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Anticancer Agents |*Hot Paper*|

Tumor Targeting with an *iso*DGR–Drug Conjugate

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Abstract: Herein we report the first example of an *iso*DGR–drug conjugate (**2**), designed to release paclitaxel selectively within cancer cells expressing integrin $\alpha_V\beta_3$. Conjugate **2** was synthesized by connecting the *iso*DGR peptidomimetic **5** with paclitaxel via the lysosomally cleavable Val–Ala dipeptide linker. Conjugate **2** displayed a low nanomolar affinity for the purified integrin $\alpha_V\beta_3$ receptor (IC_{50} = 11.0 nM). The tumor targeting ability of conjugate **2** was assessed in vitro in anti-proliferative assays on two isogenic cancer cell lines characterized by different integrin $\alpha_V\beta_3$ expression: human glioblastoma U87 ($\alpha_V\beta_3+$) and U87 β_3-KO ($\alpha_V\beta_3-$). The *iso*DGR-PTX conjugate **2** displayed a remarkable targeting index (TI = 9.9), especially when compared to the strictly related RGD-PTX conjugate **4** (TI = 2.4).

Nowadays, the development of molecular devices able to selectively deliver chemotherapeutics at the disease site has gained a central position in cancer research. In particular, such targeting agents would allow circumventing the lack of selectivity observed when administering cytotoxic agents to patients. Due to this main limitation, traditional chemotherapy requires the use of high drug dosages, with consequent severe side effects that vitiate the overall efficacy of the therapy.[1] A first approach consisted in the preparation of antibody–drug conjugates (ADCs), in which the use of monoclonal antibodies (mAbs) to target specific tumor antigens resulted in a clear discrimination of cancer cells from healthy tissues. However, this strategy presents several drawbacks, especially related to high manufacturing costs, poor pharmacokinetic properties and possible immune-system-induced alteration of drug efficiency.[2] At this stage, small molecule–drug conjugates (SMDCs) arose as an alternative to ADCs: in this case, the targeting moiety is a small molecule, such as an oligopeptide, a peptidomimetic or a vitamin, capable of interacting selectively with particular proteins overexpressed by tumor cells. Unlike ADCs, the use of a small molecule ascribes improved pharmacokinetic properties to the entire conjugate, which in principle can be synthesized by easier and more affordable synthetic strategies.[2]

In the field of SMDCs, integrin $\alpha_V\beta_3$ represents a very interesting target to be exploited for the selective delivery of anticancer agents within the tumor site. As matter of fact, the expression of this transmembrane receptor is increased in a variety of human cancer types (e.g., breast cancer, glioblastoma, pancreatic tumor, prostate carcinoma) with respect to healthy tissues. The increased expression of $\alpha_V\beta_3$ integrin in tumor cells is associated with different pathological features: angiogenesis, tumor growth, apoptosis resistance, and metastasis.[3] Integrin $\alpha_V\beta_3$ recognizes endogenous ligands by the tripeptide arginine-glycine-aspartate[4] (RGD) and also by the related sequence *iso*-aspartate-glycine-arginine[5,6] (*iso*DGR). In 2012, computational and biochemical studies showed that *iso*DGR-containing cyclopeptides act as genuine $\alpha_V\beta_3$ antagonists, blocking the ligand binding site and inhibiting integrin allosteric activation.[6a] In contrast to the RGD ligands which in some cases may cause adverse paradoxical integrin activation effects,[6a,7] compounds based on the *iso*DGR motif could become a new generation of integrin-binding drugs free from these drawbacks. For example, *iso*DGR ligand **1** (Figure 1) displays inhibitory effects on the FAK/Akt integrin-activated transduction pathway and on integrin-mediated cell infiltration processes, qualifying therefore as a true integrin antagonist.[8]

A variety of ligands containing the RGD sequence have been synthesized and reported in the literature so far, with some of them showing a very high affinity for the integrin receptor.[9]

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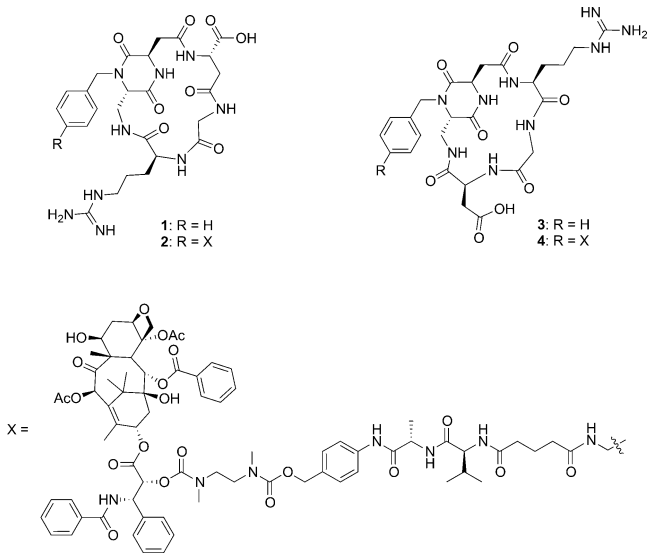


Figure 1. Structures of the integrin ligands *cyclo*[DKP-*iso*DGR] (**1**) and cyclo[DKP-RGD] (**3**), and of the corresponding SMDCs *cyclo*[DKP-*iso*DGR]-Val-Ala-PTX (**2**) and *cyclo*[DKP-RGD]-Val-Ala-PTX (**4**).

Moreover, numerous RGD–drug conjugates have been developed for tumor targeting in the past two decades,[10–12] while no example of *iso*DGR–drug conjugate has ever been reported. In fact, compared to the high binding affinity of the RGD ligands for $\alpha_v\beta_3$ integrin ($IC_{50} < 15$ nM),[9] the *iso*DGR motif displayed much lower affinity ($IC_{50} \geq 43$ nM),[13] with a single notable exception (**1**, $IC_{50} = 9.2$ nM), see Figure 1.[8]

Herein we report the first example of an *iso*DGR–drug conjugate (**2**, Figure 1), based on ligand **1**, which displays a high binding affinity for the purified integrin $\alpha_v\beta_3$ receptor ($IC_{50} =$ 11.0 nM), see Table 1.

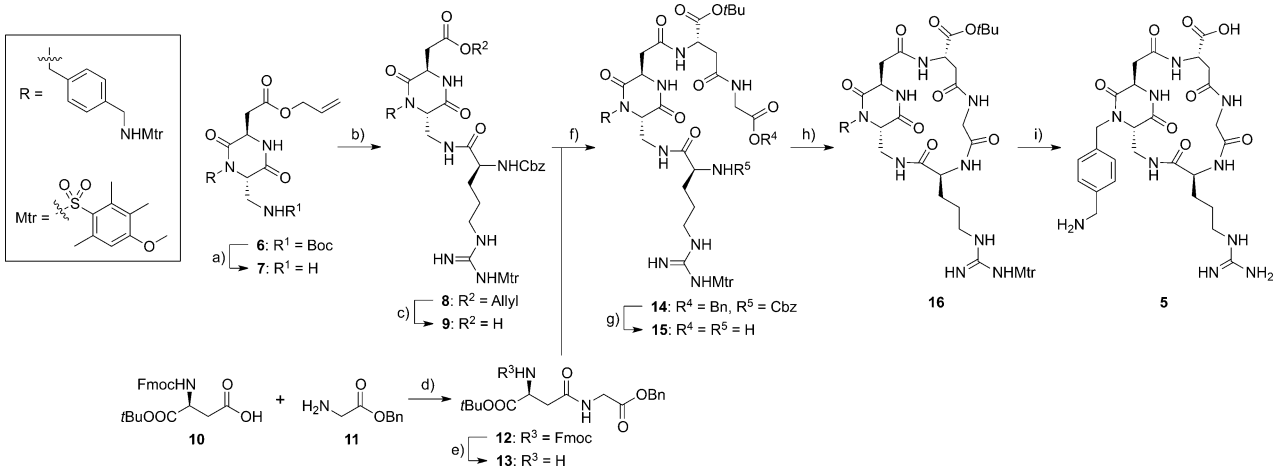
Conjugate **2** has been designed in a way similar to the corresponding RGD-drug conjugate (**4**, Figure 1),[10g] which contains the RGD integrin ligand **3**.[14] The *iso*DGR targeting moiety has been linked to the cytotoxic agent paclitaxel using the lysosomally cleavable dipeptide Val–Ala: this sequence showed high plasma stability, whereas it is rapidly cleaved by lysosomal cysteine proteases (such as cathepsins B and D) upon integrin-mediated internalization by endocytosis.[10g,15,16]

Table 1. Inhibition of biotinylated vitronectin binding to purified $\alpha_v\beta_3$ receptor.

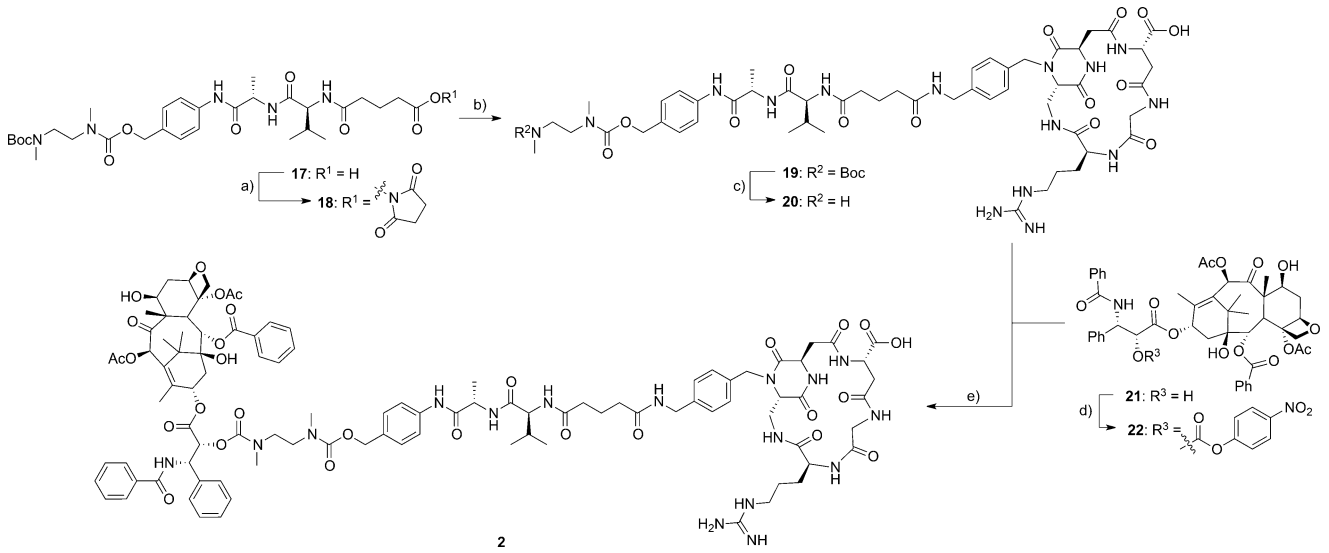
Entry	Ligand	Structure	$\alpha_v\beta_3$ IC_{50} [nM][a]
1	**1**	*cyclo*[DKP-*iso*DGR]	9.2 ± 1.1
2	**2**	*cyclo*[DKP-*iso*DGR]-Val-Ala-PTX	11.0 ± 0.2
3	**3**	*cyclo*[DKP-RGD]	4.5 ± 1.1
4	**4**	*cyclo*[DKP-RGD]-Val-Ala-PTX	13.3 ± 3.6

[a] IC_{50} values were calculated as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding. Screening assays were performed by incubating the immobilized integrin $\alpha_v\beta_3$ with increasing concentrations (10^{-12}–10^{-5} M) of the RGD or *iso*DGR ligands in the presence of biotinylated vitronectin (1 mg mL^{-1}), and measuring the concentration of bound vitronectin in the presence of the competitive ligands.

In order to synthesize conjugate **2**, first we prepared peptidomimetic **5** (Scheme 1), a derivative of the *iso*DGR ligand **1** bearing an amino functional group suitable for conjugation. Bifunctional diketopiperazine **6**[10h] was Boc-deprotected and reacted with Cbz-Arg(Mtr) to give carboxylic acid **9** upon allyl ester cleavage. Acid **9** was coupled with dipeptide **13**, obtained starting from protected aspartic acid **10** (commercially available) and benzyl glycinate **11**. The benzyl (Bn) and carboxybenzyl (Cbz) protecting groups of the resulting compound **14** were selectively removed by catalytic hydrogenolysis to afford amino acid **15**, which was cyclized under high dilution conditions (1.4 mM). Mtr- (4-methoxy-2,3,6-trimethylbenzenesulphonyl) and *tert*-butyl ester removal on macrolactam **16** afforded the desired *iso*DGR peptidomimetic **5** after HPLC purification and freeze-drying. The benzylic amine of compound **5**



Scheme 1. Synthesis *iso*DGR peptidomimetic **5**. Reagents and conditions: a) TFA/CH_2Cl_2 1:2, RT, 2 h; b) Cbz-Arg(Mtr)-OH, HATU, HOAt, *i*Pr_2NEt, DMF, 0 °C to RT, overnight, 94% over 2 steps; c) [$Pd(PPh_3)_4$], *N*-methylaniline, CH_2Cl_2, 0 °C, 1 h, 88%; d) HATU, HOAt, *i*Pr_2NEt, DMF, 0 °C to RT, overnight, 86%; e) piperidine, DMF, 2 h, RT, 67%; f) **13**, HATU, HOAt, *i*Pr_2NEt, DMF, 0 °C to RT, overnight, 95%; g) H_2, 10% Pd/C, THF/H_2O 1:1, overnight, RT, 95%; h) HATU, HOAt, *i*Pr_2NEt, DMF/CH_2Cl_2 1:1 (1.4 mM), 0 °C to RT, overnight, 79%; i) TFA/TMSBr/thioanisole/EDT/phenol 70:14:10:5:1, 2 h, RT, 47%. (TFA = trifluoroacetic acid, HATU = 1-[Bis(-dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate, TMSBr = bromotrimethylsilane, EDT = 1,2-ethanedithiol).



Scheme 2. Synthesis of *iso*DGR–drug conjugate **2**. Reagents and conditions: a) DIC, NHS, DMF, 0 °C to RT, overnight; b) **5**, CH_3CN/PBS 1:1; pH 7.3–7.6, 0 °C to RT, overnight; c) TFA/CH_2Cl_2 1:2, 1 h, RT, 55% over three steps; d) 4-Nitrophenylchloroformate, pyridine, CH_2Cl_2, −50 °C to −20 °C; 4 h, 69%; e) **22**, iPr_2NEt, DMF, 0 °C to RT, overnight, 55%. (PBS = phosphate-buffered saline).

was coupled with **18**, the *N*-hydroxysuccinimidyl ester of carboxylic acid **17**,[10g] to give compound **19** (Scheme 2). Treatment of **19** with trifluoroacetic acid in dichloromethane afforded amine **20** which was reacted with carbonate **22** to obtain the final *iso*DGR-PTX conjugate **2**.

The tumor-targeting ability of conjugate **2** was assessed in vitro against two isogenic cancer cell lines characterized by different integrin $\alpha_V\beta_3$ expression. U87 human glioblastoma cells were selected as the integrin $\alpha_V\beta_3$-expressing cell line and, at the same time, used to generate the corresponding clone U87 β_3-KO, in which the expression of the gene encoding for the β_3 integrin subunit was deleted using CRISPR-Cas9 gene editing technology.[17] Flow cytometry studies on U87 and U87 β_3-KO cells confirmed the absence of integrin $\alpha_V\beta_3$ in the U87 β_3-KO cell line (Figure 2).

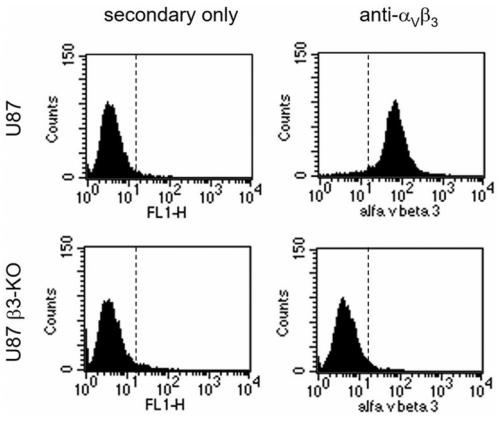


Figure 2. Flow cytometry experiments on U87 and U87 β_3-KO cells to assess the different $\alpha_V\beta_3$ integrin expression. Cells were incubated with the secondary antibody (CF488A-goat anti-mouse IgG, Biotium 20011), or with the anti-$\alpha_V\beta_3$ antibody (clone LM609-Millipore MAB 1976) followed by the secondary antibody, see the Supporting Information.

Clone U87 β_3-KO is not a perfect negative control, as the conjugates can still be actively internalized upon interaction with other integrins, for example, $\alpha_V\beta_5$. Therefore, the selectivity shown by the conjugates in cell viability experiments using U87 β_3-KO and U87 cells [$IC_{50}(\alpha_V\beta_3-)/IC_{50}(\alpha_V\beta_3+)$] should be considered as a minimum (conservative) value.[18] Cells were incubated with increasing doses of *iso*DGR–PTX conjugate **2** for 144 h, before measuring the cell viability in culture (Table 2).

Table 2. Evaluation of anti-proliferative activity of *iso*DGR-PTX conjugate **2** in U87 and U87 β_3-KO.

Structure	IC_{50} [nM][a] U87 ($\alpha_V\beta_3+$)	U87 β_3-KO ($\alpha_V\beta_3-$)	TI[b]
Paclitaxel (**21**)	0.64	0.28	1
*iso*DGR-PTX conjugate (**2**)	927.6	4003.0	9.9
RGD-PTX conjugate (**4**)	550.0	581.7	2.4

[a] IC_{50} values were calculated as the concentration of compound required for 50% inhibition of cell viability in culture, based on quantitation of the ATP present as estimated by CellTiter-GLO; cells were treated for 144 h in 96-well plates. [b] Targeting index (TI): $[IC_{50}(\alpha_V\beta_3-)/IC_{50}(\alpha_V\beta_3+)]_{conjugate}/[IC_{50}(\alpha_V\beta_3-)/IC_{50}(\alpha_V\beta_3+)]_{paclitaxel}$.

Parallel experiments were performed under the same conditions with the RGD–PTX conjugate **4** (Figure 1) and paclitaxel (PTX, **21**). Paclitaxel itself appeared to be 2.3 times more effective on U87 β_3-KO cells, possibly because the β_3-integrin-depleted cells divide faster. Taking into account the intrinsic selectivity shown by free paclitaxel, the *iso*DGR–PTX conjugate **2** displayed a remarkable targeting effect (TI = 9.9), especially when compared to the strictly related RGD–PTX conjugate **4** (TI = 2.4). Apparently, the *iso*DGR-PTX conjugate **2** is recognized more specifically by integrin $\alpha_V\beta_3$ than the related RGD-PTX conjugate **4** which can be effectively internalized also by other integrins expressed on the cell surface.

In conclusion, the first *iso*DGR–drug conjugate (**2**) has been developed for tumor targeting. Compound **2** displayed a low nanomolar affinity for the purified integrin $\alpha_V\beta_3$ receptor and a notable targeting ability when tested on two isogenic cancer cell lines expressing integrin $\alpha_V\beta_3$ at different levels. Fine tuning of the linker[19] is in progress in order to improve the potency of the conjugate while retaining the high selectivity.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antitumor agents · cancer · drug delivery · integrins · peptidomimetics

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[18] A very recent paper reports data supporting our conclusions, see: S. Roy, J. Y. Axup, J. S. Forsyth, R. K. Goswami, B. M. Hutchins, K. M. Bajuri, S. A. Kazane, V. V. Smider, B. H. Felding, S. C. Sinha, *Chem. Commun.* **2017**, *53*, 4234 – 4237. SMDCs targeting integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ were tested against human melanoma cells M21 [expressing integrin $\alpha_V\beta_3$ (++), $\alpha_V\beta_5$ (++), $\alpha_V\beta_8$ (+)] and M21-L (lacking the α_V subunit gene), and against human breast cancer cell variants MDA-MB-435 (expressing the β_3 subunit gene) and MDA-MB-435-β_3-KO (in which the β_3 gene was knocked out). While in the first case (M21 vs. M21-L lacking the α_V subunit gene) a strong selectivity was observed, in the second case (MDA-MB-435 vs. MDA-MB-435-β_3-KO) little or no selectivity was observed. It was concluded that "These results indicate that on tumor cells that express multiple target integrins recognized by the SMI (Small

Molecule Inhibitor) component of the drug conjugate, experimental knock-down of one of at least three α_V containing target integrins still left sufficient recognition sites on the tumor cells for effective cell killing. This result is remarkable in view of the fact that $\alpha_V\beta_3$, the target experimentally reduced in this cell model, is the major α_V integrin on MDA-MB-435 breast cancer cells. Thus, the threshold for target expression by tumor cells to be effectively eliminated by the SMI-drug conjugates is apparently remarkably low."

[19] For a review, see: A. Dal Corso, L. Pignataro, L. Belvisi, C. Gennari, *Curr. Top. Med. Chem.* **2016**, *16*, 314 – 329.

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