CHEMBIOCHEM

DOI: 10.1002/cbic.200800327

Synthesis and Biological Characterisation of Targeted Pro-Apoptotic Peptide

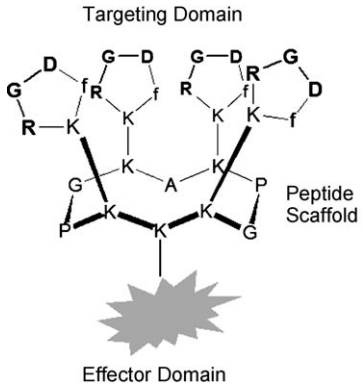
Stéphanie Foillard,[a] Zhao-hui Jin,[b] Elisabeth Garanger,[a] Didier Boturyn,*[a] Marie-Christine Favrot,[b] Jean-Luc Coll,[b] and Pascal Dumy*[a]

We report herein the synthesis and in vitro assay of new, multimeric RGD-peptide conjugates for cell-targeted drug delivery. We generated a peptide scaffold comprising two functional domains, one a tumour blood vessel "homing" motif and the other a programmed cell-death-inducing peptide sequence. RGD peptides were selected to direct the molecular conjugate to $\alpha_V\beta_3$ integrin-containing tumour cells. The pro-apoptotic (Lys-Leu-Ala-Lys-Leu-Ala-Lys)$_2$ peptide was found to be nontoxic outside cells, but toxic when internalized into targeted cells as it disrupted the mitochondrial membrane. The synthesis of these targeted pro-apoptotic conjugates was carried out by assembling three different units (that is, scaffold, RGD units and pro-apoptotic peptide) through chemoselective ligations. We show that one compound displays significant biological effect in $\alpha_V\beta_3$ integrin-containing tumour cells.

Introduction

Conventional chemotherapy causes serious side effects in patients due to its toxicity to normal cells and noncancerous tissues. The selective delivery of bioactive molecules to the site of disease might be one avenue toward better treatment.[1] However, this strategy relies on pathologic markers that are accessible from the bloodstream. The discovery of targeted molecules (ligands, monoclonal antibodies) could offer an opportunity to concentrate the drug of interest close to or inside the tumour cells. In this context, most researchers are focusing on targeting the $\alpha_V\beta_3$ integrin receptor. This protein plays a key role in cancer, especially in tumour angiogenesis and metastasis, and in regulating intracellular signalling, cell migration, cell proliferation and cell survival.[2] The $\alpha_V\beta_3$ integrin receptor binds to extracellular-matrix proteins such as vitronectin and fibronectin through recognition of the ubiquitous triad sequence Arg-Gly-Asp (RGD). This peptide sequence has served as the basis for the development of potent and selective integrin peptide ligands. In the last decade, rational screening of RGD-containing peptides has led to the discovery of the highly active peptide cyclo[-Arg-Gly-Asp-D-Phe-Val-] by Kessler and co-workers.[3,4] This peptide displays high affinity (2 nmol L^{-1}) for the $\alpha_V\beta_3$ integrin receptor of vitronectin and low affinity (>2000 nmol L^{-1}) for the $\alpha_{IIb}\beta_3$ integrin receptor (present on platelets) of fibronectin.[5] It is worth noting that the *N*-alkylated analogue cyclo[-Arg-Gly-Asp-D-Phe-(*N*-Me)Val-] (Cilengitide) is currently being investigated in phase II clinical trials as an inhibitor of angiogenesis.[5] Furthermore, it is well known that exchanging valine for lysine in the cognate cyclopentapeptide has no significant influence on biological activity.[6] Therefore, modification of the lysine side chain has led to the synthesis of numerous RGD-containing conjugates for tumour therapy[7] and imaging.[8] Besides allowing for the conjugation of bioactive molecules, the use of a multivalent ligand results in high specificity and has become a rule for the design of RGD conjugate compounds.[9]

Following this concept, we previously designed a tetrameric RGD peptide with desirable biological properties.[10–11] These compounds are based on a cyclic decapeptide scaffold containing two independent functional domains: a clustered ligand domain for cell targeting and an effector domain for detection and/or therapy in a spatially controlled manner. The re-



[a] Dr. S. Foillard, Dr. E. Garanger, Dr. D. Boturyn, Prof. Dr. P. Dumy
Département de Chimie Moléculaire, UMR CNRS-UJF 5250
ICMG FR 2607, 301, rue de la chimie, BP 53
FR 38041 Grenoble Cedex 9 (France)
Fax: (+33) 4-76-51-49-46
E-mail: didier.boturyn@ujf-grenoble.fr
pascal.dumy@ujf-grenoble.fr

[b] Dr. Z.-h. Jin, Prof. Dr. M.-C. Favrot, Dr. J.-L. Coll
Institut Albert Bonniot, INSERM, U823
Domaine de la Merci, FR 38706 La Tronche Cedex (France)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

gioselectivity of this sytem is easily tunable, and is dependent upon the protecting groups chosen for the Pro-Gly sequence.[12] Extensive studies with scaffolds containing one to 16 RGD peptide ligands have highlighted the utility of clustered ligands.[13] For instance, after modification of the effector domain with near-infrared fluorochromes, peptide scaffolds containing four RGD ligands are very efficient in specifically targeting tumour neovasculature as well as $\alpha_V\beta_3$-expressing metastases in murine animal models.[14] Cellular uptake was shown to occur in vitro through integrin-receptor-mediated endocytosis by early endosomes.[10–11] These results emphasize the interest of using our sophisticated molecules as vectors for a targeted drug delivery.

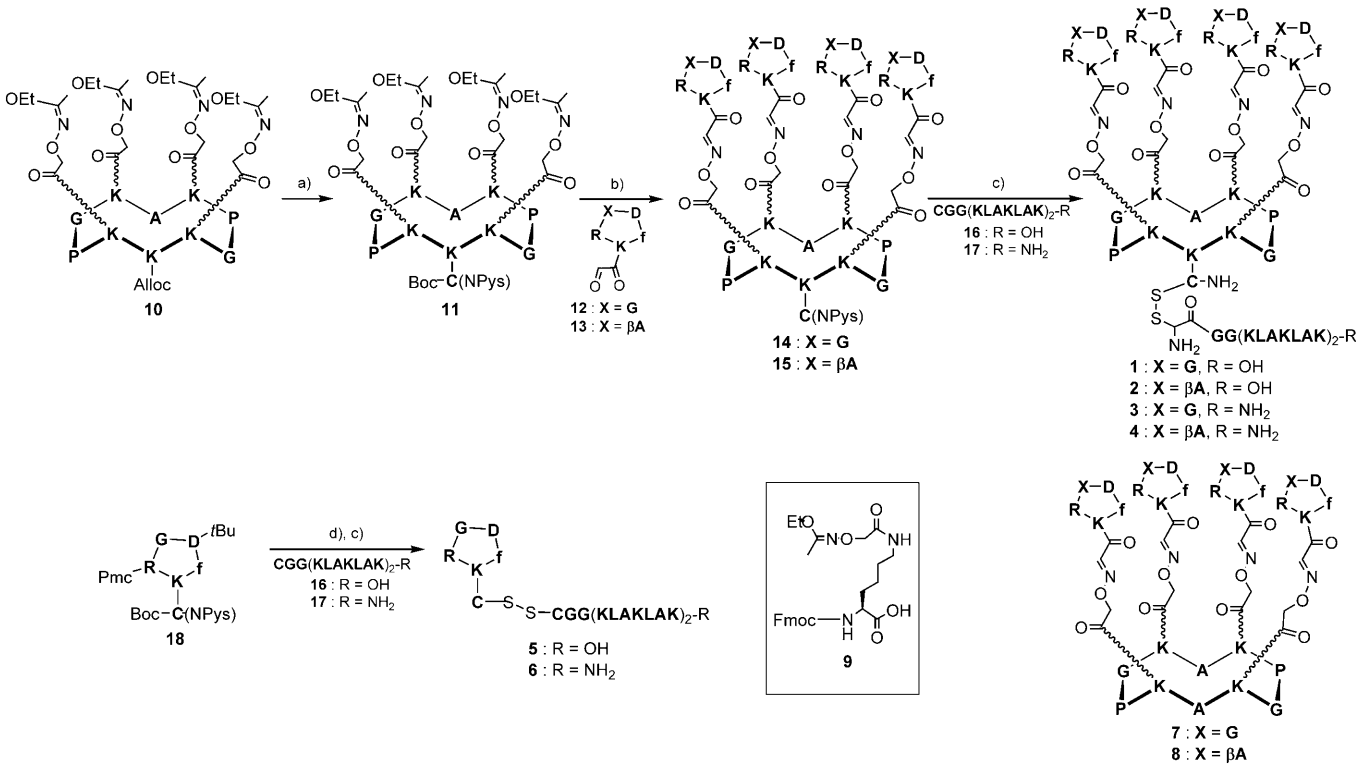
We then focused on the synthesis of monomeric and tetrameric RGD-containing drug conjugates and their biological evaluation in $\alpha_V\beta_3$ integrin-expressing cells. To this end, we envisioned using a programmed cell death-inducing peptide such as (Lys-Leu-Ala-Lys-Leu-Ala-Lys)$_2$ (KLA) as a scaffold.[15] This molecule has been shown to be nontoxic outside of cells, but is toxic when internalized by targeted cells, as it disrupts the mitochondrial membrane.[16] Synthesis of these bifunctional conjugates involves the chemoselective ligation of RGD ligands and KLA peptides to the upper and the lower faces of the scaffold, respectively (Scheme 1). With these molecules in hand, we concentrated our work on assessing activity and validating the potency of the clustered compounds.

Results and Discussion

Design and synthesis of bifunctional conjugates

Based on previous results,[10–14] we reasoned that a tetrameric RGD-containing template would be suitable for specific targeting and selective delivery of bioactive compounds. In this context, several compounds (**1**–**8**) were designed (Scheme 1): cyclodecapeptides **1**–**4** and **7**–**8** were decorated with either cyclo[-Arg-Gly-Asp-D-Phe-Lys-], an $\alpha_V\beta_3$ integrin ligand, or cyclo[-Arg-βAla-Asp-D-Phe-Lys-], a nonsense peptide. The introduction of an additional methylene group (β-alanine substitution for glycine) inhibits the adhesion of this peptide to $\alpha_V\beta_3$ integrin.[17] To evaluate the advantages of our multivalent ligand architecture over monomeric RGD-containing peptides for drug delivery, we prepared monomers **5** and **6** in parallel.

A modular synthesis was adopted to construct the bifunctional molecules (Scheme 1). We chose chemoselective reactions to ligate different moieties on the peptide scaffold. Stable oximes were utilized to connect the aldehyde-bearing "homing" RGD motif to the scaffold displaying four aminooxyacetyl residues (Aoa). Oxime formation is highly efficient, compatible with a wide variety of chemical functions and occurs between unprotected fragments without the need for coupling reagents and with minimal chemical manipulation.[18] Moreover, the oxime linkage is stable in vitro and in vivo and does not trigger any immune response.[19] At the bottom face of the peptide scaffold, a cleavable disulfide bridge was used to carry the pro-apoptotic KLA peptide inside the targeted cell.



Scheme 1. Modular synthesis of conjugates **1**–**6**. a) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, 30 min; Boc-Cys(NPys)OH, PyBOP, DIPEA, DMF, 30 min; b) 70% TFA, CH_3CN, H_2O, **12** or **13**, 30 min; c) **16** or **17**, PBS pH 4.8/DMF (1:3), 5 min; d) 90% TFA, 2.5% H_2O, 2.5% TIS, 5% EDT, 3 h.

It is well known that disulfide bridges are reduced enzymatically by thioredoxines in the cytosol and in late endocytic compartments.[20] Thus, they have been used to improve the efficiency of targeted drug delivery.[21] To avoid degradation of the pro-apoptotic peptide by proteases inside cells, we used KLA peptide **17**, which is amide protected at the C terminus. KLA peptide **16**, which contains an C terminus carboxylic acid, was used as a negative control.

All peptides were prepared through a combination of solid- and solution-phase syntheses, according to methods already developed in our group.[10,22–23] The convenient choice of lysine side-chain modifications and their relative positioning within the peptide primary sequence provide cyclodecapeptide **10**, which contains adequate protected attachment sites pointing to opposite faces of the template backbone. As illustrated in Scheme 1, four lysines bearing protected aminooxy functionality and one N^{ε}-Alloc protected lysine were appended to the upper and lower face of the scaffold backbone sequence, respectively. In order to introduce the protected aminooxy groups during solid-phase peptide synthesis (SPPS), we have recently developed building block **9** through protection of the aminooxy functionality with the 1-ethoxyethylidene group (Eei).[24] The use of the Eei group avoids side reactions such as overacylation at the amino site of the monoprotected aminooxy function, which is observed with classical Boc-protected aminooxy moieties under SPPS coupling conditions. We have shown that Eei is stable under standard SPPS conditions and can be removed by using a dilute solution of TFA.[24] Placement of glycine at the C-terminal end of the linear decapeptide H-Lys(Eei-Aoa)-Lys(Alloc)-Lys-(Eei-Aoa)-Pro-Gly-Lys(Eei-Aoa)-Ala-Lys(Eei-Aoa)-Pro-Gly-OH was necessary to prevent epimerization during the subsequent head-to-tail cyclization steps. This reaction was performed in DMF under high dilution with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) to give cyclodecapeptide **10**, as reported earlier.[10]

Removal of the Alloc group was carried out by using a well-established $Pd^0/PhSiH_3$ procedure.[25] Consecutive coupling of Boc-Cys(NPys) with PyBOP afforded the key intermediate cyclodecapeptide **11** in quantitative yield. Since Eei is not stable under RP-HPLC conditions (0.1% TFA), the crude product was used for the subsequent step. By using a similar synthesis scheme, integrin ligand **12** and nonsense peptide **13**, both bearing a glyoxylyl aldehyde group on the lysine side chain, were prepared. Linear peptide H-Asp(*t*Bu)-D-Phe-Lys[Boc-Ser-(*t*Bu)]-Arg(Pmc)-Gly-OH and its β-alanine analogue were cyclized under high dilution, followed by TFA deprotection and sodium periodate oxidation as previously described.[23]

To append targeting elements to scaffold **11**, we performed amniooxy deprotection and chemoselective ligation of peptides **12** or **13** in one pot since oxime formation occurs by an acid-catalyzed mechanistic pathway.[26] Aminooxy groups are highly reactive towards carbonyl-containing compounds such as volatile aldehydes or ketones,[27] and we preferred to trap these groups during their deprotection. Consequently, a slight excess of aldehydic compound **12** (2 equiv per aminooxy group) was added to an acidic solution containing scaffold **11**. The reaction was complete after 20 min at room temperature. RP-HPLC purification furnished the RGD-containing intermediate **14** with satisfactory yield (60% overall yield from compound **10**). The same procedure was applied to obtain the control scaffold **15**. Key intermediates **14** and **15** were characterized by ES-MS, and the observed molecular weights were in excellent agreement with the calculated values. It must be noted that these intermediates can be utilized to carry a broad panel of thiol-containing biologically relevant molecules.

To introduce a disulfide bridge and prevent undesired homodimerization, we chose to use 3-nitro-2-pyridinesulfenyl (Npys)-activated/protected cysteine.[28] This group is stable over a wide pH range, and the reaction can be monitored spectrophotometrically through titration of the released 3-nitro-2-pyridinethiol.[29] To tether homodisulfide compounds, disulfide bridges were formed under mildly acidic conditions under argon for 5 min. Reactions were carefully monitored by HPLC (Figure 1 A), and products **1**–**4** were directly recovered after RP-HPLC purification in good yields (~80%). All conjugates were characterized by ES-MS, and deconvoluted masses were in good agreement with calculated masses (Figure 2 B).

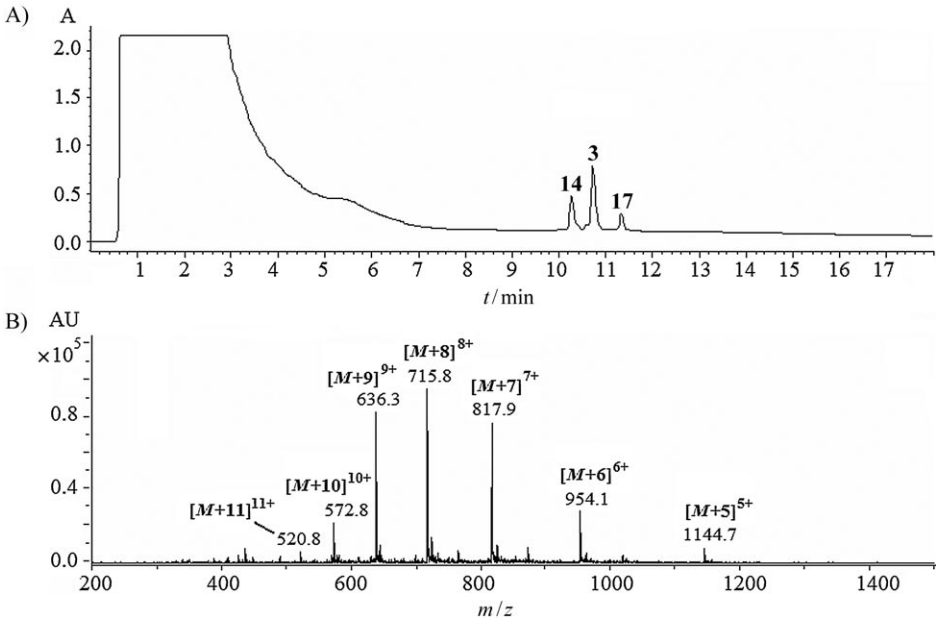


Figure 1. A) RP-HPLC profile of the reaction mixture containing compound **3** after 1 min. B) ES-MS analysis of purified compound **3**.

Control monomers **5** and **6** were synthesized using a similar method.[23] Cyclo[-Arg(Pmc)-Gly-Asp(*t*Bu)-D-Phe-Lys[Boc-Cys(NPys)]-] (**18**) was first incubated under acidic conditions, and then the deprotected cyclopentapeptide was added to the thiol-containing peptides **16** or **17**. Products **5** and **6** were obtained after RP-HPLC purification. Control compounds **7**, **8** and cyclopentapeptide cyclo[-Arg-Gly-Asp-D-Phe-Lys-] **19**, which lack KLA motifs, were prepared as previously described.[13]

Biological evaluation of RGD-peptide conjugates

The use of a tetravalent RGD-containing molecule capitalizes on the fact that multimeric ligand presentation improves ligand–receptor interactions.[13] Previous biological studies have shown that multivalent RGD compounds are clearly internalized in $\alpha_v\beta_3$ integrin-expressing cells[10] and were consistent with an integrin receptor-mediated endocytosis pathway.[11] To date, fluorescent compounds have been easily exploited to target different cells or tissues and successfully applied to the noninvasive optical imaging of metastasis and tumours in mice.[14] Therefore, we took advantage of our KLA conjugates as a scaffold for drug delivery.

All compounds were examined for cytotoxicity towards the $\alpha_v\beta_3$ integrin-expressing murine mammary adenocarcinoma cell line (Ts/Apc). Ts/Apc cells were grown for one week in culture plates and maintained at an appropriate temperature and gas mixture (37 °C, 5% CO_2) in a cell incubator. As expected, conjugates **2** and **8** containing the RβAD nonsense sequence do not induce cell death. Surprisingly, conjugate **4** triggers around 50% cell death as compared to the control PBS-treated cells (Figure 2). We hypothesize that longer exposure time (one week) of cells to compounds results in minor internalization of peptides through an integrin-independent fluid-phase uptake process (pinocytosis). Monomer RGD ligands **5**, **6** and **19** were found to be less potent than their multivalent analogues, compounds **1**, **3** and **7**, respectively, at a concentration of 5 μM; this emphasizes the beneficial effect that the RGD ligand cluster has on the scaffold. In particular, in contrast to treatment with **3**, which was lethal, after incubation with monomer **6**, more than 30% of cells were still alive. It was necessary to increase the concentration of **6** to 20 μM to get a similar result to that observed with 5 μM of **3**. Even at 20 μM **6**, as shown in the culture plates (Figure 3, inset), we still observe some living Ts/Apc colonies. In contrast, the plate containing the conjugate displaying the greatest biological activity, **3**, has no living colonies present. Moreover, **3**, which bears an amide at the C terminus of the KLA moiety, evades enzyme degradation and exhibits a longer duration of action, while unprotected **1** is rapidly degraded by proteases. It is well attested that the C terminus can be protected from protease degradation through the use of an amide instead of a carboxylic acid, or by use of D-amino acids instead of natural L-amino acids.[30] Consequently, the biological activity of compounds **1** and **5** is very low and approximately the same as those of their respective analogues, **7** and **19**, which lack the KLA moiety. Their resulting activity comes from the known cytotoxic effect of the RGD residue.[31]

Finally, to test whether apoptosis was indeed the mechanism of cell death, Ts/Apc cells were treated with Hoechst 33342 dye. Cells undergoing apoptosis display nuclear condensation and DNA fragmentation. These events can be detected by staining with Hoechst and subsequent fluorescence microscopy. As shown in Figure 3, compound **3** triggers

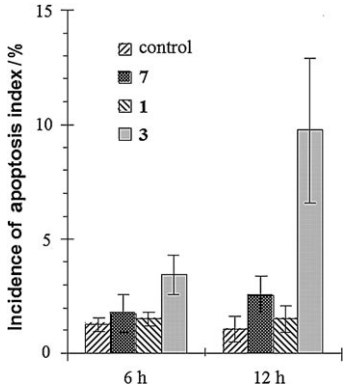


Figure 3. Apoptosis assays by Hoechst 33342 staining using 5 μM of conjugates on Ts/Apc cells; control bars represent assays without addition of conjugate.

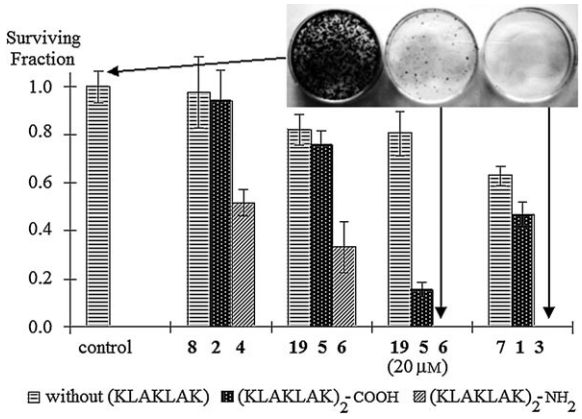


Figure 2. Evaluation of Ts/Apc colony survival by using 5 μM of conjugates (except when specified); control bar represents surviving fractions from culture plates incubated without addition of conjugate.

significant apoptosis in $\alpha_v\beta_3$ integrin-expressing Ts/Apc cells after 12 h, while cells treated with compounds **1** and **7** do not show any signs of apoptosis. As mentioned above, conjugate **1** exhibits a reduction in its biological activity due to proteolysis. Consequently, when colonies are treated with compounds **1** or **7** cell death results essentially from the RGD residues.

Altogether, these results suggest that our scaffold would be highly effective for the delivery of therapeutic agents through a specific receptor-mediated endocytosis pathway. This approach could be essential for delivery of biologically relevant biomolecules such as peptides, proteins and nucleic acids, since most of these compounds cannot cross the cellular membrane. For instance, a protein such as the highly toxic ricin A might be a good candidate for tumour-targeted drug delivery.[32] Nevertheless, chemical modifications of some bio-

molecules will be necessary to increase their stability and biological activity.

Conclusions

The chemical strategy presented here is based on peptide synthesis and chemoselective ligations. It is very flexible, modular and was adapted to prepare a series of compounds. From biological studies, we show that a tetrameric RGD-containing scaffold bearing an apoptosis-inducing peptide through a cleavable bond offers an interesting outlook for tumour-targeted drug delivery. Because the chemical assembly of these scaffolds is convergent and the scaffold domains are well separated, synthesis of more sophisticated systems that are required for in vivo studies should be facile. By adapting the corresponding domain on the scaffold, it might be possible to target different cells or tissues due to the increasing number of selective ligands selected in vivo.[33]

Experimental Section

General: Protected amino acids, Sasrin™ and chlorotrityl resins were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem and other reagents were obtained from either Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France). RP-HPLC was performed on a Waters system equipped with a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. The purity of peptide derivatives was analyzed on an analytical column (Macherey–Nagel Nucleosil 120 Å 3 mm C18 particles, 30×4.6 mm) by using the following solvent system: solvent A, water containing 0.09% TFA; solvent B, acetonitrile containing 0.09% TFA and 9.91% H_2O. A flow rate of 1.3 mL min^{-1} was employed and UV absorbance was monitored at 214 nm and 250 nm simultaneously. A preparative column (Delta-Pak 100 Å 15 mm C18 particles, 200×2.5 mm) was used to purify the crude peptides (when necessary) by using an identical solvent system at a flow rate of 22 mL min^{-1}. ESI mass spectra were recorded on an Esquire 3000 (Bruker) spectrometer. The analysis was performed in the positive mode for peptide derivatives using 50% aqueous acetonitrile as eluent.

Peptide synthesis: Assembly of all protected peptides was carried out using a Fmoc/*t*Bu strategy either manually in a glass reaction vessel fitted with a sintered glass frit or automatically on a synthesizer (348 Ω synthesizer, Advance ChemTech). Coupling reactions were performed by using, relative to the resin loading, 1.5–2 equiv of *N*-α-Fmoc-protected amino acid activated in situ with 1.5–2 equiv of PyBOP and 3–4 equiv of DIPEA in DMF (10 mL g^{-1} resin) for 30 min. Manual syntheses were controlled by Kaiser and/or TNBS tests. *N*-α-Fmoc protecting groups were removed by treatment with a piperidine/DMF solution (1:4; 10 mL g^{-1} resin) for 10 min. The process was repeated three times, and the completeness of deprotection was verified by the UV absorption of the piperidine washings at 299 nm.

Cyclization Reactions: All linear peptides (0.5 mM) were dissolved in DMF, and the pH was adjusted to 8–9 by addition of DIPEA. PyBOP (1.2 equiv) was added and the solution was stirred at room temperature for 30 min, as described earlier.[10] Solvent was removed under reduced pressure. The residue was dissolved in a minimum of CH_2Cl_2 followed by precipitation with ether to obtain the peptide. It was then triturated and washed three times with ether to yield crude material, which was used without further purification.

Building block 9: Amino acid **9** was prepared following the procedure previously described.[24]

Synthesis of decapeptide scaffold 10: The linear peptide H-Lys-(Eei-Aoa)-Lys(Alloc)-Lys-(Eei-Aoa)-Pro-Gly-Lys(Eei-Aoa)-Ala-Lys(Eei-Aoa)-Pro-Gly-OH was prepared on a 2-chlorotrityl chloride® resin (2.0 g, loading of 1.1 mmol g^{-1}) by using the building block **9** and by following the reported strategy.[24] The peptide was obtained as a white solid powder (2.27 g, 1.26 mmol). The cyclization reaction was carried out as described aboveusing the crude linear peptide (85 mg, 47 μmol). Peptide **10** was obtained as a white powder (79 mg, 47 μmol, 57%). RP-HPLC (C18, 214 nm, 5–100% B in 15 min) t_R = 10.8 min; ESI-MS calcd for $C_{75}H_{125}N_{19}O_{24}$: 1675.9, found *m/z* 1698.4 $[M+Na]^+$.

Synthesis of decapeptide scaffold 11: Peptide **11** was prepared following the previously reported procedure[24] starting from peptide **10** (20 mg, 12 μmol). The fully protected peptide **11** was obtained as a white solid powder (22.8 mg, 11.7 μmol, 98%). RP-HPLC (C18, 214 nm, 5–100% B in 15 min) t_R = 12.1 min; ESI-MS calcd for $C_{84}H_{136}N_{22}O_{27}S_2$: 1948.9, found *m/z* 1988.2 $[M+K]^+$.

Synthesis of pentapeptides 12 and 13: RGD-containing pentapeptides **12** and **13** were prepared as previously described.[23]

Synthesis of decapeptide scaffold 14: Peptides **11** (5 mg, 2.57 μmol) and **12** (18 mg, 23.3 μmol) were dissolved in TFA/H_2O (250 μL, 7:3). The mixture was stirred for 20 min and the product was purified by RP-HPLC affording the conjugate **14** as a white powder (7.3 mg, 1.55 μmol, 60%). RP-HPLC (C18, 214 nm, 5–100% B in 15 min) t_R = 7.7 min; ESI-MS calcd for $C_{179}H_{260}N_{58}O_{53}S_2$: 4133.9, found *m/z* 4134.7 $[M+H]^+$.

Synthesis of decapeptide scaffold 15: Peptides **11** (7.0 mg, 3.59 μmol) and **13** (13.7 mg, 17.4 μmol) were dissolved in TFA/H_2O (2 mL, 7:3). The mixture was stirred for 1 h and the product was purified by RP-HPLC affording the conjugate **15** as a white powder (13.5 mg, 2.84 μmol, 79%). RP-HPLC (C18, 214 nm, 5–60% B in 15 min) t_R = 10.4 min; ESI-MS calcd for $C_{183}H_{268}N_{58}O_{53}S_2$: 4189.9, found *m/z* 4191.0 $[M+H]^+$.

Synthesis of peptide 16: The linear peptide **16** was assembled on H-Lys(Boc)-2-chlorotrityl® resin (500 mg, loading of 0.8 mmol g)$^{-1}$ by using the general procedure. Peptide was released from the resin by using a solution of TFA/EDT/TIS/H_2O (90:5:2.5:2.5) for 3 h. The product was purified by RP-HPLC (5–100% B in 30 min) and obtained after lyophilization as a white powder (225 mg, 0.13 mmol, 43%). RP-HPLC (C18, 214 nm, 5–60% B in 15 min) t_R = 8.7 min; ESI-MS calcd for $C_{79}H_{149}N_{23}O_{18}S$: 1741.3, found *m/z* 871.6 $[M+2H]^{2+}$, 581.1 $[M+3H]^{3+}$, 436.2 $[M+4H]^{4+}$, 349.1 $[M+5H]^{5+}$.

Synthesis of peptide 17: The linear peptide **17** was assembled on Rink-amide resin (300 mg, loading of 0.7 mmol g)$^{-1}$ using the general procedure set. Peptide was released from the resin using a solution of TFA/EDT/TIS/H_2O (90:5:2.5:2.5) for 3 h. The product was purified by RP-HPLC (5–60% B in 30 min) and obtained after lyophilization as a white powder (159.5 mg, 60.0 μmol, 29%). RP-HPLC (C18, 214 nm, 5–60% B in 15 min) t_R = 10.7 min; ESI-MS calcd for $C_{79}H_{150}N_{24}O_{17}S$: 1739.1, found *m/z* 1739.9 $[M+H]^+$.

Synthesis of pentapeptide 18: Cyclo[-R(Pmc)-G-D(*t*Bu)-f-K-] was prepared as previously described.[23] This peptide (50 mg, 48.1 μmol) was dissolved in DMF (6 mL) and the pH was adjusted

to 9 with DIPEA. PyBOP (42.1 mg, 80 μmol) and Boc-Cys(NPys)-OH (30.4 mg, 80 μmol) were added, and the reaction mixture was stirred for 30 min at room temperature. The peptide **18** was obtained as a white powder (43.1 mg, 36.4 μmol, 76%). RP-HPLC (C18, 214 nm, 5–100% B in 15 min) $t_R = 9.5$ min; ESI-MS calcd for $C_{53}H_{74}N_{12}O_{13}S_3$: 1182.5, found *m/z* 1182.5 $[M+H]^+$.

Synthesis of conjugate 1: Compound **16** (3.3 mg, 1 μmol) was added to a solution of compound **14** (4.6 mg, 1 μmol) in DMF/phosphate buffer (3:1; 200 μL, 0.01 M, pH 4.8). The reaction mixture immediately became bright yellow. The mixture was stirred for 10 min at room temperature and immediately purified by RP-HPLC (20–60% B in 40 min). After lyophilisation, compound **1** was obtained as a white powder (6.4 mg, 0.9 μmol, 90%). RP-HPLC (C18, 214 nm, 5–60% B in 15 min) $t_R = 11.0$ min; ESI-MS calcd for $C_{253}H_{405}N_{79}O_{69}S_2$: 5718.0, found *m/z* 5719.8 $[M+H]^+$.

Synthesis of conjugate 2: Compound **16** (2.8 mg, 1.1×10^{-6} mol) was added to a solution of compound **15** (5.0 mg, 1.1×10^{-6} mol) in DMF/phosphate buffer (3:1; 200 μL, 0.01 M, pH 4.8). The reaction mixture immediately became bright yellow. The reaction was stirred for 15 min at room temperature and immediately purified by RP-HPLC (5–60% B in 30 min). After lyophilisation, compound **2** was obtained as a white powder (6.0 mg, 8.4×10^{-7} mol, 76%). RP-HPLC (C18, 214 nm, 5–100% B in 15 min) $t_R = 7.9$ min; ESI-MS calcd for $C_{257}H_{413}N_{79}O_{69}S_2$: 5774.1, found *m/z* 5776.7 $[M+H]^+$.

Synthesis of conjugate 3: Compound **14** (15 mg, 3.19 μmol) and peptide **17** (9.4 mg, 3.56 μmol) were dissolved in DMF/phosphate buffer (3:1; 700 μL, 0.01 M, pH 4.8) under argon. The reaction mixture was stirred for 5 min at room temperature under argon. The product was purified by RP-HPLC (5–60% B in 30 min), affording compound **3** as a white powder (20.3 mg, 2.82 μmol, 88%). RP-HPLC (C18, 214 nm, 5–100% B in 15 min) $t_R = 7.9$ min; ESI-MS calcd for $C_{253}H_{406}N_{80}O_{68}S_2$: 5717.0, found *m/z* 5718.1 $[M+H]^+$.

Synthesis of conjugate 4: Compound **15** (10.0 mg, 2.10 μmol) and peptide **17** (6.2 mg, 2.34 μmol) were dissolved in DMF/phosphate buffer (400 μL, 0.01 M, pH 4.8) (3:1) under argon. The reaction mixture was stirred for 5 min at room temperature under argon. The product was purified by RP-HPLC (5–60% B in 30 min), affording compound **4** as a white powder (12.3 mg, 1.70 μmol, 81%). RP-HPLC (C18, 214 nm, 5–100% B in 15 min) $t_R = 8.0$ min; ESI-MS calcd for $C_{257}H_{414}N_{80}O_{68}S_2$: 5773.1, found *m/z* 5775.8 $[M+H]^+$.

Synthesis of conjugate 5: Peptide **18** (41.8 mg, 35.4 μmol) was treated in TFA/CH_2Cl_2 (5 mL, 9:1) solution. The mixture was stirred for 50 min and the product was purified by RP-HPLC, affording Cyclo[-Arg-Gly-Asp-D-Phe-Lys[Cys(NPys)]-] as a white powder (16 mg, 14.7 μmol, 42%). RP-HPLC (C18, 214 nm, 5–100% B in 15 min) $t_R = 7.3$ min; ESI-MS calcd for $C_{35}H_{48}N_{12}O_{10}S_2$: 860.3, found *m/z* 860.4 $[M+H]^+$.

Cyclo[-Arg-Gly-Asp-D-Phe-Lys[Cys(NPys)]-] (2.3 mg, 1.9 μmol) and peptide **16** (5 mg, 2 μmol) were dissolved in DMF/phosphate buffer (400 μL, 0.01 M, pH 4.8) under argon. The reaction mixture was stirred for 5 min at room temperature under argon. The product was purified by RP-HPLC (5–60% B in 30 min), affording compound **5** as a white powder (3.7 mg, 1.1 μmol, 56%). RP-HPLC (C18, 214 nm, 5–60% B in 15 min) $t_R = 10.5$ min; ESI-MS calcd for $C_{109}H_{193}N_{33}O_{26}S_2$: 2444.4, found *m/z* 2444.2 $[M+H]^+$.

Synthesis of conjugate 6: Cyclo[-Arg-Gly-Asp-D-Phe-Lys[Cys(NPys)]-] (5 mg, 4.59 μmol) and peptide **17** (12.3 mg, 4.64 μmol) were dissolved in DMF/phosphate buffer (600 μL, 0.01 M, pH 4.8) under argon. The reaction mixture was stirred for 5 min at room temperature under argon. The product was purified by RP-HPLC (5–60% B in 30 min), affording compound **6** as a white powder (12.3 mg, 3.43 μmol, 75%). RP-HPLC (C18, 214 nm, 5–60% B in 15 min) $t_R = 10.4$ min; ESI-MS calcd for $C_{109}H_{194}N_{34}O_{25}S_2$: 2443.4, found *m/z* 2443.0 $[M+H]^+$.

Synthesis of RGD-containing decapeptides 7 and 8: RGD-containing decapeptides **7** and **8** were prepared as previously described.[13]

Synthesis of RGD-containing pentapeptide 19: cyclo[-Arg-Gly-Asp-D-Phe-Lys-] **19** was prepared as previously described.[23]

Evaluation of TSA colony survival: One day before treatment, 200 Ts/Apc cells were seeded in Petri dishes with RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), penicillin (50 U mL^{-1}), streptomycin (50 mg mL^{-1}) and β-mercaptoethanol (2.5×10^{-1} M) and incubated at 37 °C in a 5% CO_2 atmosphere. The culture medium was removed and replaced for 1 h with appropriate medium without β-mercaptoethanol, antibiotics and FBS. Cells were treated with different concentrations of $(KLAKLAK)_2$ conjugates and control peptides. After one week incubation, cell colonies were washed twice with PBS and cells were fixed with ethanol for 15 min. Cell colonies were then dried (15 min at 37 °C) and stained with 1% methylene blue in phosphate buffer (10 mM pH 8.5) for 15 min. After water washing, Petri dishes were dried and cell colonies were counted.

Apoptosis assays: Twenty-four hours before transfection, 10^4 Ts/Apc cells per well were seeded in 96-well assay plates with RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 U mL^{-1}), streptomycin (50 mg mL^{-1}) and β-mercaptoethanol (0.25 M) and incubated at 37 °C under 5% CO_2 atmosphere. Medium was removed and replaced for 1 h in appropriate medium without β-mercaptoethanol, antibiotics and FBS. Cells were treated with different concentrations of $(KLAKLAK)_2$ conjugates and control peptides. After 6 or 12 h of incubation, cells were washed twice with PBS and cells were fixed with 0.5% paraformaldehyde at room temperature for 5–10 min. Cells were then stained for 5 min with Hoechst 33342 (5 μM). Cell nuclei were observed by microscopy.

Abbreviations

Alloc: allyloxycarbonyl, Boc: *tert*-butoxycabonyl, Cy5: cyanine 5, DIPEA: diisopropyethylamine, DMF: dimethylformamide, EDT: ethanedithiol, Fmoc: 9-fluorenylmetoxycarbonyl, NPys: 3-nitro-2-pyridinesulfenyl, PMC: 2,2,5,7,8-pentamethylchroman-6-sulfonyl, PBS: phosphate buffered saline , PyBOP: 1*H*-benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate, RP-HPLC: reversed-phase, high-performance liquid chromatography, SPPS: solid-phase peptide synthesis, TFA: trifluoroacetic acid, TIS: triisopropylsilane, TNBS: 2,4,6-trinitrobenzenesulfonic acid

Acknowledgements

This work was supported by the Association pour la Recherche contre le Cancer (ARC N° 3741), the Région Rhône-Alpes (N° 0301372501 and 0301372502), the Cancéropôle (N° 032115 and 042259), the Université Joseph Fourier, the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM) and NanoBio (Grenoble).

Keywords: chemoselective ligation · drug conjugates · drug delivery · integrins · ligand-based targeting · RGD peptides

[1] J.-N. Rybak, E. Trachsel, J. Sceuermann, D. Neri, *ChemMedChem* **2007**, *2*, 22–40.
[2] D. Hanahan, R. A. Weinberg, *Cell* **2000**, *100*, 57–70.
[3] M. Aumailley, M. Gurrath, G. Müller, J. Calvete, R. Timpl, H. Kessler, *FEBS Lett.* **1991**, *291*, 50–54.
[4] M. Gurrath, G. Müller, H. Kessler, M. Aumailley, R. Timpl, *Eur. J. Biochem.* **1992**, *210*, 911–921.
[5] M. A. Dechantsreiter, E. Planker, B. Matha, E. Lohof, G. Holzemann, A. Jonczyk, S. L. Goodman, H. Kessler, *J. Med. Chem.* **1999**, *42*, 3033–3040.
[6] R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 7461–7472.
[7] R. J. Kok, A. J. Schraa, E. J. Bos, H. E. Moorlag, S. A. Asgeirsdottir, M. Everts, D. K. F. Meijer, G. Molema, *Bioconjugate Chem.* **2002**, *13*, 128–135.
[8] R. Haubner, H. J. Wester, W. A. Weber, C. Mang, S. I. Ziegler, S. L. Goodman, R. Senekowitsch-Schmidtke, H. Kessler, M. Schwaiger, *Cancer Res.* **2001**, *61*,1781–1785.
[9] a) X. Montet, M. Funovics, K. Montet-Abou, R. Weissleder, L. Josephson, *J. Med. Chem.* **2006**, *49*, 6087–6093; b) P. Chenevier, L. Bourel-Bonnet, D. Roux, *J. Am. Chem. Soc.* **2003**, *125*, 16261–16270; c) G. Thumshirn, U. Hersel, S. L. Goodman, H. Kessler, *Chem. Eur. J.* **2003**, *9*, 2717–2725; d) R. J. Kok, A. J. Schraa, E. J. Bos, H. E. Moorlag, S. A. Asgeirsdottir, M. Everts, D. K. F. Meijer, G. Molema, *Bioconjugate Chem.* **2002**, *13*, 128–135.
[10] D. Boturyn, J.-L. Coll, E. Garanger, M.-C. Favrot, P. Dumy, *J. Am. Chem. Soc.* **2004**, *126*, 5730–5739.
[11] E. Garanger, D. Boturyn, Z. Jin, P. Dumy, M.-C. Favrot, J.-L. Coll, *Mol. Ther.* **2005**, *12*, 1168–1175.
[12] D. Boturyn, E. Defrancq, G. T. Dolphin, J. Garcia, P. Labbe, O. Renaudet, P. Dumy, *J. Pept. Sci. J. Pep. Sc.* **2008**, *14*, 224–240.
[13] E. Garanger, D. Boturyn, J.-L. Coll, M.-C. Favrot, P. Dumy, *Org. Biomol. Chem.* **2006**, *4*, 1958–1965.
[14] a) J. Razkin, V. Josserand, D. Boturyn, Z.-H. Jin, P. Dumy, M. Favrot, J.-L. Coll, I. Texier, *ChemMedChem* **2006**, *1*, 1069–1072; b) Z. Jin, V. Josserand, J. Razkin, E. Garanger, D. Boturyn, M.-C. Favrot, P. Dumy, J.-L. Coll, *Mol. Imaging* **2006**, *5*, 188–197; c) Z. Jin, J. Razkin, V. Josserand, D. Boturyn, A. Grichine, I. Texier, M.-C. Favrot, P. Dumy, J.-L. Coll, *Mol. Imaging* **2007**, *6*, 43–55.
[15] M. M. Javadpour, M. M. Juban, W.-C. J. Lo, S. M. Bishop, J. Brannon Alberty, S. M. Cowell, C. L. Becker, M. L. McLaughlin, *J. Med. Chem.* **1996**, *39*, 3107–3113.
[16] H. M. Ellerby, W. Arap, L. M. Ellerby, R. Kain , R. Andrusiak, G. Del Rio, S. Krajewski, C. R. Lombardo, R. Rao, E. Ruoslahti, D. E. Bredesen, R. Pasqualini, *Nat. Med.* **1999**, *5*, 1032–1038.
[17] P. C. Brooks, A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Klier, D. A. Cheresh, *Cell* **1994**, *79*, 1157–1164.
[18] a) L. E. Canne, P. Botti, R. J. Simon, Y. Chen, E. A. Dennis, S. B. H. Kent, *J. Am. Chem. Soc.* **1999**, *121*, 8720–8727; b) Z. Miao, J. P. Tam, *J. Am. Chem. Soc.* **2000**, *122*, 4253–4260; c) L. Zhang, T. R. Torgerson, X.-Y. Liu, S. Timmons, A. D. Colosia, J. Hawiger, J. P. Tam, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 9184–9189; d) K. Rose, *J. Am. Chem. Soc.* **1994**, *116*, 30–33; e) D. Forget, D. Boturyn, E. Defrancq, J. Lhomme, P. Dumy, *Chem. Eur. J.* **2001**, *7*, 3976–3984.
[19] S. Grigalevicius, S. Chierici, O. Renaudet, R. Lo-Man, E. Deriaud, C. Leclerc, P. Dumy, *Bioconjugate Chem.* **2005**, *16*, 1149–1159.
[20] a) B. Arunachalam, U. Phan, H. J. Geuze, P. Cresswell, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 745–750; b) T. Maeda, K. Fujimoto, *Colloids Surf. B* **2006**, *49*, 15–21.
[21] a) E. P. Feener, W. C. Shen, H. J. Ryser, *J. Biol. Chem.* **1990**, *265*, 18780–18785; b) M. D. Henry, S. Wen, M. D. Silva, S. Chandra, M. Milton, P. J. Worland, *Cancer Res.* **2004**, *64*, 7995–8001; c) C. D. Austin, X. Wen, L. Gazzard, C. Nelson, R. Scheller, S. J. Scales, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 17987–17992.
[22] E. Garanger, D. Boturyn, O. Renaudet, E. Defrancq, P. Dumy, *J. Org. Chem.* **2006**, *71*, 2402–2410.
[23] D. Boturyn, P. Dumy, *Tetrahedron Lett.* **2001**, *42*, 2787–2790.
[24] S. Foillard, M. Ohsten Rasmussen, J. Razkin, D. Boturyn, P. Dumy, *J. Org. Chem.* **2008**, *73*, 983–991.
[25] N. Thieriet, J. Alsina, E. Giralt, F. Guibé, F. Albericio, *Tetrahedron Lett.* **1997**, *38*, 7275–7278.
[26] W. P. Jencks, *J. Am. Chem. Soc.* **1959**, *81*, 475–481.
[27] S. Houdier, M. Legrand, D. Boturyn, S. Croze, E. Defrancq, J. Lhomme, *Anal. Chim. Acta* **1999**, *382*, 253–263.
[28] F. Albericio, D. Andreu, E. Giralt, C. Navalpotro, E. Pedroso, B. Ponsati, M. Ruiz-Gayo, *Int. J. Pept. Protein Res.* **1989**, *34*, 124–128.
[29] T. Kimura, R. Matsueda, Y. Nakagawa, E. T. Kaisere, *Anal. Biochem.* **1982**, *122*, 274–282.
[30] C. Borghouts, C. Kunz, B. Groner, *J. Pept. Sci.* **2005**, *11*, 713–726.
[31] E. Garanger, D. Boturyn, P. Dumy, *Anticancer Agents Med. Chem.* **2007**, *27*, 552–558.
[32] T. R. Daniels, T. Delgado, G. Helguera, M. L. Penichet, *Clin. Immunol.* **2006**, *121*, 159–176.
[33] P. Monaci, F. Bartoli, G. D. Zenzo, M. Nuzzo, L. Urbanelli, *Tumor Targeting* **1999**, *4*, 129–124.

Received: May 13, 2008
Published online on August 19, 2008

