Bioorganic & Medicinal Chemistry 18 (2010) 64–72

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Novel tumor-targeted RGD peptide–camptothecin conjugates: Synthesis and biological evaluation

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ARTICLE INFO

Article history:
Received 26 May 2009
Revised 29 October 2009
Accepted 7 November 2009
Available online 12 November 2009

Keywords:
Tumor-targeting
Integrins
Camptothecin conjugates
RGD cyclopeptides

ABSTRACT

Five RGD peptide–camptothecin (CPT) conjugates were designed and synthesized with the purpose to improve the therapeutic index of this antitumoral drug family. New RGD cyclopeptides were selected on the basis of their high affinity to α_v integrin receptors overexpressed by tumor cells and their metabolic stability. The conjugates can be divided in two groups: in the first the peptide was attached to the drug through an amide bond, in the second through a hydrazone bond. The main difference between the two spacers lies in their acid stability. Affinity to the receptors was maintained for all conjugates and their internalization into tumor cells was demonstrated. The first group conjugates showed lower in vitro and in vivo activity than the parent drug, probably due to the excessive stability of the amide bond, even inside the tumor cells. Conversely, the hydrazone conjugates exhibited in vitro tumor cell inhibition similar to the parent drug, indicating high conversion in the culture medium and/or inside the cells, but their poor solubility hampered in vivo experiments. On the basis of these results, information was acquired for additional development of derivatives with different linkers and better solubility for in vivo evaluation.

1. Introduction

Traditional cancer chemotherapy is based on the assumption that rapidly proliferating cancer cells are more likely to be killed than quiescent cells of physiological tissues. However, cytotoxic agents have very poor specificity, which leads to systemic toxicity, causing severe side effects. Recent improvements in the knowledge of typical receptors overexpressed by cancer cells during their proliferation allow the exploitation of selective ligands, which, conjugated with cytotoxic agents, are able to preferentially address them to the tumors.

Advances in tumor-targeting drug conjugates include monoclonal antibodies (mAb), polyunsaturated fatty acids, hyaluronic acid, folic acid and small peptides as ligands of tumor associated receptors.[1] At present, several immunoconjugates are undergoing clinical evaluation,[2] and among them, the immunoconjugate of calicheamicin[3] (Mylotarg®) has been approved by FDA against acute myeloid leukemia. Nevertheless, the practical use of immunoconjugates is only suitable for highly potent drugs, because a limited amount of antigens are overexpressed on the tumor cell surface and a limited number of molecules can be loaded on each mAb without decreasing the binding affinity and increasing the immunogenicity.

In the last few years, conjugates of cytotoxic agents with small peptides, addressed to different receptors overexpressed by tumoral cells, have been studied as potential selective antitumoral chemotherapeutics. Somatostatin and bombesin have been intensively studied as tumor-targeting molecules in conjugation with different cytotoxic agents.[4–8]

Among selective receptor-targeting small peptides, integrin-mediated RGD peptides appear attractive candidates. The arginine–glycine–aspartic acid (RGD) is a cell adhesion motif present in many proteins of the extracellular matrix (ECM).[9] Through their RGD sequence ECM proteins recognize $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors, which play an important role in angiogenesis and tumor growing. α_v Integrins are expressed on the luminal surface of neovasculature, but are not found on the mature capillaries. In addition, they have been shown to be upregulated in tumor blood vessels that undergo continuous angiogenesis and have been implicated in metastasis.[10] Inhibition of angiogenesis has been shown to prevent tumor growth and even cause tumor regression

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doi:10.1016/j.bmc.2009.11.019

in various experimental models.[11] However, antiangiogenic therapy alone usually is not sufficient to eradicate tumors.[12] On the other hand, RGD peptides conjugated with cytotoxic agents are likely to exhibit a tumor-targeting and antiangiogenic synergetic effect. During the last few years, a number of RGD-cytotoxic drugs were developed and showed promising activities in vitro and in vivo.[13–18]

In the study described herein, we investigated the usefulness of RGD peptides as carriers for antitumoral drugs belonging to the family of camptothecins (CPT). Potent CPT analogues **1a–c** were chosen among a large series prepared in our laboratory,[19–22] bearing at 7-position functional groups suitable for conjugation (Fig. 1a). As targeting device we chose cyclic peptide analogues of c(RGDfV) developed by Kessler and co-worker.[23] We synthesized a series of novel pseudopeptides,[24] all containing at the 5-position a trifunctional pseudoamino acid, consisting of a non-proteinogenic amino acid with a side chain bearing a suitable functional group for the attachment of cytotoxic drugs. Among them, four cyclopeptides, **2a–d**, were selected, which proved to have the optimal characteristics of affinity to α_v integrins in vitro and metabolic stability, increased by the presence of the non-natural amino acid in their sequence (Fig. 1b). The linker between drug and targeting device has critical significance for the efficacy of the conjugates, its prerequisites being stability into the body circulation and lability into the tumor cell. Unlike the linkers used for the immunoconjugates, that are shielded from plasma peptidases by the bulky antibody moiety, the choice of the linker for conjugates with small peptides is very challenging. Accordingly, we synthesized two classes of conjugates, that differ in the chemical nature of the bond between drug and peptide. The first is characterized by a stable amide bond, that should guarantee stability in the body circulation; for this purpose we avoided the use of ester bonds prone to the attack of esterases. The second class of conjugates is characterized by a hydrazone bond which is supposed to be stable at neutral pH of the body fluids, but promptly cleaved at the low pH inside the tumor cells. The use of hydrazones for the release of drugs at low pH has been widely documented in the literature.[25,26]

The binding assays of the cyclopeptides and of the corresponding conjugates to the isolated α_v integrins, the study of their effect on cell adhesion and in vitro cytotoxicity are described. Furthermore, the in vivo activity of one of the conjugates was evaluated against A2780 human ovarian carcinoma in nude mice.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of camptothecin derivatives

Camptothecin derivatives **1a** (ST1968, Namitecan) and **1b** were synthesized according to methods already published.[19–21] Compound **1c** was obtained as illustrated in Scheme 1: the aldehyde **1b** was transformed into the unsaturated aldehyde **3** by a Wittig reaction and, after protection as the acetal **4**, the double bond was reduced giving **5**. Finally, acidic deprotection afforded the camptothecin derivative **1c**.

2.1.2. Synthesis of cyclopeptides

The linear peptides were synthesized on solid-phase following the standard Fmoc-protocol. The four peptides differ from each other for the pseudoamino acid at the 5-position. The commercially available pseudoaminoacids ω-benzyl Fmoc-adipic acid ester[Fmoc-Aad(Bn)-OH] and Fmoc-aminomethylphenylalanine [Fmoc-Amp(Cbz)-OH] were introduced in the cyclopeptides **2a′** and **2b′–c′**, respectively, while the pseudoaminoacid **6**, introduced in the cyclopeptide **2d**, was synthesized in our laboratory according to the method described in Scheme 2: the trioxaundecanedioic acid monobenzyl ester was submitted to hydrazinolysis and the monoacid hydrazide was protected with Boc, activated with *N*-hydroxysuccinimide and coupled with Fmoc-Amp-OH to afford **6**. After cleavage from the resin, the peptides were cyclized in solution and orthogonally deprotected at the 5-position. Then, the Amp-containing peptides were further treated with succinic anhydride or 3,6,9-trioxaundecanedioic acid to obtain **2b′** and **2c′**, respectively. Only **2d** was obtained after total deprotection, because the successive formation of the hydrazone conjugates is chemoselective.

2.1.3. Synthesis of conjugates

Five conjugates were synthesized. For the three conjugates **7a–c**, cyclopeptides in their partially protected form were attached to CPT via an amide bond, using DCC/HOAT as condensing reagents, followed by total acidic deprotection (Scheme 3). For the two conjugates **8a–b**, cyclopeptide **2d** was attached to CPT via hydrazone bond, after removal of the protecting groups from arginine and aspartic acid. Conjugates **8a** and **8b** were easily obtained by adding the corresponding CPT aldehydes to a solution containing the pep-

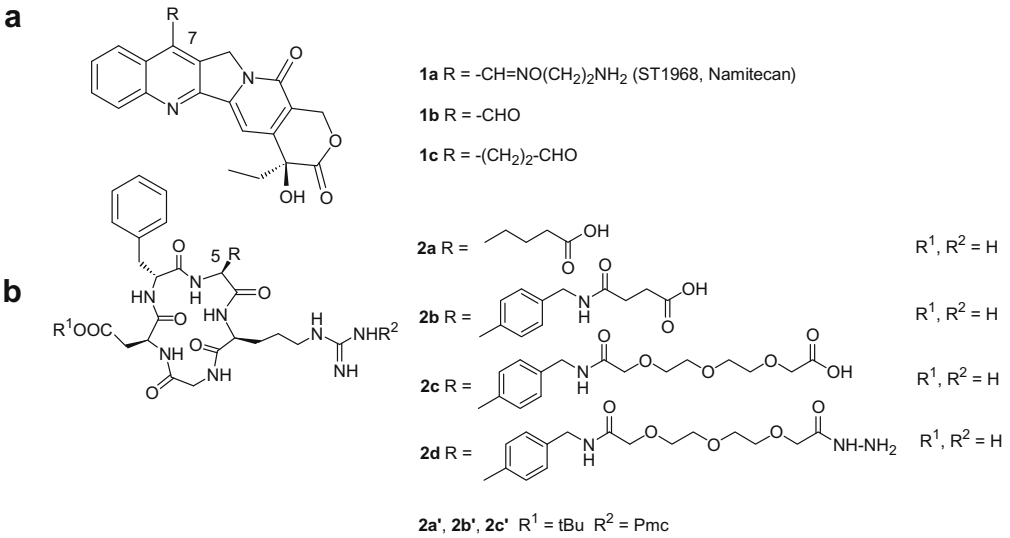
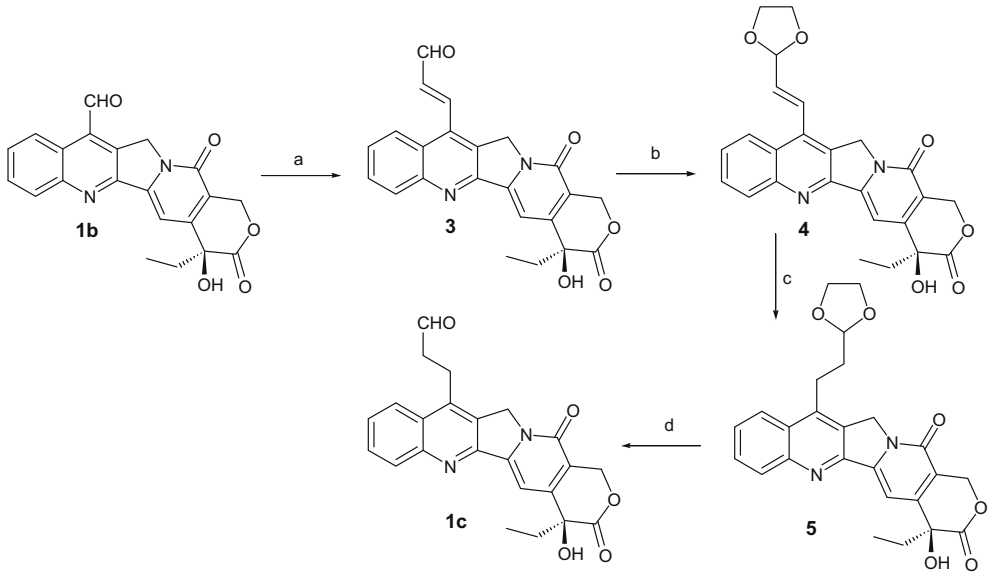
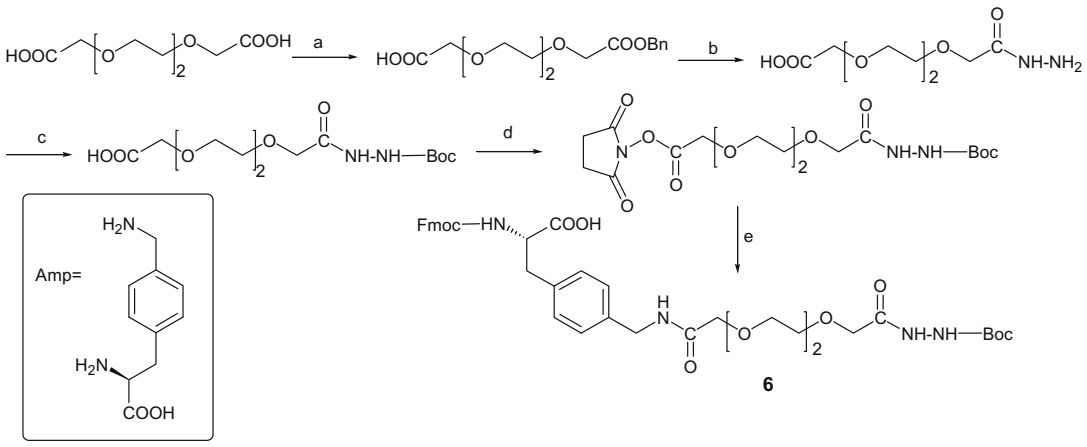


Figure 1. (a) Structure of Camptothecin analogues; (b) structure of cyclopeptides.



Scheme 1. Reagents and conditions: (a) $Ph_3P{=}CH{-}CHO$, $CHCl_3$, reflux, 68%; (b) CH_2OHCH_2OH, PTSA, $MgSO_4$, toluene, reflux Dean–Stark, 56%; (c) H_2, Pd/C 10%, MeOH, 99%; (d) 80% AcOH, 80 °C, 39%.



Scheme 2. Reagents and conditions: (a) BnOH, DCC, TEA, DMAP, DCM; (b) NH_2-NH_2.H_2O, EtOH; (c) $(Boc)_2O$, 1 N NaOH, MeOH, 76%; (d) *N*-hydroxysuccinimide, DCC, DCM, 86%; (e) Fmoc-Amp-OH, TEA, DCM/DMF, 52%.

tide hydrazide in MeOH and stirring under nitrogen at room temperature for 36 h (Scheme 4).

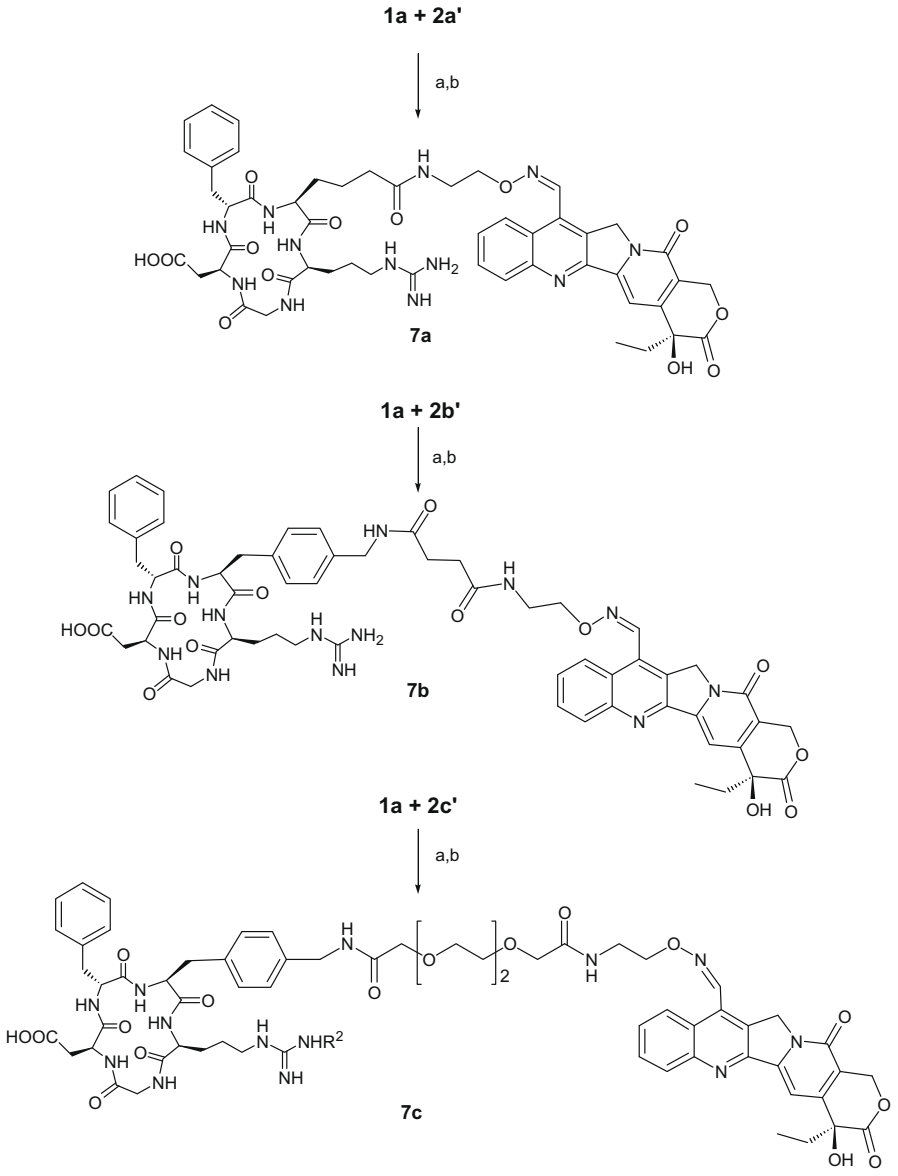
2.2. Biological evaluation

2.2.1. In vitro evaluation of 7a–c

Firstly, the binding of the three conjugates to the isolated receptors and adhesion to three tumoral cell lines (PC3 prostatic carcinoma, A498 renal carcinoma, A2780 ovarian carcinoma) were measured, showing that peptides effectively mediate the binding to the cell surface in a dose-dependent manner, whereas CPTs alone do not bind to integrins or the cell surface (Table 1).

All the conjugates maintained a good receptor affinity. Moreover, the cell adhesion resulted increased in some case, compared to the peptides alone; this behavior should be attributed to cooperative conformational effects of the whole molecule. It is interesting to notice that either peptides or the corresponding conjugates efficiently inhibit the adhesion of A2780 ovarian carcinoma cells overexpressing $\alpha_v\beta_5$ integrin as well as of A498 renal carcinoma overexpressing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, while a minor effect was observed on PC3 prostate carcinoma cells endowed with low levels of integrins.

We also demonstrated the internalization of the conjugates into the tumor cells: the compounds were isolated from the whole cell extracts and analyzed by HPLC with fluorometric detection. From the results illustrated in Figure 2, it is evident that the conjugates **7a** and **7c** accumulate inside the cell at a much lower rate than the free parent drug. In fact, CPT shows a rapid intracellular uptake with the highest value at 5 h and a slow decrease until 72 h, while the conjugates reach a concentration peak at 48 h, suggesting a mechanism dependent on integrins interaction and/or turnover. This experiment also shows that only small detectable amounts of CPT are released at the end of the treatment, proving the high stability of the conjugates both in culture medium and inside the tumor cells. This is consistent with their lower cytotoxicity compared to the free drug, as reported in Table 2. Conjugates **7a–c** cytotoxicity resulted more than one order of magnitude lower than CPT alone, and we attributed these results mainly to the amide bond, which is too stable to promptly release the drug.



Scheme 3. Synthesis of camptothecin–cyclopeptide conjugates with amide bond. Reagents: (a) DCC, HOAT, DIEA, DMF; (b) TFA/DCM, 1:1.

To overcome this problem, novel conjugates were designed containing the acid-labile hydrazone linker. Because hydrazones are known to be stable at neutral pH, these conjugates were expected to have a sufficiently long lifetime under normal physiological conditions to undergo internalization and localization in the acidic lysosomal environment, releasing the active drug. Accordingly, two CPT analogues **1b** and **1c** were synthesized and linked to the RGD cyclopeptide **2d** bearing a hydrazide group, to obtain the conjugates **8a** and **8b**.

2.2.2. Stability of the conjugates

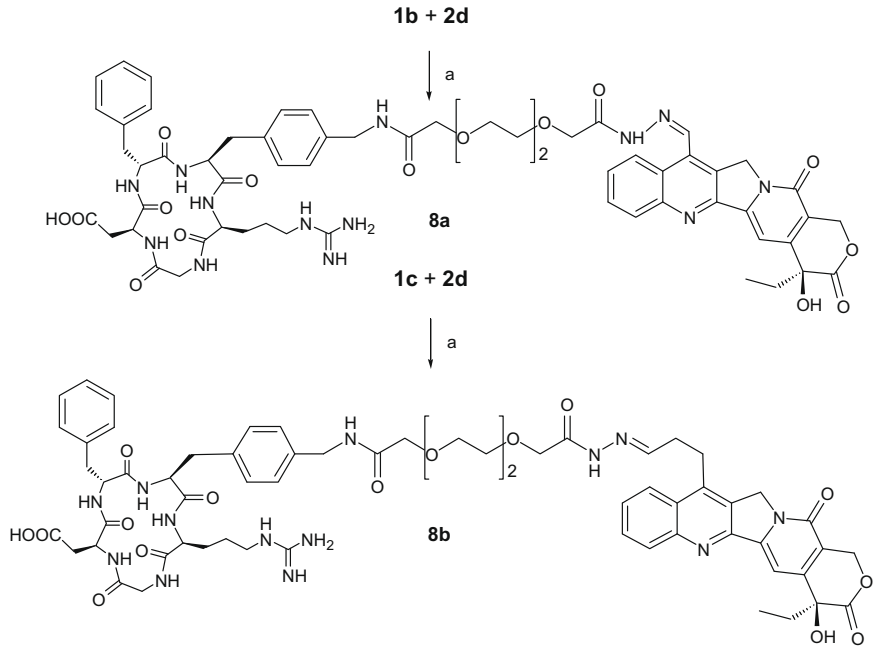
In order to assess the stability of these conjugates at different pH, they were dissolved with DMSO and diluted with phosphate or acetate buffers at 37 °C, in order to reach pH 7.4 or 5, respectively, and a concentration of 100 μM (1% DMSO). Disappearance of the starting material was monitored by HPLC (see Supplementary data). Conjugates **7a–c** proved to be stable at both pH (half-life >72 h), whereas conjugates **8a and 8b** showed half-lives of about 6 h at pH 7.4. Compound **8b** underwent rapid hydrolysis at pH 5 (half-life less than 2 min) as expected, while **8a** showed a half-life of 25 h. (Table 3) The resistance to acid hydrolysis exhibited by the latter hydrazone, derived from the aromatic aldehyde, can be attributed to the conjugation of the π-bond of the C=N– group with the π-bond of the CPT aromatic ring, as observed by Kale et al.[26] for their hydrazone derivatives.

2.2.3. In vitro evaluation of 8a–b

Biological results of the conjugates and the corresponding CPT analogues are reported in Table 4. Both conjugates showed high cytotoxic activity. Considering the poor stability found at pH 7.4, we can hypothesize that this activity is partially due to the CPT released in the culture medium during the experiments. On the other hand, the difference in cytotoxicity found between the two conjugates can be attributed to the difference in their acidic stability in lysosomal medium.

2.2.4. In vivo antitumor activity and tumor distribution

The antitumor effect of the conjugate **7c** was investigated on A2780 human ovarian carcinoma xenograft in nude mice. The molecule, delivered bis in die intraperitoneally or subcutaneously, pro-



Scheme 4. Synthesis of camptothecin–cyclopeptide conjugates with hydrazone bond. Reagents and conditions: (a) MeOH, N_2, room temperature.

Table 1
Binding of cyclopeptides **2a–c** and the corresponding conjugates **7a–c** to the isolated α_v integrins and their effect on cell adhesion in the presence of vitronectin after 3 h treatment

Entry	Binding assay, IC_{50} nM		Adhesion assay, IC_{50} μM		
	$\alpha_v\beta_3$	$\alpha_v\beta_5$	PC3	A498	A2780
1a	No binding		No adhesion		
2a	28.6 ± 0.76	0.17 ± 0.01	38.8 ± 8.5	6.5 ± 0.6	5.2 ± 0.2
7a	0.59 ± 0.01	0.37 ± 0.01	5.8 ± 0.6	3.7 ± 0.3	3.1 ± 0.5
2b	37.6 ± 0.99	5.1 ± 0.07	33.7 ± 7.8	34 ± 2.2	9.5 ± 1.9
7b	7.27 ± 0.06	8.39 ± 0.07	>100	5.4 ± 1.3	
2c	4.0 ± 0.1	0.35 ± 0.09	18.8 ± 4.4	10.7 ± 0.4	12.3 ± 0.9
7c	12.5 ± 2.1	6.5 ± 0.03	8.3 ± 1.0	3.1 ± 0.3	1.8 ± 0.2

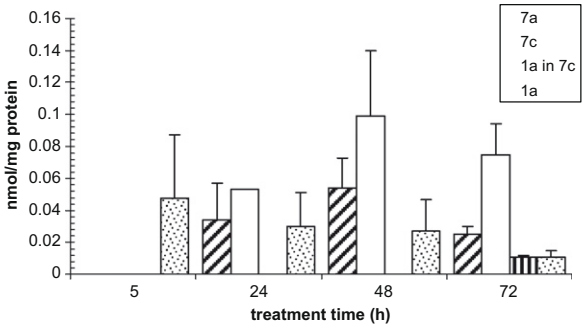


Figure 2. Uptake of conjugates **7a**, **7c** and **1a** in A498 tumor cells.

duced a tumor volume inhibition of about 40%. Moreover, the product showed to be well tolerated at a dose of 48 mg/Kg, since no body weight loss or lethal toxicity occured (Table 5). Nevertheless, CPT **1a** given alone at 16.3 mg/Kg, corresponding to the equimolar dose present in the conjugate, resulted to be toxic, because all mice died. In a subsequent experiment, we used a non toxic dose of **1a** comparable to the free drug found in plasma after administration of the conjugate. This dose was estimated from measurements of AUC_{INF} (area under plasma concentration curve

Table 2
Cytotoxicity of the conjugates **7a–c** and the corresponding cyclopeptides **2a–c** on different tumor cell lines after 72 h treatment, IC_{50}, μM

Entry	PC3	A498	A2780
1a	0.36 ± 0.06	0.064 ± 0.01	0.0049 ± 0.001
2a	>1000	>1000	>1000
7a	15 ± 1.5	3.2 ± 0.4	0.4 ± 0.03
2b	>1000	>1000	>1000
7b	4.7 ± 0.4	4.3 ± 0.1	0.16 ± 0.001
2c	>1000	>1000	>1000
7c	27 ± 3.9	0.74 ± 0.1	0.18 ± 0.01

Table 3
Half-lives of hydrazone conjugates **8a** and **8b**

Entry	pH 7.4, 37 °C $t_{1/2}$, h	pH 5, 37 °C $t_{1/2}$, h
8a	6	25
8b	5.8	<2 min

extrapolated to infinity) for **7c** and **1a**: 82 and 2 μg/h/mL were found, respectively. This means that 2.5% of the free drug is released from the conjugate in the blood circulation. In fact, after administration of 0.42 mg/Kg of **1a**, equivalent to the amount of free drug, the tumor volume inhibition of 43% and a body loss of 0% were found. These data strongly suggest that the antitumor activity found for **7c** could be due to the small circulating plasma levels of free CPT released from the conjugate and to CPT tumor distribution.

The same xenograft model was used to evaluate pharmacokinetics and tumor distribution. As shown in Figure 3, high levels of compound **7c** were found both in plasma and in tumor, demonstrating that the conjugate attains in vivo tumor homing and releases free CPT inside tumors. Moreover, **7c** concentration profiles versus time were similar in plasma and tissue and suggested a good and comparable apparent half-life, as evident by the curves slope. As formerly observed during the in vitro uptake

Table 4
Binding to α_v integrins, adhesion to tumor cells and cytotoxicity of the cyclopeptide **2d**, CPT analogues **1b** and **1c**, and the corresponding conjugates **8a** and **8b**

Entry	Binding assay IC_{50} nM		Adhesion assay IC_{50} μM		Cytotoxicity IC_{50} μM	
	$\alpha_v\beta_3$	$\alpha_v\beta_5$	PC3	A2780	PC3	A2780
2d	3.8 ± 0.1	0.65 ± 0.1	15.6 ± 2.4	11.8 ± 0.9	>1000	>1000
1b					0.13 ± 0.04	0.003 ± 0.00006
8a	11.66 ± 2.5	59.9 ± 1.07	4.8 ± 0.5	6.7 ± 0.7	0.22 ± 0.02	0.09 ± 0.01
1c					0.12 ± 0.04	0.015 ± 0.002
8b	6.01 ± 0.22	22.95 ± 1.18	11 ± 0.8	12.9 ± 0.5	0.42 ± 0.03	0.017 ± 0.002

Table 5
Antitumor activity of the novel conjugate 7c and CPT 1a delivered subcutaneously or intraperitoneally bis in die against A2780 human ovarian carcinoma xenografted in nude mice

Compound	Single dose mg/Kg	Route	Treatment schedule	TVI%[a] ± SE	BWL%[b]	Lethal toxicity[c]
7c	48	ip	q4 d × 4	43 ± 6* (+16)	2	0/8
7c	48	sc	qd × 5	37 ± 5* (+17)	0	0/8
1a	16.3	sc	qd × 5	ne	30	8/8
1a	0.42	sc	qd × 5	43 ± 12* (+17)	0	0/8

ne = not evaluable for lethal toxicity.
[a] Tumor volume inhibition percentage versus control mice (in parenthesis the day upon the tumor injection).
[b] Maximum body weight loss percentage due to the drug treatment.
[c] Dead/treated animals.
* $P \leqslant 0.05$ versus vehicle-treated group (Mann–Whitney).

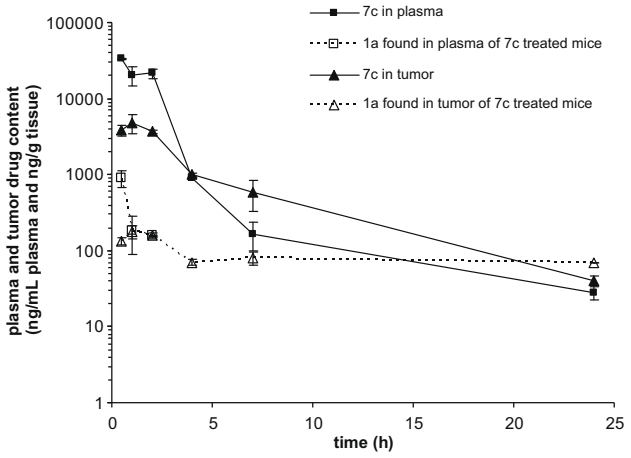


Figure 3. Plasma and tumor concentration profiles versus time of conjugate **7c** in A2780 human ovarian carcinoma xenografted nude mice treated at a dose of 48 mg/Kg.

study, free CPT is very slowly released by the conjugate, confirming also in vivo its stability.

3. Conclusions

Our studies demonstrate the potential applicability of suitable RGD peptides for CPTs tumor-targeting. We obtained conjugates with high affinity to α_v integrin receptors overexpressed by tumor cells and demonstrated their internalization through the cell membrane. Amide bond containing conjugates are very stable in buffer solution at pH 7.4 and in culture medium, but are less active than the parent drug either in vitro or in vivo, most probably because the linker between the targeting peptide and CPT is too stable and does not release sufficient amounts of the drug inside the tumor cells. The in vivo experiments were carried out using only one dose of **7c**, which corresponds to the maximum solubility of the compound. Since none toxicity was observed, we may argue that its antitumor profile could be enhanced using a dose corresponding to the MTD (Maximum Tolerated Dose). Hydrazone-containing conjugates demonstrated poor stability in buffer solution at pH 7.4 (half-life 6 h). Hydrazone linkers have been often used for conjugating drugs with macromolecules such as antibodies or biopolymers, which, thanks to their steric hindrance, can shield the bond from hydrolysis. This is not the case of conjugates containing a small molecule, as our compounds, being the linker between drug and targeting device much more accessible. As expected, at pH 5 the conjugate **8b** was totally hydrolyzed within 2 min, while **8a**, synthesized from the aromatic aldehyde **1b**, was hydrolyzed more slowly in the same conditions. Considering in vitro cytotoxicity of these two conjugates, we can hypothesize that the high activity found is partially due to the CPT released in the culture medium during the experiment. Further studies or in vivo experiments were hampered by the high insolubility of these molecules. Based on the information provided by the experiments described in this article, the future research will aim to systematically design and evaluate new RGD-CPT conjugates in order to find the optimal linker that warrants acceptable stability in the body circulation and prompt release inside the tumor cell, together with improved solubility.

4. Experimental

4.1. Materials and methods

All reagents and solvents were reagent grade and were distilled prior to use. All amino acids and resin were purchased from Bachem (Switzerland). Flash chromatography was carried out on silica gel (Merck 230–400 mesh). TLC analysis was conducted on silica gel plates (Merck $60F_{254}$). Products were characterized by HPLC, Waters 600 instrument, with an analytical column Purosphere, STAR®, Merck, C18, 5 μ, 4.6 × 250 mm, using acetonitrile/water buffered with 0.1% trifluoroacetic acid as mobile phase. UV detection was performed at $\lambda = 220$ nm for peptides and 360 nm for conjugates. When necessary, compounds were purified by semi-preparative HPLC system equipped with column Alltima®, Alltech, RP18, 10 μ, 22 × 250 mm. ^{1}H NMR spectra were recorded on AC300 Bruker instrument. Chemical shifts (δ values) are given in ppm. Mass spectra were recorded on Bruker Autoflex Maldi-Tof or Micro-Tof Q. α_v integrins were purchased from Chemicon.

^{125}I-Echistatin was purchased from Amersham. Vitronectin was purchased from Cal-biochem.

4.2. Synthesis of the conjugates 7a–c

General procedure. To a solution of the cyclopeptides **2a′–c′** in DMF at 0 °C, **1a** (1.5 equiv) was added followed by 2.2 equiv of DIEA, HOAT and DCC and the mixture was stirred at room temperature for 24 h. After evaporation of the solvent under reduced pressure, the residue was dissolved with DCM and the solution washed with water, dehydrated (Na_2SO_4) and evaporated to dryness. The residue was purified by standard flash chromatography (DCM/MeOH, 95:5→80:20) and total deprotection was carried out with TFA/DCM, 1:1 for 2 h.

After evaporation of the solvent, the residue was redissolved in a minimum quantity of TFA and precipitated with anhydrous ether, repeating the operation several times, to obtain the final conjugates as trifluoroacetate salts with high purity and good yield.

4.2.1. Conjugate 7a

Total yield 75% with >98% HPLC purity (34% CH_3CN in H_2O + 0.1% TFA): R_t = 6.92 and 9.65 min, corresponding to *E*/*Z* isomers of CPT derivatives; Maldi mass = 1035.40 $[MH]^+$; ^{1}H NMR (DMSO-d_6) δ: 9.29 (s, 1 H), 8.60 (d, 1H), 8.22 (d, 1H), 7.91 (t, 1H), 7.76 (t, 1H), 7.37 (s, 1H), 7.20–7.10 (m, 5H), 5.39 (d, 4H), 4.60 (m, 1H), 4.44 (m, 1H), 4.36 (t, 2H), 4.10–3.95 (m, 4H), 3.53 (d, 2H), 3.08–2.30 (m, 8H), 2.05 (t, 2H), 2.03–1.88 (m, 2H), 1.87–1.65 (m, 1H), 1.60–1.37 (m, 5H), 0.88 (t, 3H).

4.2.2. Conjugate 7b

Total yield 61% with 95% HPLC purity (38% CH_3CN in H_20 + 0.1% TFA): R_t = 3.20 and 3.98 min; Maldi mass = 1168.24 $[MH]^+$; ^{1}H NMR (DMSO-d_6) δ: 9.30 (s, 1H), 8.62 (m, 1H), 7.61–8.40 (m, 11H), 7.35 (s, 1H), 6.82–7.25 (m, 12H), 6.55 (s, 1H), 5.30–5.50 (m, 4H), 4.02–4.60 (m, 10H), 3.50 (m, 2H), 2.20–3.25 (m, 12H), 1.20–1.95 (m, 6H), 0.89 (t, 3H).

4.2.3. Conjugate 7c

Total yield 72% with 97% HPLC purity (34% CH_3CN in H_2O + 0.1% TFA): R_t = 6.6 and 9.33 min; ESI mass = 1272.27; ^{1}H NMR (DMSO-d_6) δ 9.39 (s, 1H), 8.68 (d, 1H), 8.35 (d, 1H), 8.01 (t, 1H), 7.87 (t, 1H), 7.48 (s, 1H), 7.23–7.12 (m, 9H), 5.50 (d, 4H), 4.65 (m, 1H), 4.51 (t, 2H), 4.36–4.10 (m, 7H), 4.01 (d, 4H), 3.75–3.59 (m, 10H), 3.19 (t, 2H), 3.10–2.68 (m, 6H), 2.02–1.90 (m, 2H), 1.89–1.40 (m, 4H), 0.99 (t, 3H).

4.3. Synthesis of the conjugates 8a and 8b

To a solution of the cyclopeptide **2d** in MeOH, CPT aldehydes were added under stirring and the solution kept at room temperature under N_2 for 36 h. Dilution with diethylether gave a precipitate, which was filtered and repeatedly triturated with diethylether/DCM to give the final conjugates.

4.3.1. Conjugate 8a

Yield 52% with 92% HPLC purity (28% CH_3CN in H_2O): R_t = 13.33 min; Maldi mass = 1228.13 $[MH]^+$; ^{1}H NMR (DMSO-d_6) δ: 8.09–8.40 (m, 7H), 7.78–8.00 (m, 5H), 7.84 (s, 1H), 6.81–7.75 (m, 13H), 6.55 (s, 1H), 5.39–5.55 (m, 4H), 3.85–4.70 (m, 16H), 3.75–2.78 (m, 10H), 2.03–1.02 (m, 8H), 0.90 (t, 3H).

4.3.2. Conjugate 8b

Yield 88% with 86% HPLC purity (28% CH_3CN in H_2O): R_t = 8.87 min; Maldi mass = 1255.77 $[MH]^+$; ^{1}H NMR (DMSO-d_6) δ: 8.45–8.11 (m, 7H), 7.96–7.63 (m, 5H), 7.35 (s, 1H), 7.25–6.80 (m, 13H), 6.55 (s, 1H), 5.85–5.60 (m, 4H), 4.56–3.81 (m, 20H), 3.70–2.79 (m, 10H), 2.05–1.10 (m, 8H), 0.91(t, 3H).

4.4. Synthesis of 1c

To a suspension of **3** (obtained from **1b** by Wittig reaction,[27] 40 mg, 0.1 mmol) in 5 mL of dry toluene in a flask equipped with a Dean–Stark apparatus, $MgSO_4$ (24 mg, 0.2 mmol), PTSA (5 mg, 0.026 mmol) and ethylene glycol (40 mg, 0.72 mmol) were added. The reaction was kept under reflux for 5 h and diluted with DCM, washed with water and dehydrated with Na_2SO_4. After removal of the solvent, the residue was purified by flash chromatography (DCM/MeOH 95:5) to give the acetal **4** with 56% yield. To a solution of **4** (25 mg, 0.055 mmol) in 10 mL of MeOH, 8 mg of Pd/C 10% were added and the suspension was hydrogenated for 3 h. Evaporation of the solvent followed by flash chromatography (DCM/MeOH 97:3) gave compound **5** with 99% yield. Deprotection of the acetal was carried out in 80% acetic acid under reflux for 6 h. After evaporation and flash chromatography (DCM/MeOH 95:5) **1c** was obtained with 39% yield. ESI mass $[MH]^+$: 403.12; ^{1}H NMR ($CDCl_3$) δ: 9.83 (s, 1H), 8.22 (d, 1H), 8.00 (d, 1H), 7.79 (m, 1H), 7.66 (m, 2H), 5.53 (m, 2H), 5.73–5.29 (m, 4H), 3.46 (m, 2H), 3.02 (m, 2H), 1.88 (m, 2H), 1.01 (t, 3H).

4.5. Synthesis of cyclopeptides

General procedure. The linear peptides were synthesized on solid-phase starting from Fmoc-Gly-Sasrin®. Protected amino acids were added to the resin in the following order: Fmoc-Arg(Pmc)-OH, psuedoamino acid, Fmoc-D-Phe-OH, Fmoc-Asp(OtBu)-OH. Each coupling step was monitored for completion by the Kaiser test. Fmoc deprotection was carried out with 20% piperidine. Cleavage from the resin was performed with 1% TFA in DCM; the suspension of the resin was stirred for 15 min at room temperature, filtered, the filtrate immediately neutralized with pyridine and this operation repeated for 5 times. The collected filtrates were concentrated under vacuum until 5% of the initial volume and the final linear peptide isolated by precipitation with cold water. After drying, the residue was dissolved with acetonitrile containing 1% DIEA to obtain a 1.5×10^{-3} M solution. Three equivalents of TBTU/HOBT were added and the solution stirred for 1 h. After evaporation of the acetonitrile, the residue was dissolved with DCM and washed (water, 0.1 M HCl, water). Solvent was evaporated and the totally protected cyclopeptides were purified by flash chromatography.

4.5.1. c[Arg(Pmc)-Gly-Asp(OtBu)-D-Phe-Aad-OH] (2a′)

The protected cyclopeptide was purified by flash chromatography (DCM/MeOH 95:5→92:8), total yield 47.5%. 713 mg (0.69 mmol) were dissolved with 30 mL DMF/MeOH 1:1, under Ar, and selectively deprotected by Catalytic Transfer Hydrogenation (CTH), with ammonium formate (3.45 mmol) and 0.8 g of Pd/C 10%, during 2 h. The suspension was filtered through Celite, the filtrate evaporated to dryness and the residue triturated several times with water. The title compound (650 mg) was obtained as a white solid, 99% purity, with 91% yield. HPLC (50% CH_3CN in H_2O + 0.1% TFA,): R_t = 16.49 min; ESI mass: $[M-H^+]$ 939.4. ^{1}H NMR (DMSO-d_6) δ: 8.16 (br, 1H), 8.05–7.80 (m, 3H), 7.65 (d, 1H), 7.25–7.15 (m, 5H), 6.90 (br, 1H), 6.51 (br, 2H), 4.58 (q, 1H), 4.50 (q, 1H), 4.10–3.85 (m, 4H), 3.10–2.25 (m, 16H), 2.08 (t, 2H), 2.02 (s, 3H), 1.80–1.35 (m, 8H), 1.34 (s, 9H), 1.21 (s, 6H).

4.5.2. c[Arg(Pmc)-Gly-Asp(OtBu)-D-Phe-Amp(CO–CH_2–CH_2–COOH)] (2b′)

The protected cyclopeptide c[Arg(Pmc)-Gly-Asp(OtBu)-D-Phe-Amp(Cbz)] was selectively deprotected with CTH as described for **2a′**, and the product purified on preparative HPLC (50% CH_3CN in

H_2O + 0.1% TFA) with 44% yield. 120 mg (0.11 mmol) of the latter were dissolved with 4 mL of DCM/DMF 2:1, succinic anhydride (11 mg, 0.11 mmol) and TEA (0.11 mmol) were added and the reaction mixture stirred at room temperature for 1 h. After dilution with 30 mL DCM, the solution was washed with water (4 × 20 mL) and taken to dryness. 100 mg of a white solid were obtained with 84.7% yield, HPLC (50% CH_3CN in H_2O + 0.1% TFA): R_t = 11.75 min; Maldi mass $[MH]^+$: 1074.47, ^{1}H-NMR (DMSO-d_6) δ: 8.30–8.25 (m, 2H), 8.01 (m, 2H), 7.87–7.79 (m, 2H), 7.17–7.03 (m, 9H), 6.79 (b, 1H), 6.51 (br, 1H), 4.60 (q, 1H), 4.46 (q, 1H), 4.32 (q, 1H), 4.22 (d, 2H), 4.07–3.99 (m, 2H), 3.22 (s, 1H), 3.05–2.31 (m, 14H), 2.43 (s, 6H), 2.01 (s, 3H), 1.75 (t, 2H), 1.73–1.30 (m, 4H), 1.34 (s, 9H), 1.24 (s, 6H).

4.5.3. c[Arg(Pmc)-Gly-Asp(OtBu)-D-Phe-Amp(CO–CH_2–(OCH_2–CH_2)$_2$–O–CH_2–COOH)] (2c′)

The same cyclopeptide described for **2b′**, after selective deprotection, (800 mg, 0.737 mmol) and DIEA (2.2 mmol) were dissolved with DCM/DMF 3:1 and excess of 3,6,9-trioxaundecanedioic acid (5 g, 24.3 mmol) was added followed by DCC (304 mg, 1.472 mmol). The reaction mixture was stirred at room temperature overnight, then diluted with 250 mL of $CHCl_3$, washed with water (6 × 30 mL), dehydrated and concentrated to dryness. The crude residue was purified by flash chromatography (DCM/MeOH 7:3→DCM/MeOH 7:3 + 1% AcOH). 628 mg of pure **2c′** were obtained with 72.4% yield. HPLC (50% CH_3CN in H_2O+0.1% TFA): R_t = 13.96 min; Maldi mass: $[MH]^+$ 1178, ^{1}H NMR (DMSO-d_6) δ: 8.04–7.89 (m, 5H), 7.15–6.99 (m, 9H), 4.58 (q, 1H), 4.44–4.30 (m, 4H), 4.11–3.99 (m, 3H), 3.91 (d, 4H), 3.60–3.51 (m, 8H), 3.00 (t, 2H), 2.98–2.28 (m, 14H), 2.01 (s, 3H), 1.76–1.72 (m, 3H), 1.54–1.45 (m, 1H), 1.40–1.31 (m, 4H), 1.33 (s, 9H), 1.24 (s, 6H).

4.5.4. c[Arg-Gly-Asp-D-Phe-Amp(CO–CH_2–(OCH_2–CH_2)$_2$–O–CH_2–CO–NH–NH_2)] (2d)

The protected cyclopeptide was obtained with 45% yield after flash chromatography (DCM/MeOH 94:6→92:8). Total deprotection was performed with 95% TFA in water for 1 h. After concentration to small volume, the product was precipitated with dry and cool diethylether, affording pure **2d** with 99% purity and 97.8% yield. HPLC (20% CH_3CN in H_2O + 0.1% TFA): R_t = 9.14 min; Maldi mass $[MH]^+$ = 870.13. ^{1}H NMR (DMSO-d_6 + D_2O) δ: 7.14–7.09 (m, 5H), 7.02–6.97 (m, 4H), 4.57–4.52 (m, 2H), 4.46–4.44 (m, 2H), 4.26 (s, 2H), 4.00 (d, 1H), 3.95–3.90 (m, 2H), 3.57–3.51 (m, 8H), 3.26 (d, 1H), 3.04 (t, 2H), 2.87–2.64 (m, 5H), 2.38–2.33 (m, 1H), 1.75–1.72 (m, 1H), 1.51–1.47 (m, IH), 1.34–1.32 (m, 2H).

4.6. Fmoc-Amp[CO–CH_2–(O–CH_2–CH_2)$_2$–O–CH_2–CO–NH–NH_2]–OH (6) (pseudoamino acid incorporated in 2d)

To a solution of 3,6,9-trioxaundecanedioic acid (22 mg, 100 mmol) and DCC (12.4 g, 60 mmol) in 600 mL of DCM, at 0 °C, benzyl alcohol (4.32 g, 40 mmol), DMAP (977 mg, 8 mmol) and TEA (14 mL, 100 mmol) were added and the reaction mixture stirred at room temperature for 1 h, then filtered. The filtrate was washed with water (1 × 50 mL) 0.1 N HCl (1 × 50 mL) and water (3 × 50 mL). After evaporation of the solvent, the crude residue was dissolved with 500 mL of dry EtOH and NH_2–NH_2·H_2O (13.6 mL, 280 mmol) was added. After refluxing for 1 h, the solution was evaporated under vacuo until excess hydrazine was removed. The residue was dissolved with 300 mL MeOH containing 50 mL of 1 N NaOH; then a solution of $(Boc)_2O$ (26 mg, 120 mmol) in 30 mL MeOH was added dropwise and the reaction mixture stirred at room temperature for 4 h, maintaining the pH around 8 with additions of NaOH. After evaporation of methanol, the residue was dissolved with 300 mL of EtOAc and extracted with water (2 × 100 mL). The collected aqueous phases were washed back with EtOAc (5 × 30 mL) to remove organic impurities, then acidified with cold 0.5 N HCl to pH 2 and extracted with EtOAC (5 × 250 mL). 10.2 g of trioxaundecanedioic acid mono-Boc-hydrazide were obtained with 76% yield.

To the latter monoacid (8.47 g, 25 mmol), dissolved with 260 mL DCM at 0 °C, hydroxysuccinimide (3.47 g, 30.2 mmol) and DCC (6.24 g, 30.2 mmol) were added. After 3 h at room temperature, the suspension was filtered, washed with water (3 × 40 mL) and solvent evaporated to give the activated ester with 86% yield. This ester was dissolved with 135 mL DCM together with TEA (26 mmol) and added to a solution containing Fmoc-Amp-OH (12.06 g, 22 mmol) in 42 mL in DMF. After 2.5 h at room temperature, the solvent was evaporated, the residue dissolved with 400 mL DCM and washed with water (1 × 50 mL), 0.1 N HCl (1 × 30 mL) and water (3 × 50 mL). The crude product was purified by flash chromatography (DCM/MeOH 95:5→90:10) to give 8.34 g of pure **6** with 52% yield. HPLC (52% CH_3CN in H_2O + 0.1% TFA): R_t = 9.06 min; Maldi mass [M+Na] 756.9; ^{1}H NMR (DMSO-d_6) δ: 8.65 (m, 1H), 8.09 (t, 1H), 7.94 (s, 1H), 7.86 (d, 2H), 7.64–7.57 (m, 2H), 7.43 (t, 2H), 7.30 (t, 2H), 7.17 (s, 4H), 4.28–4.20 (m, 6H), 3.92 (d, 4H), 3.57–3.55 (m, 8H), 3.10–2.80 (m, 2H), 1.38 (s, 9H).

4.7. Affinity to α_v integrin receptors

96-Well plates were subjected overnight to coating with 0.5 μg/mL of integrin $\alpha_v\beta_3$ or 1 μg/mL of $\alpha_v\beta_5$, respectively. On the next day, the wells were washed and incubation was performed with 0.05 nM ^{125}I-Echistatin in the presence or absence of the sample compounds. After 3 h incubation and washing, the integrins from each well were solubilized with 2 N NaOH and the radioactivity measured on a gamma counter. Non-specific binding was determined in the presence of 1 μM Echistatin. The affinity of the samples was expressed as IC_{50} ± SD value (nM), elaborated using 'ALLFIT' software.

4.8. Cell adhesion assay

96-well plates were pretreated with 5 μg/ml of human vitronectin for 2 h at room temperature. Vitronectin was removed and 1% bovine serum albumin (BSA) was added and the plates left for 1 h at room temperature. Plates were washed with medium culture without fetal calf serum (FCS). The molecules were assayed at scalar concentrations ranging from 0.1 to 100 μM. Tumor cells were re-suspended in medium culture containing 1% BSA without FCS and plated on vitronectin substrate. After 1 h of incubation, the cells were washed once with PBS. The adhering cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature and then stained with a 1% toluidine blue solution for 10 min at room temperature. After staining, the cells were washed with double-distilled water, dried and solubilized with a 1% SDS solution, and quantified by means of absorbance on a Victor multilabel plate counter (Wal-lac) at 600 nM. The effect of tumor cell adhesion was expressed as IC_{50} ± SD value (μM), elaborated using 'ALLFIT' software.

4.9. Drug uptake

6.2×10^5 A-498 cells were seeded in 100 × 20 mm cell culture dishes. After 24 h, cells were treated for several times (5 h, 24 h, 48 h and 72 h) using as dose their IC_{50} evaluated after 72 h of treatment. At each time point, each drug-containing medium was collected and stored at −20 °C, and cells were washed with phosphate buffered saline (PBS), collected by scraping, and centrifuged at 1000*g* at 4 °C for 10′. Pellets were re-suspended in 100 μL of PBS and lysed with five cycles of freeze-and-thaw (3 min each). After centrifugation at 14,000*g* for 10 min, supernatant was placed

in new eppendorf tubes and processed adding 200 μL of methanol/acetonitrile mixture (1:1 v/v). After vortexing, samples were placed on ice for 10 min, then centrifuged at 14,000*g* for 20′ at 4 °C and supernatants were transferred into auto-sampler vials. Protein concentration of cell lysates was determined using Comassie (Bradford) Protein Assay kit (Pierce). Drug containing cell culture medium was processed as described adding 700 μL of extraction mixture. Supernatants were analyzed by HPLC-FL (Beckman Instruments) and analytical data were acquired and processed by a computer system (32Karat Software, Beckman-Coulter). The separation was performed by isocratic elution (30% CH_3CN, 70% 0.1 M AcOH containing 0.1%TEA, pH ~3.5) with a flow rate of 1 mL/min on a discovery HS-F5 column, 100 × 4.6 mm, 5 μm (Supelco). The detection was performed with a spectrofluorometric detector (RF-10AXL, Shimadzu) at λ_{ex} 370 nm and λ_{em} 510 nm. All data were expressed as mean (nmol of drug/mg of total proteins) ± SE.

4.10. Cytotoxicity assay in vitro

Tumor cells were seeded in 96-well tissue culture plates. The day after, different concentrations of drugs were added to each well. The plates were incubated for 72 h. The number of surviving cells was determined by staining with sulforodamine B. The cytotoxic activity of the compounds was expressed as IC_{50} ± SD value (μM), elaborated using 'ALLFIT' software.

4.11. Nude mouse xenograft assay

A2780 ovarian carcinoma cells (3×10^6/mouse) were implanted subcutaneously into right flank of nude mice on day 0. Compound **7c** was dissolved in 12% 1-methyl-2-pyrrolidinone and was intraperitoneally administered at a concentration of 48 mg/kg bis in die every fourth day for four times (q4 d × 4) or subcutaneously bis in die every day for 5 days (qd × 5/w) in a volume of 10 mL/kg, starting two days after tumor injection. Tumor volumes TV (mm^3) were estimated as $d \times D/2$, where d and D are the shortest diameter and the longest diameter measured in mm upon 16 or 17 days after tumor injection. To determine toxicity of the compound, the body weight of tumor-bearing mice was also measured. All data are presented as means ± SE. Mice were sacrificed when tumor volumes reached around 2 cm^3. The statistical differences compared with vehicle-treated group were calculated by Mann–Whitney's test. Animal experiments were performed under the permission in accordance with European Directives no. 86/609, and with Italian DL 116, January 27th, 1992.

4.12. In vivo plasma concentrations and tumor distribution

Tumors were established in nude mice as described above. Animals (3 mice/group) were treated intravenously with the conjugate **7c** at a dose of 45 mg/kg and sacrificed at different times (0.5, 1, 2, 4, 7, 24 h) after intravenous injection. Plasma (100 μL) was processed by adding 400 μL of a cold mixture of 0.1% acetic acid and methanol (1:5, vol/vol). Samples were kept on ice for 10 min, then centrifuged at 1000*g* for 10 min at 4 °C. Tumors were homogenized with a Potter using the same extraction mixture and applying a tissue weight/mixture volume ratio of 1:5. Homogenized samples were kept on ice for 10 min and then centrifuged at 4000*g* for 10 min at 4 °C. Supernatants were analyzed by HPLC-FL as described above. All data were expressed as mean (ng of drug/mL plasma or ng/g tissue) ± SE.

Acknowledgements

This work was supported by Sigma-Tau, Roma, Italy. We thank Patrizia Tobia and Matilde Cati for technical assistance.

Supplementary data

Figures S1 and S2 describing decreasing rates of **8a** at different pHs. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.019.

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