Targeting Integrin $\alpha_V\beta_3$ with Theranostic RGD-Camptothecin Conjugates Bearing a Disulfide Linker: Biological Evaluation Reveals a Complex Scenario

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Abstract: Theranostic RGD-camptothecin conjugates, possessing a disulfide linker and a fluorescent naphthalimide moiety, were synthesized and biologically evaluated. The conjugates showed nanomolar affinity for the purified $\alpha_V\beta_3$-integrin receptor. For antiproliferative assays, the U87 human glioblastoma were chosen as $\alpha_V\beta_3$-expressing cells, whereas a non $\alpha_V\beta_3$-expressing clone (U87 β_3-KO) was generated as negative control. Although the U87 β_3-KO cells treated with the conjugates showed a statistically significant reduced fluorescence intensity (in the range 7-12%) compared to the parental U87, internalization of the conjugates was clearly observed in both cell lines. Stability studies showed premature cleavage of the disulfide linker in the cell media, with consequent release of free camptothecin. Consistent with the results of the internalization and stability studies, the conjugates did not show significant selectivity against the U87 cells compared to the U87 β_3-KO clone.

Introduction

Conjugation of cytotoxic agents to different carriers, capable of selectively binding to tumor-associated antigens, represents a successful strategy for the treatment of cancer. This “active targeting” approach allows the specific accumulation of anticancer agents at the site of disease, thus reducing side effects and enhancing the overall efficacy of the pharmacological treatment.[1] Antibody-drug conjugates (ADCs) are currently at the cutting-edge of this targeting technology: research in this field has led to the recent market approval of two ADCs, and more than 30 candidates are now in various clinical stages.[2,3] Although drug conjugation to antibodies appears an efficacious approach, the use of smaller targeting vehicles would overcome some remarkable limitations of ADCs. Indeed, the big dimensions of antibodies (especially when IgG formats are used) typically result in long circulation times and in the difficult diffusion of the ADC product within the tumor mass. Moreover, the high manufacturing costs and their possible immunogenicity are well-known drawbacks of ADCs.[4,5] For these reasons, vitamins such as folic acid,[6,7,8] biotin,[9,10,11] peptides,[12,13] and other small ligands[14,15,16] that showed high affinity for tumor-overexpressed antigens have been linked to different anticancer agents to form the so-called small molecule-drug conjugates (SMDCs). The tripeptide sequence Arg-Gly-Asp (RGD) in endogenous ligands has been found to efficiently bind to several integrins (including $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$), divalent cation-dependent heterodimeric membrane glycoproteins formed by non-covalently associated α- and β-subunits. Integrins regulate a variety of cellular processes such as cell growth, differentiation, proliferation and migration. Among these glycoproteins, integrin $\alpha_V\beta_3$ plays a fundamental role in the regulation of angiogenesis.[17] As a consequence, this integrin is widely expressed on the blood vessels of several human tumors (for instance breast cancer, glioblastoma, pancreatic tumor, prostate carcinoma), but not on the vasculature of normal tissues.[18,19] These findings promoted the investigation of $\alpha_V\beta_3$ integrin ligands as potential targeting moieties in SMDCs. The most significant examples of SMDCs containing integrin $\alpha_V\beta_3$ ligands have been recently reviewed.[20] In general, upon binding to the target antigen, the ligand-receptor complex becomes folded into a plasma membrane vesicle, which is internalized into the tumor cell. Depending on the receptor’s physiological functions, the vesicle is driven to a specific intracellular compartment (such as an early endosome or a lysosome). After the receptor-mediated endocytosis, the SMDC is subjected to the cleavage of the linker, which results in the release of the cytotoxic agent in the active form.[21] Thus, the linker group plays a key role to avoid premature drug release in circulation and, on the other hand, to release efficiently the payload at the tumor site.[22,23]

Since 2009, our group has been working on the synthesis of cyclic peptidomimetic integrin ligands based on the RGD sequence and a bifunctional 2,5-diketopiperazine (DKP) scaffold (compound **1**, Figure 1).[24] The latter was included in the ligand’s structure to modify its chemical-physical properties and to impart conformational rigidity to the RGD binding motif. Later on, the *cyclo*[DKP-RGD] ligand was

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functionalized with a benzylamino moiety (compound **2**, Figure 1),[25] which was used as anchoring point for the conjugation to different bioactive molecules. For instance, the integrin ligand was coupled to a pro-apoptotic SMAC (Second Mitochondria-derived Activator of Caspases) mimetic compound and to an antiangiogenic VEGFR-targeting peptide.[26,27] Moreover, the ligand was conjugated to the anticancer drug paclitaxel (PTX) through both ester and peptide linkers.[25,28] In particular, compounds **3** and **4** feature linkers containing the lysosomally-cleavable dipeptides Val-Ala and Phe-Lys, respectively (Figure 1).

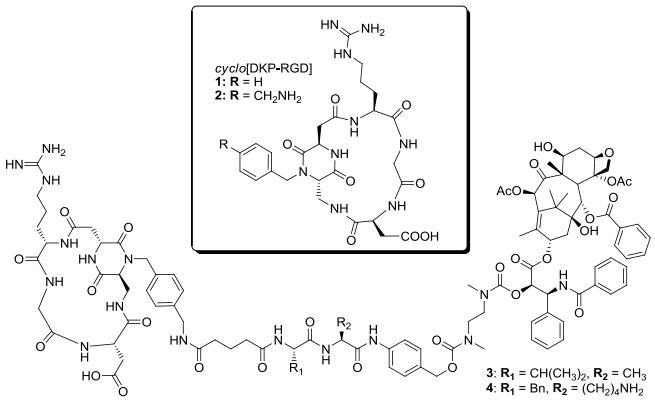


Figure 1. Molecular structures of the integrin ligand *cyclo*[DKP-RGD] **1,** the functionalized analogue *cyclo*[DKP-RGD]-CH_2NH_2 **2**, the SMDCs *cyclo*[DKP-RGD]-Val-Ala-PTX **3** and *cyclo*[DKP-RGD]-Phe-Lys-PTX **4**.[24,25,28]

The latter compounds were tested in vitro against two isogenic cancer cell lines expressing $\alpha_V\beta_3$ integrin at different levels to evaluate their ability to selectively target $\alpha_V\beta_3$-positive tumor cells. Although this kind of comparative assay is a common technique to screen the selective cytotoxicity of ADCs and vitamin-based SMDCs against antigen-expressing cell lines,[6,29] this evaluation has been rarely carried out for conjugates targeting integrin $\alpha_V\beta_3$.[20]

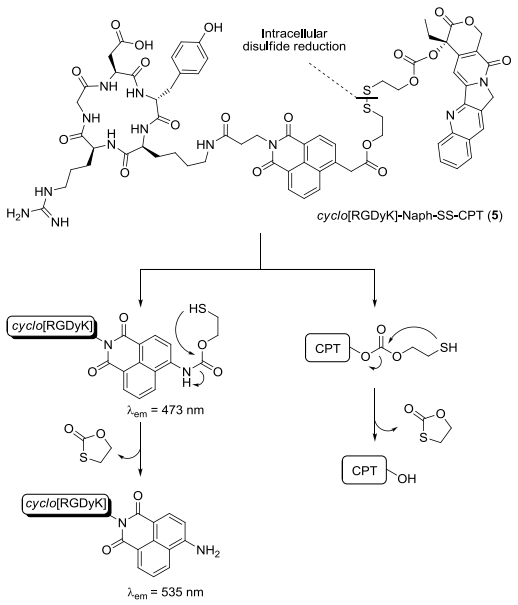


Figure 2. The theranostic *cyclo*[RGDyK]-CPT conjugate **5** and its mechanism of action: upon activation by intracellular reduction, the free thiol functional groups undergo ring closing onto a carbonate and a carbamate bonds, resulting in drug release and red-shifting the fluorescence emission of the naphtalimide moiety.[30a]

Our experiment demonstrated the crucial role of the linker moiety in mediating the in vitro selectivity of RGD-based SMDCs. Indeed, while compound **4** was only marginally able to discriminate between cell lines with different receptor expression, presumably due to a premature cleavage before the endocytic process, compound **3** was found to be 67-fold more active against the $\alpha_V\beta_3$-expressing subclone compared to $\alpha_V\beta_3$-non expressing parental CCRF-CEM acute lymphoblastic leukemia cell line. Encouraged by the above result, we decided to explore different linkers in the drug delivery system. Surprisingly, disulfide linkers have been scarcely employed so far in integrin-targeted SMDCs, with a notable exception: in 2012, Kim and coworkers designed a theranostic RGD-camptothecin (CPT) conjugate (compound **5** in Figure 2) possessing a disulfide linker and a fluorescent naphthalimide moiety.[30] In particular, the disulfide linker of compound **5** was shown to be selectively cleaved in the tumor microenvironment,[30a] where the thiol-disulfide exchange with endogenous thiols, e.g. glutathione (GSH) and thioredoxin, can take place.[31] Besides promoting the release of CPT, the intracellular disulfide reduction results in a red-shifted emission of

the fluorescent probe (see Figure 2): this interesting feature allowed a real-time monitoring of the SMDC's endocytosis, mediated by integrin $\alpha_V\beta_3$.[30a]

Results and Discussion

In this paper, we report our own efforts to exploit disulfide likers for conjugating a $\alpha_V\beta_3$-integrin ligand to a cytotoxic drug and then to evaluate the anticancer properties of disulfide-bearing SMDCs in vitro. We prepared the known compound **5**[30a] and its analog *cyclo*[DKP-RGD]-Naph-SS-CPT (compound **6**, Figure 3), in which our ligand *cyclo*[DKP-RGD] is used as the targeting moiety.

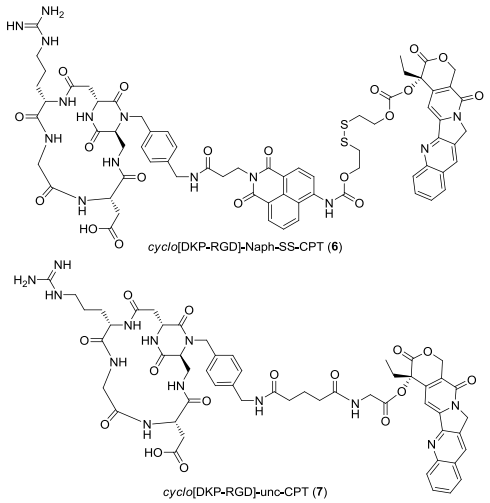
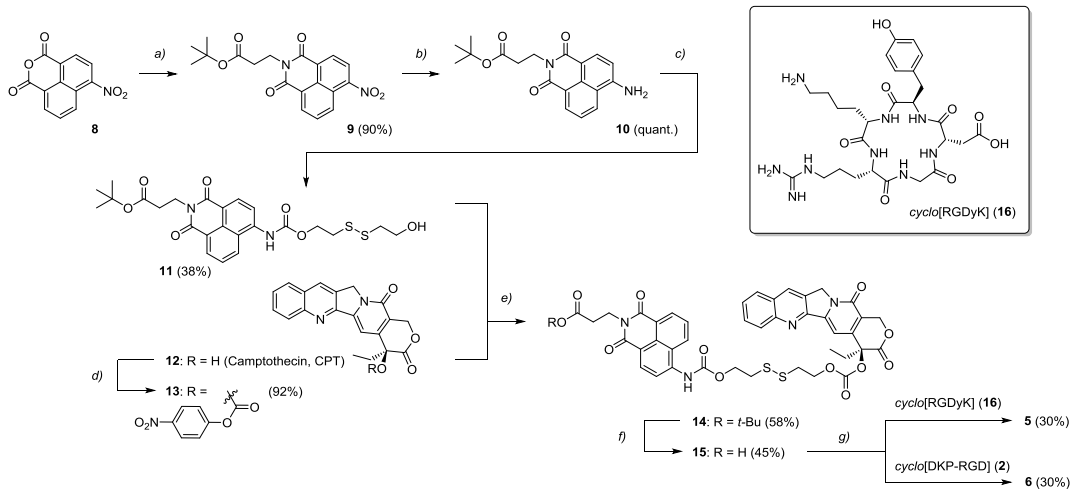


Figure 3. Molecular structures of the SMDC *cyclo*[DKP-RGD]-Naph-SS-CPT **6** and the corresponding "uncleavable" *cyclo*[DKP-RGD]-unc-CPT conjugate **7**.

In order to test these conjugates for their ability to selectively target $\alpha_V\beta_3$-expressing cancer cells, a new cellular model was devised. In particular, the U87 human glioblastoma cells were chosen as $\alpha_V\beta_3$ +, whereas the same cell line was used to generate a non $\alpha_V\beta_3$-expressing clone (U87 β_3-KO), by deletion of the β_3 gene expression through CRISPR-Cas9 gene editing technology.[32]



Scheme 1. Synthesis of RGD-CPT conjugates **5** and **6**. Reagents and conditions: a) β-Alanine *tert*-butyl ester hydrochloride, Et_3N, EtOH reflux, 3 h; b) $SnCl_2$, EtOH, r.t., 1 h; c) i. triphosgene, *i*Pr_2NEt, toluene, r.t., 2 h; ii. 2,2'-dithiodiethanol, 1:1 THF/CH_2Cl_2, r.t., overnight; d) 4-nitrophenyl chloroformate, DMAP, CH_2Cl_2, 0 °C, 4 h; e) DMAP, CH_2Cl_2, r.t., overnight; f) 1:2 TFA/CH_2Cl_2, r.t., 45 min; g) i. DIC, NHS, DMF, r.t., overnight; ii. **16**, MeCN/PBS/DMF (1:1:0.5; pH 7.5), r.t., overnight (**5**); **2**, MeCN/PBS/DMF (1:1:0.5; pH 7.5), r.t., overnight (**6**). DMAP: 4-dimethylaminopyridine; DIC: diisopropylcarbodiimide; NHS: *N*-hydroxysuccinimide; PBS: phosphate-buffered saline.

Moreover, the fluorescent moiety present in compounds **5** and **6** allowed us to gain insight into the internalization process, possibly mediated by the *cyclo*[DKP-RGD] ligand. Indeed, accumulation of the fluorescent compounds on the cell membrane and internalization were investigated by confocal microscopy and immunofluorescence studies. Finally, the "uncleavable"

conjugate *cyclo*[DKP-RGD]-unc-CPT (compound **7**, Figure 3), characterized by the lack of the disulfide linker, was synthesized and used as a negative control.

Synthesis of conjugates 5, 6 and 7

The SMDCs *cyclo*[RGDyK]-Naph-SS-CPT (**5**) and *cyclo*[DKP-RGD]-Naph-SS-CPT (**6**) were prepared according to the synthetic strategy shown in Scheme 1. Several steps of the original synthesis of **5** were optimized, and all the intermediates were fully characterized by NMR spectroscopy (see the Supporting Information). Commercially available 4-nitro-1,8-naphthalic anhydride **8** was reacted with β-alanine *tert*-butyl ester affording naphtalimide **9** in high yield. While Kim and coworkers reported the reduction of the nitro group by catalytic hydrogenation, in our hands this methodology led to significant formation of byproducts. We eventually found that using an excess of tin(II) chloride, amine **10** could be obtained quantitatively after filtration over silica gel. Amine **10** was then transformed into a carbamate by treatment with triphosgene followed by reaction with an excess of 2,2'-dithiodiethanol. The resulting primary alcohol **11** was reacted with (4-nitrophenoxycarbonyl)-camptothecin (compound **13**), prepared from camptothecin **12** as described in the literature,[33] affording compound **14**. The *tert*-butyl ester protecting group was then removed and the free carboxylic acid was coupled with *cyclo*[RGDyK] (**16**) and *cyclo*[DKP-RGD]-CH_2NH_2 (**2**) affording SMDC **5** and **6**, respectively. The two conjugates *cyclo*[RGDyK]-Naph-SS-CPT (**5**) and *cyclo*[DKP-RGD]-Naph-SS-CPT (**6**) were purified by semipreparative HPLC and lyophilized, before being subjected to biological assays. The synthesis of the "uncleavable" conjugate *cyclo*[DKP-RGD]-unc-CPT (compound **7**, Figure 3) is reported in the Supporting Information.

Competitive Binding Assays to the $\alpha_V\beta_3$ integrin receptor

SMDC **5**, **6** and **7** were examined in vitro for their ability to inhibit the binding of biotinylated vitronectin to the purified $\alpha_V\beta_3$ receptor. The calculated IC_{50} values are listed in Table 1.

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

Compound	Structure	$\alpha_V\beta_3$ IC_{50} (nM)[a]
5	*cyclo*[RGDyK]-Naph-SS-CPT	5.3 ± 0.5
6	*cyclo*[DKP-RGD]-Naph-SS-CPT	21.4 ± 2.3
7	*cyclo*[DKP-RGD]-unc-CPT	7.4 ± 1.1
1	*cyclo*[DKP-RGD]	4.5 ± 1.1

[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 ± SD of triplicate determinations.

The screening assays were performed incubating the immobilized integrin receptor with solutions of the RGD-CPT conjugates at different concentrations (10^{-12}-10^{-5} M) in the presence of biotinylated vitronectin (1 μg/mL), and measuring the concentration of bound vitronectin. Overall, these results show that, despite their remarkable steric bulk, all the RGD-CPT conjugates (**5**-**7**) are able to bind to $\alpha_V\beta_3$ integrin at low-nanomolar concentrations, similarly to what observed for the free *cyclo*[DKP-RGD] ligand (**1**) and for other SMDCs developed by our group.[25,26,28]

Generation and characterization of U87 β3-KO cell line

The CRISPR/Cas9 genome editing technology[32] was employed to knockout the integrin β_3 gene (ITGB3) in U87 cells, generating the U87 β_3-KO cell line (the experimental details are provided in the Supporting Information). To confirm ITGB3 gene disruption in U87 β_3-KO cells, integrin $\alpha_V\beta_3$ protein expression was analyzed by immunofluorescence (flow cytometry and microscopy): as expected, no $\alpha_V\beta_3$ expression was detected in U87 β_3-KO cells, while the expression of an unrelated reference integrin receptor (i.e., $\alpha_V\beta_5$) was comparable to that of the parental cell line (see Figure 4 and the Supporting Information).

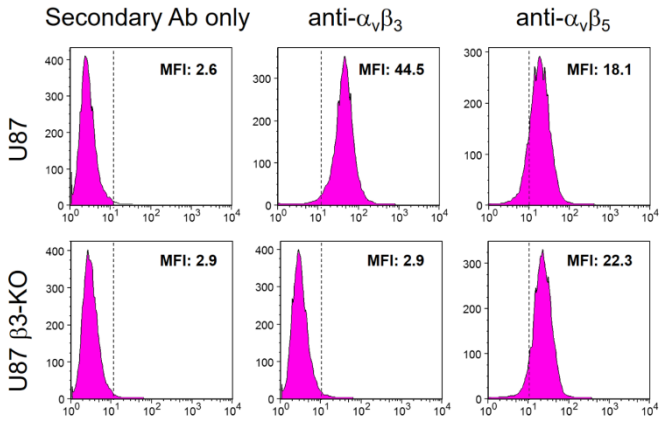


Figure 4. Flow cytometry analysis of integrin $\alpha_V\beta_3$ and integrin $\alpha_V\beta_5$ in U87 and U87 β3-KO cell lines. MFI: Median of Fluorescence Intensity.

The specific sensitivity to camptothecin of the U87 β_3-KO cell line (IC_{50} = 11.2 nM, treatment for 144 h) resulted similar to that of the U87 parental cell line (IC_{50} = 7.2 nM, treatment for 144 h, see the Supporting Information). These data support the use of the newly generated ITGB3+/– U87 isogenic cell line pair as a suitable model for the in vitro biological investigation of the RGD conjugates.

Confocal microscopy analysis and quantitative imaging (ArrayScan)

The U87 human glioblastoma cells ($\alpha_V\beta_3$ +) and the corresponding non $\alpha_V\beta_3$-expressing clone (U87 β_3-KO) were incubated with the fluorescent RGD-CPT conjugates **5** and **6** (5 μM). After incubation for 3 h, the cells were washed with phosphate buffer solution (PBS), fixed with paraformaldehyde in the presence of 1 μg/mL Hoechst 33342 (Sigma Aldrich) and analyzed by confocal microscopy (confocal laser scanning microscope SP2, Leica) equipped with a 63× objective (oil immersion).

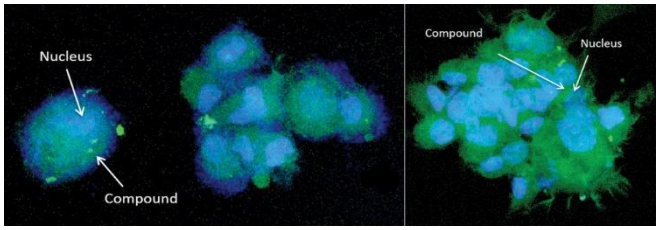


Figure 5. Images of parental U87 ($\alpha_V\beta_3$ +, left) and non $\alpha_V\beta_3$-expressing clone (U87 β_3-KO, right) treated for 3 h with compound **5** (5 μM). Blue: nuclei (Hoechst); green: fluorescent RGD-CPT conjugate.

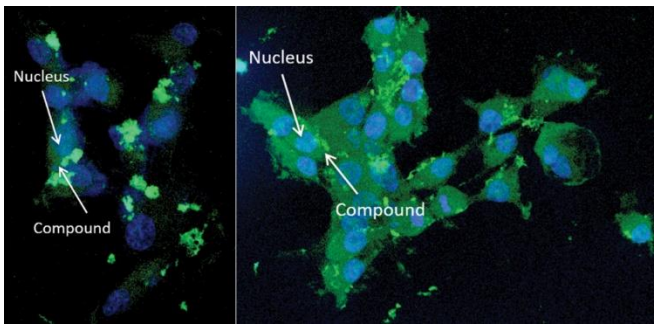


Figure 6. Images of parental U87 ($\alpha_V\beta_3$ +, left) and non $\alpha_V\beta_3$-expressing clone (U87 β_3-KO, right) treated for 3 h with compound **6** (5 μM). Blue: nuclei (Hoechst); green: fluorescent RGD-CPT conjugate.

As can be seen in Figures 5 and 6, internalization of conjugates **5** and **6** was clearly observed in both cell lines, irrespective of the level of $\alpha_V\beta_3$ integrin expression. The fluorescence pattern of the probe indicated a diffuse cytoplasmic and nuclear localization with the presence of bright intracellular structures (putatively endocytic vesicles).

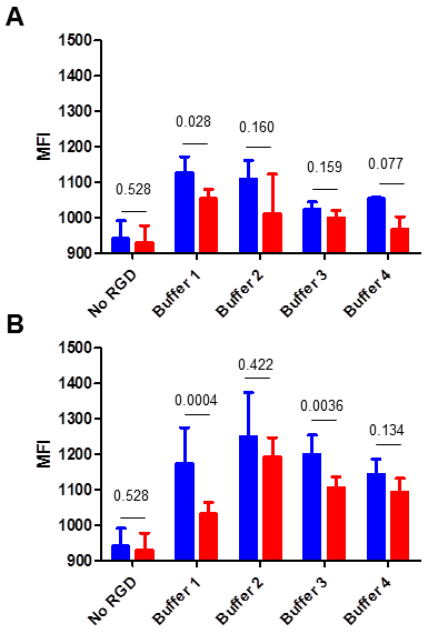


Figure 7. Mean fluorescence intensity (MFI) of the U87 cells (blue ■) and their β_3-KO clones (red ■) treated with compound **5** (A) and **6** (B), as a function of medium composition. Cells were cultured (cellular density = 25000 c/w) for 48 h in 96-well plate, then the medium was replaced with fresh medium containing buffers 1-4 (see below) and 5 μM compound **5** (A) or **6** (B), followed by 6 h incubation at 37 °C. Buffer 1: Phosphate Buffer Solution (PBS); Buffer 2: Eagle's Minimum Essential Medium (EMEM); Buffer 3: Coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, see ref. [25]; Buffer 4: Phosphate Buffer containing additionally 157 mM NaCl, 6 mM KCl and 4 mM $CaCl_2$, see ref. [34]. All values are the arithmetic mean ± SD (N ≥ 2).

The cells were also analyzed by quantitative imaging using the ArrayScan high-content screening reader (Thermo Fisher Scientific), in order to quantify the mean fluorescence intensity after 6 h treatment with conjugates **5** and **6**. At least 700 cells were acquired in each sample using the *Cytoplasm to Nuclear Translocation* BioApplication in two fluorescence channels: individual cells were identified based on nuclear staining (Hoechst 33342, blue channel) and their respective RGD probe fluorescence was quantified in the green channel. We first investigated how some variables reported to influence internalization impact on the total fluorescence.[14,35] On the one hand, the effect of different metal ions on the extent of internalization was studied and found to be relatively minor (see Figure 7). On the other hand, cell density was varied (c/w = 2500, 10000, 25000), and the internalization was found to be more effective at higher cell density (c/w = 25000) (not shown), as already reported by Moncelet et al. in 2013.[35] At c/w = 25000, the non $\alpha_V\beta_3$-expressing cells (U87 β_3-KO) treated with either conjugate **5** (Figure 7, A) or **6** (Figure 7, B) showed a statistically significant reduced fluorescence intensity compared to the parental U87 cells ($\alpha_V\beta_3$ +). The maximum observed reduction was 7% with compound **5** (A, buffer 1, p = 0.028) and 12% with compound **6** (B, buffer 1, p = 0.0004). Although this reduction is definitely observable, it is much smaller than expected, considering that the clone U87 cells do not express integrin $\alpha_V\beta_3$. This finding led us to conclude that internalization of conjugates **5** and **6** in the non $\alpha_V\beta_3$-expressing cells (U87 β_3-KO) can occur passively or actively by RGD binding to other integrins, e.g. $\alpha_V\beta_5$.[36,37]

Stability assays

In order to investigate the role of the disulfide linker in the conjugates *cyclo*[RGDyK]-Naph-SS-CPT (**5**) and *cyclo*[DKP-RGD]-Naph-SS-CPT (**6**), stability tests in vitro were performed. The $\alpha_V\beta_3$-expressing ($\alpha_V\beta_3$ +) U87 cells and their non $\alpha_V\beta_3$-expressing clone (U87 β_3-KO) were cultured for 24 h in a 96-well plate, then the medium was replaced with fresh medium containing compound **5**, **6** and **7** (5 μM), followed incubation at 37 °C for 6 h. The cell medium was withdrawn and analyzed by RP-HPLC to evaluate the stability of the RGD-CPT conjugates in cell media. In the case of compounds **5** and **6**, an HPLC peak showing the same retention time of CPT was observed, indicating that release of the free drug in the cell media had taken place (see the Supporting Information). In order to confirm that the observed HPLC peak really corresponds to the free drug rather than to CPT still connected to the self immolative spacer, derivatives **5** and **6** were treated with a reducing agent - dithiothreitol (DTT) - and analyzed by MALDI-ToF-ToF MS. After reduction with DTT, both conjugates clearly release the free drug and no traces of fragment CPT-self immolative spacer (see Fig 2) were detected. In sharp contrast with compounds **5** and **6**, the “uncleavable” conjugate *cyclo*[DKP-RGD]-unc-CPT (compound **7**) showed a good stability both in the cell media and in the presence of the reducing agent.

Taken together, these results suggest that reduction of the disulfide bond present in the linker of **5** and **6** takes place in the cell media, with consequent release of free camptothecin. Thus, we discovered that the RGD-SS-CPT conjugates **5** and **6** are not completely stable in the cell media even after a relatively short incubation time (6 h) in both the U87 cells ($\alpha_V\beta_3$ +) and the non $\alpha_V\beta_3$-expressing U87 clone (β_3-KO). It is well known that the stability of the disulfide bond depends on the steric hindrance at the alpha carbons.[38]

Cell proliferation analysis

Conjugates **5** and **6** were also subjected to a set of biological tests, aimed at evaluating their antiproliferative activity. U87 and U87 β_3-KO cells were seeded in 96-well plates (c/w = 2000) and incubated for 48 h. Cells were treated with increasing compound concentrations for 6 h, then the supernatant was washed out and replaced with fresh medium. The plates were incubated for additional 138 h and finally processed for cell proliferation luminescent readout (CellTiter-Glo® Luminescent Cell Viability Assay, Promega). The calculated IC_{50} values are listed in Table 2. Consistent with the results of the above-described internalization and stability studies, the compounds did not show significant selectivity against the U87 cells ($\alpha_V\beta_3$ +) compared to the non $\alpha_V\beta_3$-expressing U87 clone (β_3-KO). Indeed, the antiproliferative activity of the two conjugates *cyclo*[RGDyK]-Naph-SS-CPT (**5**) and *cyclo*[DKP-RGD]-Naph-SS-CPT (**6**) is comparable to that of free camptothecin and the targeting index calculated for both conjugates is relatively small (1.3-1.7). In fact, the premature cleavage of the disulfide linker outside of the cell produces two species (see Figure 2): the RGD-naphthalimide moiety, which is able to enter the cell by integrin-mediated endocytosis, and free CPT, that crosses the cell membrane through passive diffusion.

Table 2. Antiproliferative activity of RGD-CPT conjugates in U87 and U87 β_3-KO cell lines.

Structure	IC_{50} (nM)[a]		S[b]	T.I.[c]
	U87	U87 β_3-KO		
Camptothecin (CPT)	725	434	0.6	1
cyclo[RGDyK]-Naph-SS-CPT (**5**)	205	210	1.0	1.7
cyclo[DKP-RGD]-Naph-SS-CPT (**6**)	220	170	0.8	1.3
cyclo[DKP-RGD] (**1**)	>10000	>10000	-	-

[a] IC_{50} values were calculated as the concentration of compound required for 50% inhibition of cell viability, as estimated by GraphPad Prism software. All values are the arithmetic mean of two replicates. Cells were treated for 6 hours in U-bottom 96-w plates, then washed and incubated for 138 hours in compound-free medium in 96-w flat bottom plates. [b] Selectivity (S): IC_{50}(U87 β_3-KO)/IC_{50}(U87). [c] Targeting index (T.I.): selectivity/selectivity observed with free camptothecin.

Conclusions

Inspired by a recent paper by Kim and co-workers,[30a] we have synthesized and biologically evaluated in vitro two theranostic RGD-camptothecin conjugates (**5**) and (**6**), possessing a disulfide linker and a fluorescent naphthalimide moiety. The conjugates were able to inhibit biotinylated vitronectin binding to the purified $\alpha_V\beta_3$-integrin receptor at nanomolar concentrations. As cellular model for testing the ability of the conjugates to selectively target $\alpha_V\beta_3$-expressing cancer cells, the U87 human glioblastoma cells were chosen as $\alpha_V\beta_3$ +, whereas the same cell line was used to generate a non $\alpha_V\beta_3$-expressing clone (U87 β_3-KO), by deletion of the β_3 gene expression through CRISPR-Cas9 gene editing technology. The fluorescent moiety present in the conjugates allowed us to gain insight into the internalization process: accumulation of the fluorescent compounds on the cell membrane and internalization were investigated by confocal microscopy and immunofluorescence studies. Internalization of conjugates **5** and **6** was clearly observed in both cell lines, irrespective of the level of $\alpha_V\beta_3$ integrin expression. The non $\alpha_V\beta_3$-expressing cells (U87 β_3-KO) treated with the conjugates showed a statistically significant reduced fluorescence intensity compared to the parental U87 cells ($\alpha_V\beta_3$ +). The maximum observed reduction (in the range 7-12%) is much smaller than expected, considering that the β_3-KO clone does not express integrin $\alpha_V\beta_3$. This finding led us to conclude that internalization of the conjugates in the non $\alpha_V\beta_3$-expressing cells (U87 β_3-KO) can occur passively or actively by RGD binding to other integrins, e.g. $\alpha_V\beta_5$. Stability studies showed that the RGD-SS-CPT conjugates **5** and **6** are not completely stable in the cell media even after a relatively short incubation time (6 h) in both the U87 cells ($\alpha_V\beta_3$ +) and the non $\alpha_V\beta_3$-expressing U87 clone (β_3-KO), due to premature cleavage of the disulfide linker, with consequent release of free camptothecin. Consistent with the results of the internalization and stability studies, both conjugates **5** and **6** did not show significant selectivity against the U87 cells ($\alpha_V\beta_3$ +) compared to the non $\alpha_V\beta_3$-expressing U87 clone (Targeting Index = 1.3-1.7). Altogether, a complex scenario is revealed by our studies, which deserves further and deeper investigations. It should be noted that, although there are many reports where RGD-drug conjugates have been tested in vitro against $\alpha_V\beta_3$-expressing tumor cell lines,[20] only in a few cases negative controls have been described. In these cases, the negative control is often a different non $\alpha_V\beta_3$-expressing tumor cell line, a protocol which is not very rigorous given the strong differences between the two species. Ideally, one should use as negative control the isogenic

non $\alpha_V\beta_3$-expressing tumor cell line,[28] as proposed here with U87 β_3-KO. However, the limited success of the present approach tells us that the search for a suitable isogenic negative control is not yet over, and possibly a α_V-KO cancer cell line is a better option for this goal.[36]

Supporting Information Summary

For the preparation of the three conjugates (*cyclo*[RGDyK]-Naph-SS-CPT **5**, *cyclo*[DKP-RGD]-Naph-SS-CPT **6** and *cyclo*[DKP-RGD]-unc-CPT 7), see the Supporting Information. Depletion of integrin β_3 gene (ITGB3) in U87 human glioblastoma cells by CRISPR/Cas9 technique, competitive binding assays to the $\alpha_V\beta_3$ integrin receptor, immunofluorescence analysis of integrin $\alpha_V\beta_3$ expression, cell proliferation assays and stability assays are also described in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: CRISPR-Cas9 technology • $\alpha_V\beta_3$-integrin • internalization • RGD-drug conjugates • theranostic conjugates

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