reduced binding ability (3-fold and 6-fold increase of IC\textsubscript{50} respectively) compared to the free ligand 1 (entry 6). To our delight, when the number of cyclo[DKP-RGD] ligand moieties in the conjugates increases from 1 to 3, a clear trend of IC\textsubscript{50} decrease can be observed (entries 1–2 $\rightarrow$ 3–4), to reach an IC\textsubscript{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.

![Figure 2. A) General structure of the conjugates. B) Molecular structures of monomeric conjugates (5, 6). C) Molecular structures of multimeric conjugates (7–9).](image-url)
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] αvβ3 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 αvβ3 (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

![Scheme 1. Synthesis of (cyclo[DKP-RGD]-)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), iPr2NET (1.7 equiv), DMF, RT, overnight (16a–16e); b) 1) 1:2 TFA/CH2Cl2, 45 min; 2) 17 (1.5 equiv), iPr2NET (4 equiv), DMF, RT, overnight; c) 19 (1 equiv), 18a or 18b (1.5 equiv), CuSO4·5H2O (0.5 equiv), sodium ascorbate (0.6 equiv), 1:1 DMF/H2O, 30 °C, overnight; d) 18c (1 equiv), 19 (3 equiv) CuSO4·5H2O (1 equiv), sodium ascorbate (1.2 equiv), 1:1 DMF/H2O, 30 °C, overnight; e) 18d (1 equiv), 19 (3.6 equiv) CuSO4·5H2O (1.5 equiv), sodium ascorbate (1.8 equiv), 1:1 DMF/H2O, 30 °C, overnight; f) 18e (1 equiv), 19 (4.8 equiv) CuSO4·5H2O (2 equiv), sodium ascorbate (2.4 equiv), 1:1 DMF/H2O, 30 °C, overnight.]

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

![Figure 4. Inhibition of the binding of biotinylated vitronectin to αvβ3 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.]
those amenable to extracellular cleavage\cite{16} (for example, by matrix metalloproteinases\cite{17} or elastases\cite{18}).

**Experimental Section**

Cyclo(DKP-RGD)-CH2NH2 (2). Fmoc-Val-Ala-N[4-[[N-(Boc)-N-di-methylethylenediamine]carbonyl][oxy][methyl][phenyl] (15)\cite{15} and 2’-(4-nitrophenoxycarbonyl)paclitaxel (17)\cite{17} 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 α5β3 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

\[\text{Table 1. Inhibition of biotinylated vitronectin binding to the } \alpha_5\beta_3 \text{ receptor.}\]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Cpd</th>
<th>Structure</th>
<th>(\alpha_5 \beta_3 \text{ IC}_{50} \text{ (nM)})</th>
<th>Rp/n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>cyclo(DKP-RGD)-Val-Ala-PTX (aliphatic scaffold)</td>
<td>14.8 ± 3.9</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>cyclo(DKP-RGD)-Val-Ala-PTX (aromatic scaffold)</td>
<td>27.3 ± 9.8</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>cyclo(DKP-RGD)3-Val-Ala-PTX</td>
<td>4.0 ± 0.1</td>
<td>3.4</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>cyclo(DKP-RGD)2-Val-Ala-PTX</td>
<td>1.2 ± 0.5</td>
<td>7.6</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>cyclo(DKP-RGD)2-Val-Ala-PTX</td>
<td>1.3 ± 0.3</td>
<td>5.3</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>cyclo(DKP-RGD)4-Val-Ala-PTX</td>
<td>4.5 ± 0.1</td>
<td>–</td>
</tr>
</tbody>
</table>

[a] \(\text{IC}_{50}\) 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 \(\text{IC}_{50}\) of the monovalent reference 6 by the \(\text{IC}_{50}\) of each multivalent conjugate. Rp/n values were calculated by dividing Rp of the multivalent conjugates by the valency (n) of each conjugate.\cite{19}


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


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These are not the final page numbers!
Unity makes strength: Multivalent binding can effectively increase the strength of ligand–receptor interactions. Newly synthesized \((\text{cyclo[DKP-RGD]})_n\)-Val-Ala-Paclitaxel \((n=1–4)\) compounds show an affinity for integrin \(\alpha_\text{v}\beta_3\) which increases with the number of cyclo[DKP-RGD] ligands.

Multivalent binding is often used to increase the strength of ligand–receptor interactions. In oncology, the development of multimeric conjugates aims at promoting a more efficient recognition of target antigens, possibly leading to better therapeutic performance. The affinity for integrin \(\alpha_\text{v}\beta_3\) of multimeric conjugates \((\text{cyclo[DKP-RGD]})_n\)-Val-Ala-PTX \((n=1–4)\) increases with the number of cyclo[DKP-RGD] ligands up to \(n=3\). For more information, see the Communication by L. Pignataro, C. Gennari et al. on page ff.