PAPER

Synthesis and biological evaluation of dual action *cyclo*-RGD/SMAC mimetic conjugates targeting αvβ3/αvβ5 integrins and IAP proteins†

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The rational design, synthesis and *in vitro* biological evaluation of dual action conjugates **11–13**, containing a tumour targeting, integrin αvβ3/αvβ5 ligand portion and a pro-apoptotic SMAC mimetic portion (*cyclo*-RGD/SMAC mimetic conjugates) are reported. The binding strength of the two separate units is generally maintained by these dual action conjugates. In particular, the connection between the separate units (anchor points on each unit; nature, length and stability of the linker) influences the activity of each portion against its molecular targets (integrins αvβ3/αvβ5 for *cyclo*-RGD, IAP proteins for SMAC mimetics). Each conjugate portion tolerates different substitutions while preserving the binding affinity for each target.

Introduction

In the pharmaceutical industry, inhibition of a molecular target sometimes leads to a bypass of the target itself by the diseased cells or organs (*e.g.* penicillins and resistance to beta-lactamases[1]). Thus, there is an increasing need for multi-targeted therapies.[2]

Multi-targeting may be achieved by administering a cocktail of active ingredients, and cocktails active against HIV are an example of clinical success.[3] However, effective drug combinations may require different administration routes, or different residence times in the human body.[4] Well tolerated drugs may become harmful in combination with other active principles, due to drug–drug interactions.[5]

A dual action compound contains the chemical elements required to interact with two molecular targets.[6] A connection is chosen for each pharmacophore unit, and a suitable spacer separates the two units without disturbing their biological activities.

Cancer implies a huge variety of pathologically altered mechanisms in the body, and cancer research works on hundreds of putative molecular targets.[7] Cancer cells either mutate the drug target or transport the drug outside the cancer cell (imatinib[8] and taxol[9] are known examples of resistance to marketed drugs). Cancer cells are less likely to develop resistance against a dual action compound simultaneously directed against two essential molecular targets. We chose integrins/angiogenesis[10] and inhibition of apoptosis proteins (IAPs)/apoptosis[11] because our research group has accumulated significant experience on both target classes in the past.

Project rationale

Integrins αvβ3, αvβ5 and α5β1 play a key role in angiogenesis and tumour development, and are accessible as cell surface receptors interacting with extracellular ligands.[12] They are involved in angiogenesis, tumour progression and metastasis.[13] The tripeptide sequence Arg-Gly-Asp (RGD) is the common motif used by endogenous ligands to recognize and bind such integrins.

Cyclo[Arg-Gly-Asp-Phe-*N*(Me)-Val] (cilengitide)[14] (**1**, Fig. 1) reached phase III clinical trials against *glioblastoma multiforme*. We reported cyclic RGD-based peptidomimetics, either built on 4-substituted aza-bicyclo[4.3.0]nonanes (ABN) (**2**, Fig. 1),[15,16] or built on bifunctional diketopiperazines (DKP) (**3**, Fig. 1),[17] as potent inhibitors of the purified αvβ3 receptor.

Apoptosis is started by stimuli received from within the cell, or from the external environment.[18] Degradation of cellular protein components is carried out by initiator and execu-

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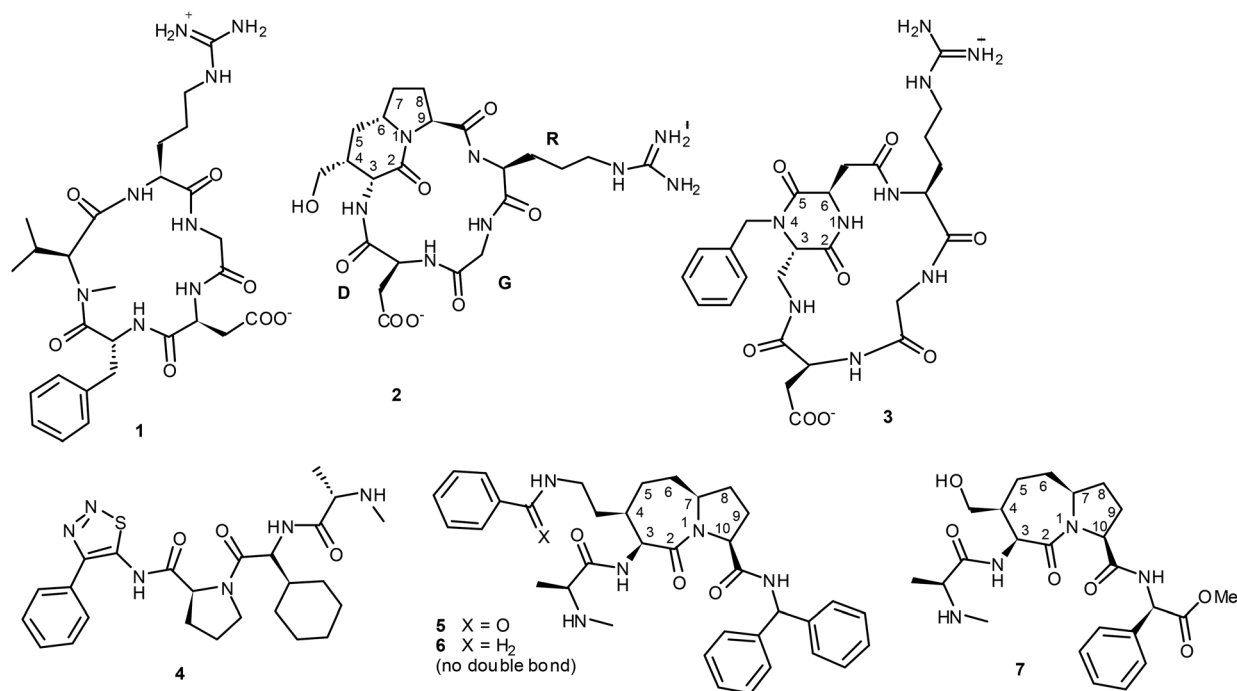This journal is © The Royal Society of Chemistry 2014

Fig. 1 Cyclic RGD ligands of $\alpha_V\beta_3$ integrin {Cilengitide (**1**, 15-membered), *cyclo*[ABN-RGD] (**2**, 15-membered) and *cyclo*[DKP-RGD] (**3**, 17-membered)} and pro-apoptotic SMAC mimetics **4** (GDC-0152), **5–7**.

tioner Cysteine ASPartic acid-specific proteASES (caspases).[19] IAPs[20] bind through their baculovirus inhibitor repeat (BIR) domains to the initiator CASP-9 (BIR3 domain, primary binding site) and to the executioner CASP-3 and CASP-7 (linker-BIR2 domain, secondary site), block caspases in their inactive forms and antagonize apoptosis.[21] Endogenous SMAC protein[22,23] (Second Mitochondria-derived Activator of Caspases) binds to the BIR3/linker-BIR2 domains of IAPs[24] and prevents CASP-3/-7/-9 inactivation, restoring caspase-dependent apoptosis.

Structural studies[25,26] showed that the AVPI N-terminal sequence of SMAC binds to IAPs with nanomolar affinity. Pro-apoptotic AVPI mimetics such as **4** (Fig. 1) are undergoing clinical evaluation as anticancer agents.[27] We introduced 4-substituted aza-bicyclo[5.3.0]decane (ABD) derivatives (*e.g.*, **5–7**, Fig. 1), endowed with good cell-free potency against IAPs and moderate cytotoxicity.[28,29]

Since α_V integrins are overexpressed on the surface of cancer cells, their ligands can be used as tumour-homing/anti-angiogenic peptidomimetics for site-directed delivery of cytotoxic drugs.[30] For example, the cyclic RGD ligand–doxorubicin conjugate **8**[31] (Fig. 2) is highly cytotoxic against cancer cells overexpressing integrins $\alpha_V\beta_3/\alpha_V\beta_5$ on their membrane. We reported good stability and *in vivo* efficacy in a mouse model of human ovarian cancer for the *cyclo*[ABN-RGD]paclitaxel conjugate **9**[32] and the *cyclo*[DKP-RGD]paclitaxel conjugate **10**[33] (Fig. 2).

We reasoned that, by connecting the AVPI/SMAC mimetic unit (**5–7**) with a cyclic RGD ligand such as **2** or **3**, dual action

conjugates targeting both angiogenesis and apoptosis could be created.

Herein we present the synthesis and biological evaluation of a small series of cyclic RGD ligand–ABD SMAC mimetic dual action conjugates (Fig. 3). We explored (i) the influence of 4- (**11**, **12**) and C-terminus (**13**) connections on the ABD SMAC mimetic unit, (ii) the influence of the ring size of the cyclic RGD ligand unit (15-membered compounds **11**, **12**, 17-membered compound **13**), and (iii) the presence of an ester- (**11**) or of an amide-containing (**12**, **13**) linker connecting the cyclic RGD and ABD SMAC portions.

Results and discussion

Chemistry

Synthetic targets – rationale. Compounds **11** and **12** (Fig. 3) are dual action conjugates where the *cyclo*[DKP-RGD] integrin ligand unit **3** is coupled with the ABD SMAC mimetic unit **6**. The former unit bears a *p*-aminomethyl group on the DKP-nitrogen benzyl substituent, while the latter unit is functionalised either with a *p*-hydroxymethyl (**11**, X = O) or with a *p*-aminomethyl group (**12**, X = NH) on the 4-benzylaminoethyl substituent. Ideally, both substitutions on the *cyclo*[DKP-RGD] ligand **3**[33] and on the ABD SMAC mimetic **6**[29] should not affect their binding affinity to integrin $\alpha_V\beta_3$ and to IAPs, respectively. 1,4-Butanedioic acid is used as a relatively short linker, because the distance between the DKP and the ABD scaffolds (21 atoms, including two phenyl groups) is largely determined
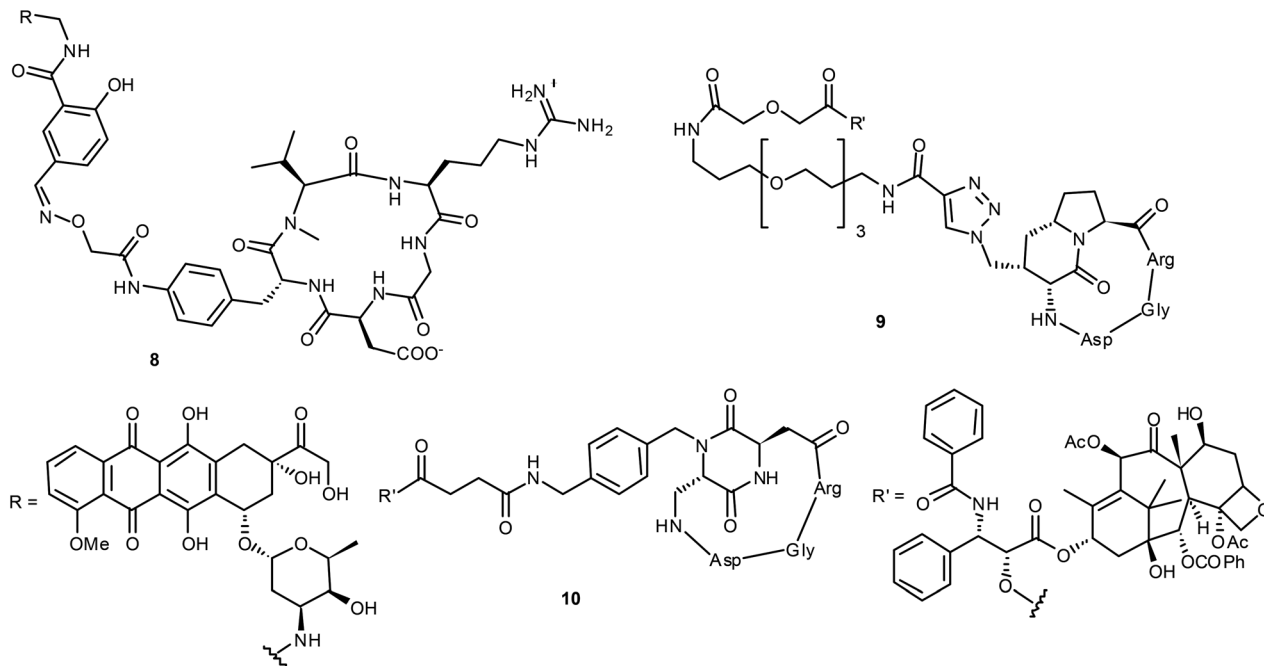
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Fig. 2 Cyclic RGD ligand–doxorubicin conjugate **8**, *cyclo*[ABN-RGD]paclitaxel **9** and *cyclo*[DKP-RGD]paclitaxel **10**.
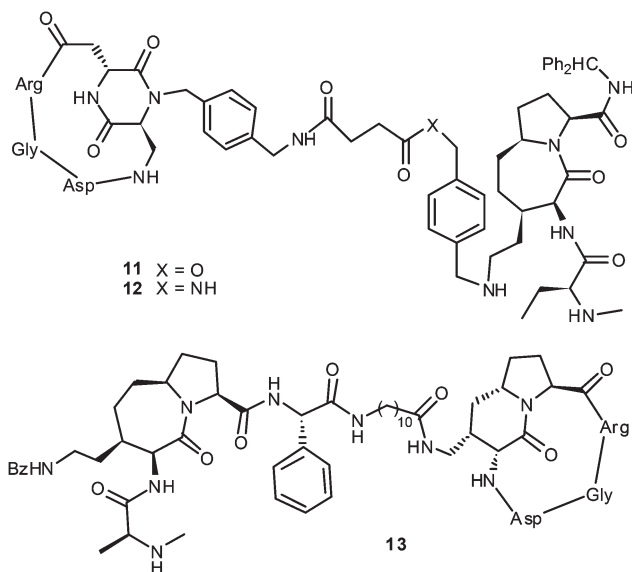

Fig. 3 Heterodimeric cyclic RGD ligand-ABD SMAC mimetic dual action conjugates: 4-connected *cyclo*[DKP-RGD]-ABD compounds **11–12** and C-terminus-connected *cyclo*[ABN-RGD]-ABD compound **13**.

by the DKP-nitrogen *p*-aminomethylbenzyl and the ABD 4-(*p*-X-methylbenzylaminoethyl) substitutions.

Compound **13** (Fig. 3) was made by coupling a *cyclo* [ABN-RGD] integrin ligand unit **2**, where the original 4-hydroxymethyl substitution was replaced by 4-aminomethyl group, with the ABD SMAC mimetic **5**, where the pro-(*S*) phenyl of the original C-terminal diphenylmethylamide was replaced by a carboxylic group. Once again, ideally both substitutions should be well tolerated in terms of binding affinities to the

respective molecular targets.[16,34] 11-Aminoundecanoic acid was used as a relatively long linker, to keep a suitable distance (18 atoms) between the ABN and ABD scaffolds.

***Cyclo*[DKP-RGD]-ABD dual action compounds 11 and 12.** Our synthetic strategy to the ester-connected conjugate **11** started from previously reported *N*-Boc protected amine **16** (Scheme 1).[28] Its C-terminal CONH–CHPh₂ amide contributes to BIR/IAP binding,[28,35] while its 4-aminoethyl function is used as a connection for the linker, which was built as shown in Scheme 1.

Namely, amine **16**[28] was submitted to a reductive alkylation (steps b, c, Scheme 1) with 4-hydroxymethyl benzaldehyde **15** (obtained by selective reduction with sodium borohydride of terephthalaldehyde **14**, step a). The resulting aminoalcohol **17** was chemoselectively *N*-Boc protected (step d), and finally bis-*N*-Boc protected benzyl alcohol **18** was acylated with succinic anhydride (step e, Scheme 1) to yield the ester-connected construct **19**. The overall yield for the reaction steps from **16** to **19** was a rather good 62%. A single direct phase chromatographic column (step c) was needed to purify the reaction products.

Construct **19** was then coupled to the previously reported *cyclo*[DKP-RGD] derivative **20**[33] (steps a, b, Scheme 1), to give the bis-*N*-Boc protected, ester-connected conjugate **21**. The multi-functional nature of both coupling partners and the presence of potentially reactive functions (*e.g.*, the guanidine and carboxylate functions in **20**) required the initial activation of the carboxylic group of **19** (step f), followed by its coupling with the amino group of **20** (step g) under carefully controlled conditions. Namely, coupling at pH 7.5 largely prevented side reactions involving the unprotected functionalities of **20**, and provided an acceptable 40% overall yield. Finally, acidic hydrolysis of the *N*-Boc groups led to quantitative deprotection and
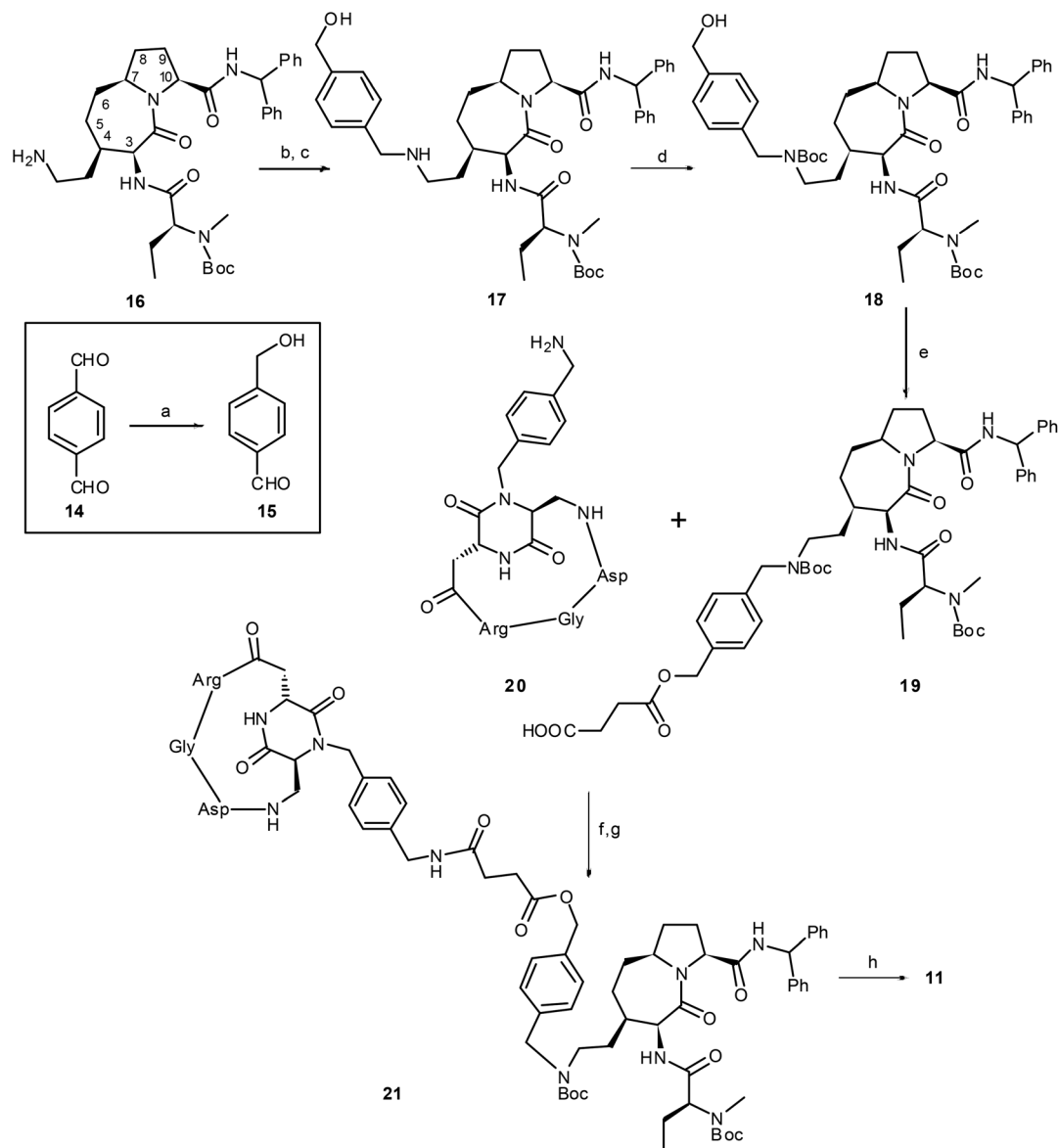
Scheme 1 Synthesis of *cyclo*[DKP-RGD]-ABD SMAC mimetic, ester-connected dual action compound **11**. Reagents and conditions: (a) NaBH$_4$, EtOH−THF−H$_2$O, −5° to 0 °C, 6 h, 83%; (b) **15**, DIPEA, dry MeOH, rt, 5 h; (c) NaBH$_4$, rt, 30 min, 80% (two steps); (d) Boc$_2$O, DIPEA, dry CH$_2$Cl$_2$, 0 °C to rt, 24 h, 87%; (e) succinic anhydride, DIPEA, DMAP, dry CH$_2$Cl$_2$, 0 °C to rt, 2 h, 90%; (f) **19**, *N*-hydroxysulfosuccinimide, DIC, dry DMF, rt, 18 h; (g) **20**, CH$_3$CN, phosphate buffer, pH 7.5, 0 °C, 18 h, 40% over two steps; (h) TFA, CH$_2$Cl$_2$, rt, 50 min, 99%.
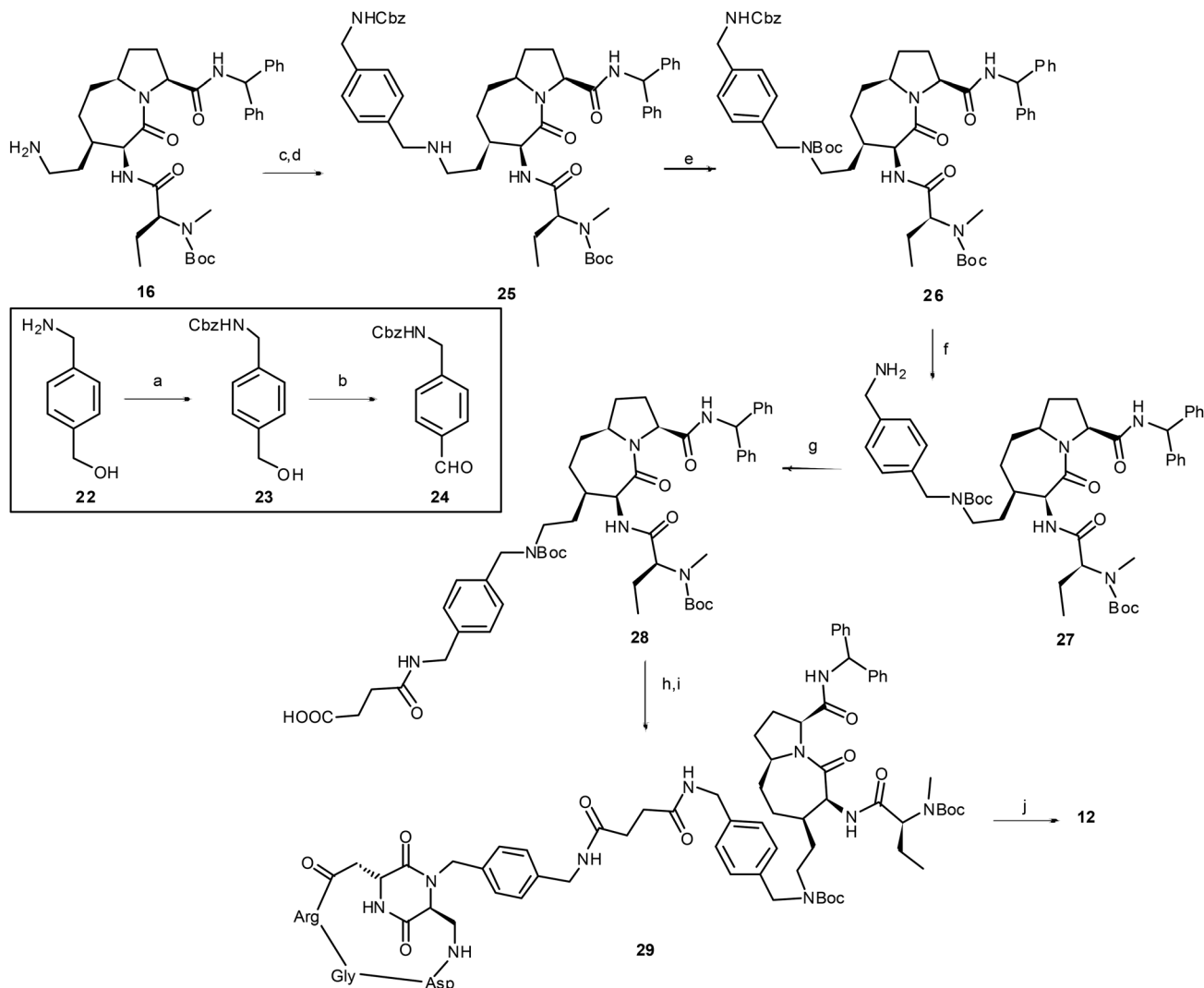
isolation of target *cyclo*[DKP-RGD]-ABD, ester-connected dual action conjugate **11** (Scheme 1). HPLC purification was required to obtain pure compounds **21** and **11**.

Our synthetic strategy to the amide-connected conjugate **12**, and in particular to the key bis-*N*-Boc protected carboxylate **28**, is shown in Scheme 2. Amine **16**[28] was submitted to a reductive alkylation (steps c, d, Scheme 2) with (*p*-aminomethyl) benzaldehyde **24**, obtained in turn by chemoselective *N*-Cbz protection of (*p*-aminomethyl)phenyl methanol **22** (step a), followed by partial oxidation with manganese dioxide of (*p*-Cbz-aminomethyl)phenyl methanol **23** (step b). The resulting *N*-Cbz, *N*-Boc protected diamine **25** was *N*-Boc protected on its free secondary amine (step e), its primary amine was depro-

tected by hydrogenolysis (step f), and finally bis-*N*-Boc protected diamine **27** was acylated with succinic anhydride (step g, Scheme 2) to yield the amide-connected construct **28**. The overall yield for the reaction steps from **16** to **28** was an excellent 82%. A direct phase chromatographic column (step d) and a reverse phase HPLC purification (step g) were required to obtain pure **28**.

Construct **28** was then coupled to the previously reported *cyclo*[DKP-RGD] derivative **20**[33] (steps h, i, Scheme 2), to give the bis-*N*-Boc protected, amide-connected conjugate **29**. Final acidic *N*-Boc deprotection (step j, Scheme 2) led to target *cyclo* [DKP-RGD]-ABD, amide-connected dual action conjugate **12** in a good 49% overall yield.

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Scheme 2 Synthesis of *cyclo*[DKP-RGD]–ABD SMAC mimetic, amide-connected dual action compound **12**. Reagents and conditions: (a) DIPEA, Cbz-Cl, dry THF, 0 °C to rt, 18 h, 71%; (b) MnO₂, dry THF, rt, 18 h, 97%; (c) **24**, DIPEA, dry MeOH, rt, 5 h; (d) NaBH₄, rt, 30 min, 83% over two steps; (e) Boc₂O, DIPEA, dry CH₂Cl₂, 0 °C to rt, 24 h, 100%; (f) H₂, Pd/C 10%, THF–H₂O, rt, 18 h, 100%; (g) succinic anhydride, DIPEA, DMAP, dry CH₂Cl₂, 0 °C to rt, 2 h, 99%; (h) *N*-hydroxysulfosuccinimide, DIC, dry DMF, rt, 18 h; (i) **20**, CH₃CN, phosphate buffer, pH 7.5, 0 °C, 18 h, 49% over two steps; (j) TFA, CH₂Cl₂, rt, 50 min, 100%.
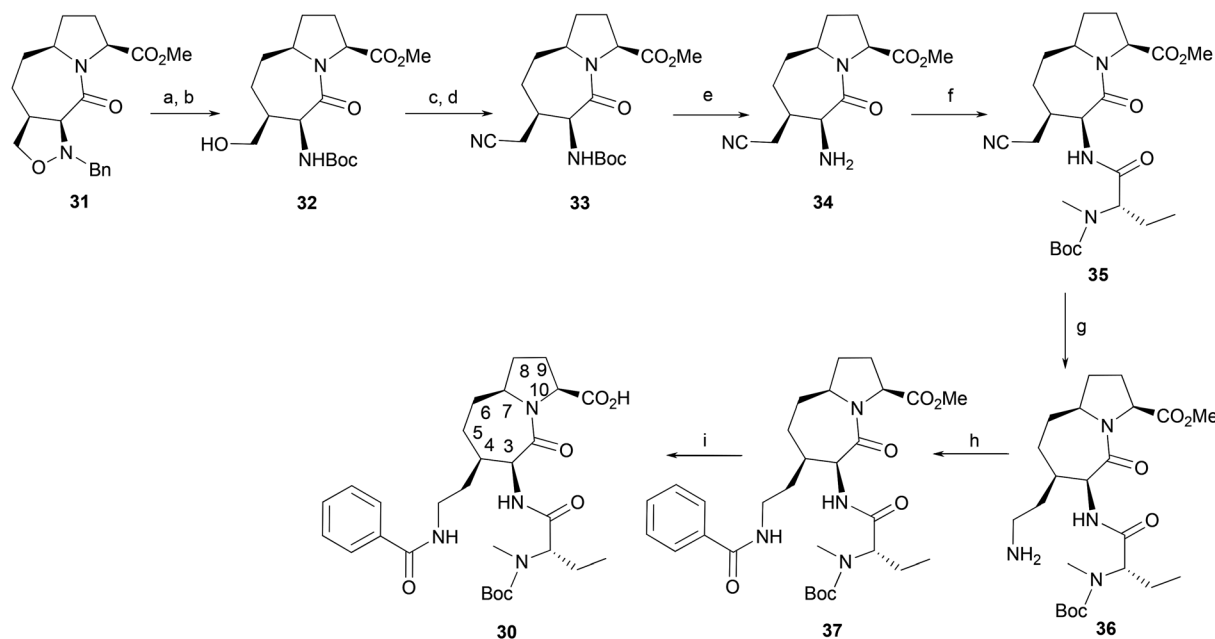
The experimental conditions and purification protocols for each reaction step in Scheme 4 leading to conjugate **12** are identical to the corresponding steps described in Scheme 1. A comparison between the yields of corresponding steps (steps b–e, Scheme 1, and steps c–g, Scheme 2; steps a–c, Scheme 1 and 2) shows better results for amide-connected conjugate **12**. It is reasonable to hypothesize a lower stability of the ester bond in ester-connected conjugate **11**, and in intermediates **19** and **21**, when compared with the amide bond in amide-connected conjugate **12**, and in intermediates **28** and **29**. This may lead to lower overall yields either under the reaction conditions, or during the work up-purification protocols.

***trans*-Cyclo[ABN-RGD]-ABD dual action compound 13.** Our synthetic strategy to the amide-connected conjugate **13** required the synthesis of *N*-Boc protected carboxylic acid **30**

(Scheme 3). Its 4-(2-benzoylaminoethyl) substituent contributes to BIR/IAP binding.[28] The C-terminal carboxylic acid is the anchoring point for the linker.

The synthesis of *N*-Boc protected carboxylic acid **30** started from the already reported tricyclic ester **31**.[28] Namely, simultaneous hydrogenolytic isoxazolidine opening and benzyl deprotection (step a, Scheme 3) and chemoselective *N*-Boc protection (step b) provided N-protected alcohol **32**. Mesylation (step c) and microwave-assisted nucleophilic substitution (step d) led to N-protected aminonitrile **33**. Acidic deprotection (step e, aminonitrile **34**) was followed by amidation of the 3-amino group with N-protected/methylated (*S*)-aminobutyric acid (step f), yielding nitrile **35**. Nitrile reduction was carried out using the H-Cube™ continuous flow hydrogenation apparatus (step g), and the amino function of compound **36** was

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Scheme 3 Synthesis of the *N*-Boc protected carboxylic acid intermediate **30**. Reagents and conditions: (a) HCOONH$_4$, Pd(OH)$_2$/C, THF–H$_2$O 4/1, 2% AcOH, 70 °C, 6 h, then rt, 18 h; (b) Boc$_2$O, TEA, dry CH$_2$Cl$_2$, rt, 20 h, 59% over two steps; (c) MsCl, TEA, dry CH$_2$Cl$_2$, 0 °C to rt, 2 h; (d) *n*BuN$_4$CN, dry DMF, MicroWave, 80 °C, 2 h, 84% over two steps; (e) AcCl, dry MeOH, rt, 5 min, 100%; (f) *N*-Boc, *N*-Me-(*S*)-2-aminobutyric acid (Abu), EDC·HCl, HOBt, DIPEA, dry CH$_2$Cl$_2$, rt, 22 h, 82%; (g) H$_2$, 60 bar, Ni-Ra cartridge, citric acid, EtOH–H$_2$O, 60 °C, 5 h, continuous flow, 0.5 mL min^{-1}, 24 h, 98%; (h) BzCl, TEA, dry CH$_2$Cl$_2$, rt, 5 h, 92%; (i) LiOH, THF–H$_2$O, 0 °C to rt, 5 h, 88%.

benzoylated (step h). Finally, methyl ester **37** was hydrolysed under basic conditions (step i, Scheme 3) to provide *N*-Boc protected carboxylic acid **30**. The overall yield for the nine reaction steps from **31** to **30** was an acceptable 32% (average reaction yield = 89%). Four direct phase chromatographic separations (steps b, d, f, h) were needed to purify the reaction products.

The synthetic strategy designed to prepare the amide-connected linker construct **38** is reported in Scheme 4. 11-Aminoundecanoic acid **39** was esterified (step a, Scheme 4) and the resulting aminoester **40** was coupled with *N*-Boc-(*S*)-phenylglycine using classical peptide coupling conditions (step b). Acidic deprotection (step c) of *N*-Boc protected **41** led to the aminoester linker **42** as a hydrochloride salt. The linker was then coupled to *N*-Boc protected carboxylic acid **30** (step d) to provide ester **43**. Hydrolysis of the methyl ester (step e) yielded the expected amide-connected construct **38**. The ester **43** was also N-deprotected (step f, Scheme 4) to provide the standard compound **44** for biological purposes (*vide infra*, Table 1). The overall yield of the reaction steps involving ABD SMAC mimetic intermediates (steps d, e; Scheme 4) was a moderate 37%. Two direct phase chromatographic separations (steps b, d) were required to purify the reaction products. The carboxylic acid construct **38** was then coupled to previously reported *cyclo* [ABN-RGD]methylamine **45**[16] (step g, Scheme 4), to give the *N*-Boc protected, amide-connected conjugate **46**. Final acidic *N*-Boc deprotection (step h, Scheme 4) led to target *cyclo*-[ABN-RGD]-ABD, amide-connected dual action conjugate **13** in a low, unoptimized two-step 17% yield.

Biology

Compound profiling – rationale. The cell-free potency of the dual action conjugates **11–13** on the BIR domains of IAPs (SMAC mimetic unit), and on integrins α$_v$β$_3$ and α$_v$β$_5$ (cyclic RGD ligand unit) was investigated to confirm the affinity for their molecular targets. Then, their cytotoxicity on tumour cell lines characterized by various levels of cytoplasmic IAPs and membrane integrins was investigated.

Cell-free assays – BIR domains/IAPs. Three BIR domain-based IAP portions were used to test the affinity of the dual action conjugates **11–13**. The primary BIR3 binding site/domain from XIAP and cIAP2 was selected to measure the affinity for two of the most therapeutically relevant IAPs.[28,29,36] The bi-functional linker-BIR2-BIR3 portion from XIAP was selected to determine any SMAC dimer-like behaviour of the dual action conjugates **11–13**.[37,38] The standard monomeric SMAC mimetic amides **5** and **6** (Fig. 1), and esters **7** (Fig. 1) and **44** (Scheme 4) were tested for comparison. Compound **5** bears a **11**, **12**-like 4-benzylaminoethyl substituent, compound **6** bears a **13**-like 4-benzoylaminoethyl substituent, compound **7** bears a C-terminus phenylglycinamide ester, while compound **44** bears a C-terminus phenylglycinamido-linker ester substituent. Their IC$_{50}$ values are reported in Table 1. The results clearly show that the attachment of a linker and a cyclic RGD ligand onto the SMAC mimetic unit does not significantly affect its affinity for IAPs. Indeed, 4-connected dual action conjugates **11** and **12** and reference compounds **5** and **6** show comparable affinity for mono- and bi-functional domains from
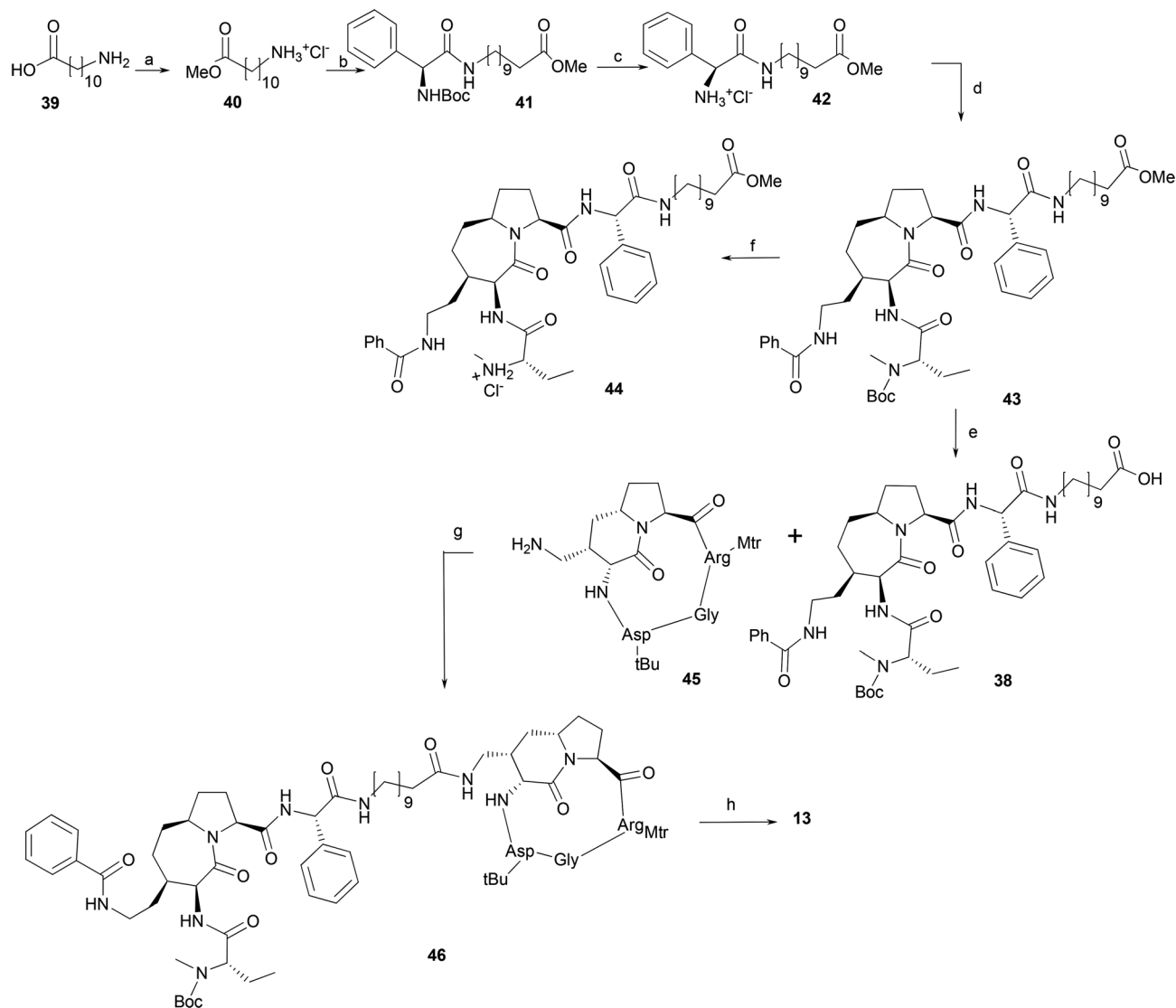
Scheme 4 Synthesis of *cyclo*[ABN-RGD]-ABD SMAC mimetic, amide-connected dual action compound **13**. Reagents and conditions: (a) SOCl₂, MeOH, rt to 80 °C, 4 h, 98%; (b) *N*-Boc-(*S*)-PheGly, EDC·HCl, HOBt, DIPEA, dry CH₂Cl₂, rt, 18 h, 72%; (c) 3 N HCl/dry MeOH, rt, 18 h, 97%; (d) **30**, EDC·HCl, HOBt, DIPEA, dry CH₂Cl₂, rt, 18 h, 63%; (e) LiOH, THF–H₂O, 0 °C to rt, 5 h, 59%; (f) 3 N HCl/dry MeOH, rt, 4 h, quantitative; (g) HATU, HOAt, DIPEA, dry DMF, rt, 10 min, then **45**, rt, 72 h, 30%; (h): TFA–thioanisole–1,2-ethanedithiol–anisole 90 : 5 : 3 : 2, rt, 2 h, 57%.

Table 1 IC₅₀s of compounds **2**, **3**, **5–7**, **11–13**, **44** on BIR portions from IAPs

Compound	BIR3, XIAP (nM)	l-BIR2-BIR3, XIAP (nM)	BIR3, cIAP2 (nM)
11	52.0 ± 4.5	50.5 ± 7.8	1.33 ± 0.3
12	25.5 ± 1.7	57.5 ± 5.8	0.73 ± 0.1
13	66.0 ± 5.4	175.8 ± 27.5	1.48 ± 0.2
5	120.0 ± 18.6	55.0 ± 13.1	NT
6	110.0 ± 26.4	27.0 ± 12.4	NT
7	760.0 ± 99.2	190.0 ± 49.1	NT
44	90.4 ± 12.2	109.8 ± 27.1	1.57 ± 0.2
2	>10 000	>10 000	NT
3	>10 000	>10 000	NT

XIAP. The same is true for C-terminus connected dual action conjugate **13** and reference compounds **7** and **44**. As expected, cyclic RGD-based reference compounds **2** and **3** did not show affinity for any BIR construct from IAPs.

The higher affinity of dual action conjugates **11–13** for cIAP proteins is consistent with what is generally observed for monomeric SMAC mimetics.[29]

Cell-free assays – αᵥβ₃/αᵥβ₅ integrins. The dual action conjugates **11–13** were tested *in vitro* for their ability to inhibit biotinylated vitronectin binding to the purified αᵥβ₃ and αᵥβ₅ receptors.[32,33] The standard monomeric, integrin ligands **2** and **3** (Fig. 1), built respectively on a *cyclo*[ABN-RGD], **13**-like scaffold, and on a *cyclo*[DKP-RGD], **11**, **12**-like scaffold, were tested for comparison. Their IC₅₀ values are reported in Table 2. The three dual action conjugates **11–13** show nano-

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Table 2 Inhibition of biotinylated vitronectin binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors by compounds **2**, **3**, **5–7**, **11–13**, **44**

Compound	$\alpha_v\beta_3$ IC$_{50}$ (nM)	$\alpha_v\beta_5$ IC$_{50}$ (nM)
11	70.1 ± 0.1	900 ± 380
12	36.5 ± 0.6	1500 ± 700
13	105.2 ± 3.4	649 ± 25
2	20.2 ± 1.9	205 ± 34
3	4.5 ± 1.1	149 ± 25
5	>10 000	>10 000
6	>10 000	>10 000
7	>10 000	>10 000
44	>10 000	>10 000

molar affinity for both $\alpha_v\beta_3$ (stronger interaction) and $\alpha_v\beta_5$ integrin (weaker interaction), with a limited loss in binding strength with respect to the reference compounds **2** and **3**. However, their residual affinity (nanomolar) should be sufficient to target integrin-expressing tumour cells. As expected, reference SMAC mimetics **5–7** and **44** did not show affinity for the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors. Based on these promising cell-free results, we decided to examine the cytotoxic activity of the three dual action conjugates **11–13** on two different tumour cell lines.

Cytotoxicity – tumour cells. The dual action conjugates **11–13** were tested in cellular assays for their ability to kill tumour cells, either expressing integrin receptors on their surface or relying on IAP proteins to impair the physiological apoptotic process. We selected ovarian IGROV-1 carcinoma cells as representatives for the former, and breast cancer MDA-MB-231 cells for the latter group. The standard monomeric, integrin ligands **2** and **3** (Fig. 1) and IAP inhibitors **5–7** and **44** (Fig. 1 and Scheme 4), were tested for comparison. Their IC$_{50}$ values are reported in Table 3. The results clearly show that dual action conjugates **11–13** are endowed with moderate cytotoxic activity. Namely, 4-ester-connected conjugate **11** and reference SMAC mimetic **5** are similarly potent against MDA-MB-231 cells. The same is true for C-terminus-connected conjugate **13** and reference SMAC mimetic **6**. The loss of cytotoxicity for 4-amide-connected conjugate **12** with respect to 4-ester-connected conjugate **11** may be due to ester hydrolysis and SMAC mimetic unit release for the latter compound, as observed by us with cyclic RGD ligand–paclitaxel conjugates.[33] Surprisingly, C-terminus-amide connected conjugate **13** shows moderate cytotoxicity on MDA-MB-231 cells,

Table 3 Cytotoxicity of compounds **11–13**, **2–3**, **5–7**, **44**

Compound	MDA-MB-231 (μM)	IGROV-1 (μM)
11	9.7 ± 1.6	>25
12	>25	>25
13	20.5 ± 2.2	11.5 ± 2.5
2	>25	>25
3	>25	>25
5	13.5 ± 1.6	>25
6	8.1 ± 0.9	>25
7	>25	>25
44	>25	>25

although no hydrolysis or SMAC unit release can easily take place.

As expected, reference cyclic RGD ligands **2** and **3** *per se* do not show any cytotoxicity against integrin-rich IGROV-1 cells.[32,33] The same is true for reference SMAC mimetics **5–7** and **44**. Thus, we assumed that dual action conjugates **11–13** should also be inactive against IGROV-1 cells. Surprisingly, C-terminus-amide-connected conjugate **13** shows cytotoxicity on IGROV-1 cells, possibly suggesting an advantage for C-terminus connected dual action compounds in terms of cellular activity.

Conclusions

In this paper we reported the rational design, synthesis and *in vitro* evaluation of cyclic RGD ligand–SMAC mimetic conjugates **11–13**. We successfully confirmed their affinity for the molecular target of each monomeric unit, and we ascertained that the moderate cytotoxicity of their monomeric precursors can be maintained. Some preliminary results (*e.g.*, the cytotoxic activity for C-terminus-amide connected conjugate **13** on IGROV-1 cells) indicate that the combination of a cyclic RGD ligand-based tumour targeting/anti-angiogenic unit with a SMAC mimetic-based pro-apoptotic unit may lead to synergistic/enhanced anticancer effects.

More generally, we plan to investigate the influence of linker composition (*e.g.*, PEG-based or amides) and hindrance (*e.g.*, rigid/constrained linkers, short linkers), and of overall physico-chemical (*e.g.*, lipophilicity) and electronic properties (*e.g.*, 4-substituents on the ABD SMAC mimetic unit) on biological activity. We aim to establish a detailed SAR, in order to evaluate the potential for cyclic RGD ligand–SMAC mimetic conjugates as anticancer agents *in vitro* and *in vivo*.

Experimental

Materials and methods

Reactions requiring anhydrous conditions were carried out in flame-dried glassware, with magnetic stirring and under a nitrogen atmosphere. Commercially available reagents were used as received. Anhydrous solvents were purchased from commercial sources and withdrawn from the container by syringe, under a slight positive pressure of nitrogen. *N*-Boc, *N*-Me-(*S*)-2-aminobutyric acid,[28] SMAC amine **16**,[28] *cyclo*[DKP-RGD]benzylamine **20**,[33] SMAC tricyclic ester **31**,[28] and *cyclo*[ABN-RGD]methylamine **45**[16] were prepared according to literature procedures. Their analytical data were in agreement with those already published. Reactions were monitored by analytical thin-layer chromatography (TLC) using silica gel 60 F$_{254}$ pre-coated glass plates (0.25 mm thickness). Visualization was accomplished by irradiation with a UV lamp and/or staining with a potassium permanganate alkaline solution or ninhydrin. Flash column chromatography was performed according to the method of Still and co-workers[39] using Chromagel

60 ACC (40–63 μm) silica gel. Proton NMR spectra were recorded on a spectrometer operating at 400.16 MHz. Proton chemical shifts are reported in ppm (δ) with the solvent reference relative to tetramethylsilane (TMS) employed as the internal standard. The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad signal, dd = doublet of doublets. Carbon NMR spectra were recorded on a spectrometer operating at 100.63 MHz, with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard. HPLC purifications were performed on a Dionex Ultimate 3000 instrument equipped with a Dionex RS Variable Wavelength Detector (column: Atlantis® Prep T3 OBD™ 5 μm 19 × 100 mm). High resolution mass spectra (HRMS) were performed on a Fourier Transform Ion Cyclotron Resonance (FT-ICR) Mass Spectrometer APEX II & Xmass software (Bruker Daltonics) – 4.7 T Magnet (Magnex) equipped with ESI source, available at CIGA (Centro Interdipartimentale Grandi Apparecchiature) c/o Università degli Studi di Milano. Low resolution mass spectra (MS) were recorded on a Waters Acquity™ UPLC-MS instrument (ESI source).

Chemistry

General procedure for the synthesis of compound 11

4-Hydroxymethylbenzaldehyde **15**. Terephthalaldehyde **14** (1.0 g, 7.45 mmol, 1 eq.) was dissolved in 12.5 mL of EtOH–H$_2$O (95 : 5), and then 17.5 mL of THF were added. To the resulting stirred solution, NaBH$_4$ (70 mg, 1.86 mmol, 0.25 eq.) was added at −5 °C in small portions over 30 minutes. The mixture was stirred for 6 h, while the temperature was maintained at 0 °C. The reaction mixture was then adjusted to pH = 5 with 2 M HCl. The solvent was evaporated at reduced pressure, then H$_2$O (20 mL) was added and the solution was extracted three times with AcOEt. The resulting organic phase was dried over Na$_2$SO$_4$ and the solvent was evaporated at reduced pressure. The crude product was purified by flash chromatography on silica gel (*n*-hexane–AcOEt, 1 : 1 as eluent) to afford the pure desired product **15** as a white solid (840 mg, 6.18 mmol, 83%).

R_f = 0.5 (Hex–AcOEt, 4 : 6); **^{1}H NMR** (400 MHz, CDCl$_3$) δ 9.82 (s, 1H), 7.71 (d, J = 7.8 Hz, 2H), 7.39 (d, J = 7.9 Hz, 2H), 4.65 (s, 2H), 2.07 (s, 1H); **^{13}C NMR** (101 MHz, CD$_2$Cl$_2$) δ 192.6, 148.8, 135.9, 130.2, 127.3, 64.5.

tert-Butyl ((S)-1-(((3S,6S,7R,9aS)-3-(benzhydrylcarbamoyl)-7-(2-((4 (hydroxymethyl)benzyl)amino)ethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxobutan-2-yl)(methyl)carbamate **17**. A solution of SMAC amine **16**[28] (50 mg, 0.074 mmol, 1.05 eq.), DIPEA (18 μL, 0.105 mmol, 1.5 eq.) in dry MeOH (0.5 mL) was added to a flask containing compound **15** (9.5 mg, 0.074 mmol, 1 eq.) under nitrogen atmosphere. The mixture was left stirring at room temperature for 5 h. NaBH$_4$ (5.3 mg, 0.14 mmol, 2 eq.) was then added stepwise (5 additions within 3 min), and the mixture was stirred at room temperature under nitrogen for 30 minutes. The mixture was then concentrated under vacuum. 5 mL of a saturated

solution of NaHCO$_3$ were added, then the mixture was extracted with AcOEt (7 mL, three times). The organic phase was dried over Na$_2$SO$_4$ and the solvent was evaporated at reduced pressure. The crude product was purified by flash chromatography on silica gel (CH$_2$Cl$_2$–MeOH, 9 : 1 as eluent) to afford the pure desired product **17** as a white foam (41 mg, 0.059 mmol, 80% yield).

R_f = 0.3 (CH$_2$Cl$_2$–MeOH, 9 : 1); **^{1}H NMR** (400 MHz, THF-d_8) δ 8.27 (bs, 1H), 7.54 (bs, 1H), 7.40–7.00 (m, 14H), 6.21 (d, J = 8.81 Hz, 1H) 4.62 (d, J = 6.53 Hz, 1H), 4.60–4.35 (m, 4H), 4.00–3.70 (m, 4H), 2.84 (bs, 3H), 2.66 (bs, 1H), 2.52 (bs, 1H), 2.15 (m, 4H), 1.91 (m, 2H), 1.85–2.57 (m, 4H), 1.46 (s, 9H), 1.47–1.31 (m, 4H), 0.87 (t, J = 7.32 Hz, 3H); **^{13}C NMR** (400 MHz, THF-d_8) δ 172.3, 171.5, 170.6, 157.0, 143.5, 143.3, 142.7, 129.2, 129.0, 128.9, 128.2, 128.1, 127.6, 127.5, 127.0, 80.2, 80.1, 79.7, 67.9, 67.7, 67.4, 67.2, 66.9, 66.7, 66.6, 64.6, 64.4, 53.4, 53.1, 49.3, 48.4, 47.1, 35.0, 34.9, 34.6, 34.4, 34.3, 33.9, 33.7, 33.5, 31.8, 27.7, 27.4, 27.1, 25.5, 25.3, 25.1, 24.9, 24.7, 22.4, 22.2, 22.2; **MS (ESI)** m/z calcd for [C$_{43}$H$_{58}$N$_5$O$_6$]$^+$: 740.44 [M + H]$^+$; found: 739.75.

tert-Butyl ((S)-1-(((3S,6S,7R,9aS)-3-(benzhydrylcarbamoyl)-7-(2-((tert-butoxycarbonyl)(4-(hydroxymethyl)benzyl)amino)ethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxobutan-2-yl)-(methyl)carbamate **18**. A solution of compound **17** (41 mg, 0.056 mmol, 1 eq.) and DIPEA (19 μL, 0.112 mmol, 2 eq.) in dry CH$_2$Cl$_2$ (0.6 mL) was stirred in an ice bath under nitrogen atmosphere. A solution of Boc$_2$O (17 mg, 0.079 mmol, 1.4 eq.) in dry CH$_2$Cl$_2$ (0.2 mL) was added. The mixture was stirred at 0 °C for 1 h under nitrogen atmosphere, then stirring was continued overnight at room temperature. Then, additional Boc$_2$O (6.7 mg, 0.030 mmol, 0.5 eq.) was dissolved in CH$_2$Cl$_2$ (0.2 mL) and added, and stirring continued for 4 h. The solvent was removed at reduced pressure, and the crude product was passed through a plug of silica gel (CH$_2$Cl$_2$–MeOH, 95 : 5 as eluent) to afford the desired pure product **18** as a white foam (41 mg, 0.049 mmol, 87% yield).

R_f = 0.7 (CH$_2$Cl$_2$–MeOH, 9 : 1); **^{1}H NMR** (400 MHz, acetone-d_6) δ 8.18 (d, J = 8.70 Hz, 1H), 7.40–7.08 (m, 14H), 6.19 (d, J = 8.66 Hz, 1H), 4.69 (d, J = 7.92 Hz, 1H), 4.61 (d, J = 5.76 Hz, 2H), 4.58–4.30 (m, 4H), 4.13 (t, J = 5.76 Hz, 1H), 3.99 (m, 1H), 3.25 (m, 1H), 2.25 (m, 1H), 2.17 (bs, 1H), 2.00–1.58 (m, 7H), 1.50 (s, 18H), 1.46 (m, 4H), 1.32 (m, 2H), 0.90 (t, J = 7.38 Hz, 3H); **MS (ESI)** m/z calcd for [C$_{48}$H$_{66}$N$_5$O$_8$]$^+$: 840.49 [M + H]$^+$; found: 840.45.

4-((4-(((2-((3S,6S,7R,9aS)-3-(Benzhydrylcarbamoyl)-6-((S)-2-((tert-butoxycarbonyl)(methyl)amino)butanamido)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-7-yl)ethyl)(tert-butoxycarbonyl)amino)methyl)-benzyl)oxy)-4-oxobutanoic acid **19**. Compound **18** (17 mg, 0.020 mmol, 1 eq.) was dissolved in dry CH$_2$Cl$_2$ (0.12 mL). Then DIPEA (4.1 μL, 0.024 mmol, 1.2 eq.) was added and the mixture was stirred in an ice bath. DMAP (1.46 mg, 0.012 mmol, 0.6 eq.) and succinic anhydride (3.6 mg, 0.036 mmol, 1.8 eq.) were added. The reaction was stirred at room temperature under nitrogen atmosphere for 2 h. CH$_2$Cl$_2$ (15 mL) was then added and the solution was washed with a solution of 1 M KHSO$_4$ (7 mL, twice). The aqueous phase was

extracted with CH_2Cl_2 (7 mL, once), then the collected organic phases were dried over Na_2SO_4. The solvent was evaporated at reduced pressure to afford the desired product **19** as a white foam that was used without further purification (17 mg, 0.018 mmol, 90% yield).

R_f = 0.6 (CH_2Cl_2–MeOH, 9 : 1); ^{1}H NMR (400 MHz, acetone-d_6) δ 10.72 (bs, 1H), 8.19 (d, J = 8.4 Hz, 1H), 7.48–6.98 (m, 15H), 6.19 (d, J = 8.4 Hz, 1H), 5.12 (s, 2H), 4.69 (d, J = 7.1 Hz, 1H), 4.63–4.26 (m, 3H), 3.99 (d, J = 7.1 Hz, 1H), 3.27 (s, 1H), 3.03–2.93 (m, 2H), 2.80 (s, 3H), 2.70–2.54 (m, 2H), 2.36–2.21 (m, 1H), 2.16 (s, 1H), 2.02–1.57 (m, 6H), 1.49 (s, 18H), 1.45–1.35 (m, 6H), 0.88 (t, J = 7.4 Hz, 3H). ^{13}C NMR (400 MHz, acetone-d_6) δ 173.6, 172.7, 170.8, 143.6, 143.3, 139.7, 136.3, 129.4, 129.3, 129.1, 128.1, 128., 127.9, 79.8, 66.3, 62.1, 60.7, 59.2, 57.3, 55.8, 49.6, 45.4, 34.6, 34.0, 33.6, 30.1 (overlapping with solvent signal), 29.8 (overlapping with solvent signal), 29.2, 28.6, 27.3, 22.2, 11.0; **MS (ESI)** m/z calcd for $[C_{52}H_{70}N_5O_{11}]^+$: 940.51 $[M + H]^+$; found: 940.33.

2-((1S,5S,11S,15R)-18-(4-((4-((4-(((2-((3S,6S,7R,9aS)-3-(Benz-hydrylcarbamoyl)-6-((S)-2-((tert-butoxycarbonyl)(methyl)amino)-butanamido)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-7-yl)ethyl)-(tert-butoxycarbonyl)amino)methyl)benzyl)oxy)-4-oxobutanamido)-methyl)benzyl)-11-(3-guanidinopropyl)-4,7,10,13,17,19-hexaoxo-3,6,9,12,16,18-hexaazabicyclo[13.2.2]nonadecan-5-yl)acetic acid 21. Compound **19** (17 mg, 0.018 mmol, 1 eq.) was dissolved in 1 mL of DMF under nitrogen atmosphere in a flame dried Schlenk tube. *N*-Hydroxysulfosuccinimide (5 mg, 0.023 mmol, 1.24 eq.) and *N,N'*-diisopropylcarbodiimide (4.18 μL, 0.027 mmol, 1.52 eq.) were then added at room temperature. The solution was stirred overnight, then DMF was removed under vacuum. The residue was then dissolved within the same Schlenk tube in 1.2 mL of CH_3CN, and a solution of compound **20**[33] (12.4 mg, 0.0144 mmol, 0.8 eq.) in 1 mL of phosphate buffer solution (the pH was adjusted to 7.5 with 0.2 M NaOH) was then added. The resulting mixture was cooled to 0 °C, adjusting the pH to 7.5 with 0.2 M NaOH, and stirring was continued overnight at room temperature. The reaction mixture was then concentrated at reduced pressure and purified by HPLC (gradient: from 90% H_2O + 0.2% HCOOH–10% acetonitrile + 0.2% HCOOH to 30% H_2O + 0.2% HCOOH–70% acetonitrile + 0.2% HCOOH in 15 minutes). The purified product was then freeze-dried to give the desired pure compound **21** as a white foam (9 mg, 0.072 mmol, 40% yield).

t_R = 12.7 min; ^{1}H NMR (400 MHz, CD₃OD) δ 8.80 (d, J = 8.3 Hz, 1H), 8.40 (t, J = 5.5 Hz, 1H), 8.34–8.15 (br m, 1H), 7.39–7.12 (m, 18H), 6.12 (d, J = 8.3 Hz, 1H), 5.21–5.03 (m, 3H), 4.84–4.73 (m, 2H), 4.61 (t, J = 5.8 Hz, 1H), 4.58–4.27 (m, 7H), 4.24–4.15 (m, 1H), 4.07–3.91 (m, 3H), 3.88 (d, J = 6.3 Hz, 1H), 3.52 (d, J = 17.2 Hz, 1H), 3.45 (dd, J = 14.7, 6.8 Hz, 1H), 3.29–3.14 (m, 3H), 3.14–2.89 (m, 1H), 2.80 (s, 3H), 2.74–2.57 (m, 6H), 2.54 (t, J = 6.7 Hz, 2H), 2.30–2.16 (m, 1H), 2.15–1.33 (m, 33H), 0.92 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, CD₃OD) δ 174.2, 174.0, 173.7, 173.6, 173.0, 172.9, 172.3, 172.2, 172.2, 171.3, 171.2, 171.1, 158.6, 143.1, 140.2, 136.7, 135.9, 135.9, 129.7, 129.5, 129.3, 128.7, 128.6, 128.5, 128.3, 81.4, 67.1, 62.8, 62.2, 60.6, 59.7, 58.3, 56.9, 54.3, 53.2, 51.3, 50.6, 48.1, 46.0,

43.9, 43.8, 42.2, 40.1, 40.1, 39.9, 37.8, 34.0, 33.6, 31.4, 31.1, 31.0, 30.8, 30.4, 28.9, 28.8, 27.7, 26.5, 23.0, 11.1; **MS (ESI)** m/z calcd for $[C_{79}H_{106}N_{15}O_{18}]^+$: 1552.78 $[M + H]^+$; found: 1552.85.

2-((1S,5S,11S,15R)-18-(4-((4-((4-((2-((3S,6S,7R,9aS)-3-(Benzhy-drylcarbamoyl)-6-((S)-2-(methylamino)butanamido)-5-oxoocta-hydro-1H-pyrrolo[1,2-a]azepin-7-yl)ethyl)amino)methyl)benzyl)-oxy)-4-oxobutanamido)methyl)benzyl)-11-(3-guanidinopropyl)-4,7,10,13,17,19-hexaoxo-3,6,9,12,16,18-hexaazabicyclo[13.2.2]-nonadecan-5-yl)acetic acid 11. Compound **21** (9 mg, 0.0058 mmol, 1 eq.) was dissolved in CH_2Cl_2 (100 μL). TFA (66 μL, 0.87 mmol, 150 eq.) was then added, and stirring was continued at room temperature for 50 minutes. The solvent and TFA were removed at reduced pressure, and the crude product was purified by HPLC (gradient: from 90% H_2O + 0.1% CF_3COOH–10% acetonitrile + 0.1% CF_3COOH to 30% H_2O + 0.1% CF_3COOH–70% acetonitrile + 0.1% CF_3COOH in 14 min). The purified product was then freeze-dried to give the trifluoroacetate salt of the desired pure compound **11** as a white foam (9.7 mg, 0.0057 mmol, 99% yield).

t_R = 8.7 min; ^{1}H NMR (400 MHz, CD₃CN) δ 7.97 (d, J = 8.1 Hz, 1H), 7.86 (dd, J = 8.4, 3.6 Hz, 1H), 7.44–7.13 (m, 18H), 6.05 (d, J = 8.1 Hz, 1H), 5.08 (s, 2H), 4.99 (d, J = 15.2 Hz, 1H), 4.75 (t, J = 6.8 Hz, 1H), 4.54 (d, J = 9.4 Hz, 1H), 4.50 (dd, J = 7.9, 4.2 Hz, 1H), 4.45 (dd, J = 9.0, 4.3 Hz, 1H), 4.31–4.23 (m, 3H), 4.10 (s, 2H), 4.04–3.89 (m, 3H), 3.89–3.78 (m, 3H), 3.46 (d, J = 17.1 Hz, 1H), 3.34 (dd, J = 15.0, 6.5 Hz, 1H), 3.12 (t, J = 6.5 Hz, 2H), 3.07–2.98 (m, 1H), 2.82–2.66 (m, 2H), 2.65–2.54 (m, 6H), 2.55–2.43 (m, 3H), 2.26–2.14 (m, 1H), 2.13–1.42 (m, 15H, overlapping with solvent signal), 0.92 (t, J = 7.5 Hz, 3H); ^{13}C NMR (101 MHz, CD₃CN) δ 173.9, 173.2, 173.1, 173.0, 172.2, 171.2, 170.8, 170.6, 170.6, 170.0, 168.6, 168.5, 157.9, 143.1, 142.9, 140.1, 138.7, 135.4, 131.8, 131.2, 129.7, 129.3, 129.1, 128.8, 128.4, 128.3, 128.0, 66.4, 63.2, 62.4, 60.3, 59.2, 58.0, 56.5, 54.9, 52.8, 51.7, 50.0, 48.2, 46.4, 43.4, 43.2, 41.8, 39.8, 38.9, 37.3, 35.5, 33.6, 32.9, 32.5, 32.3, 31.1, 30.2, 29.0, 28.6, 26.9, 26.0, 23.9, 9.1; **MS (ESI)** m/z calcd for $[C_{69}H_{90}N_{15}O_{14}]^+$: 1352.68 $[M + H]^+$; found: 1353.41; **HRMS (ESI)** m/z calcd for $[C_{69}H_{90}N_{15}O_{14}]^+$: 1352.67862 $[M + H]^+$; found: 1352.68112.

General procedure for the synthesis of compound 12. The general procedure for the synthesis of compound **12** is reported in the ESI.†

General procedure for the synthesis of compound 13

(3S,6S,7S,9aS)-Methyl 6-(tert-butoxycarbonylamino)-7-(hydroxy-methyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate 32. Compound **31**[28] (1.06 g, 3.07 mmol, 1 eq.), ammonium formate (2.23 g, 34.36 mmol, 11.2 eq.) and palladium hydroxide on carbon (320 mg, 30%) were dissolved in a mixture of THF–H_2O 4 : 1 + 2% acetic acid (300 mL). The reaction mixture was stirred at 70 °C for 6 h, then at rt overnight. The reaction mixture was then filtered over celite, washing with THF and CH_2Cl_2. The solvent was then removed at reduced pressure, obtaining 1.12 g of crude aminoalcohol that was used without any further purification.

A solution of Boc_2O (1.73 g, 7.67 mmol, 2.5 eq.) in 10 mL of dry CH_2Cl_2 was prepared and stirred at room temperature under nitrogen atmosphere. The previously prepared crude

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aminoalcohol (theoretical 3.07 mmol, 1 eq.) and dry TEA (1.1 mL, 7.89 mmol, 2.6 eq.) in 20 mL of dry CH_2Cl_2 were then added, and the reaction mixture was left under stirring at room temperature for 20 h. The reaction mixture was then diluted with CH_2Cl_2 and washed with 2 × 30 mL of 5% citric acid solution, with 2 × 30 mL of saturated $NaHCO_3$ and 2 × 30 mL of brine. The aqueous phase was washed with 2 × 20 mL of CH_2Cl_2, the organic phases were dried with Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (n-hexane–AcOEt 6 : 4 as eluent), yielding 643 mg of pure compound **32** as a white solid (1.80 mmol, 59% yield over two steps).

¹H NMR (400 MHz, $CDCl_3$) δ 6.14 (bd, 1H), 4.58 (dd, 1H, J = 4.6 Hz, J = 8.1), 4.25 (dd, 1H, J = 6.2 Hz, J = 10.5 Hz), 3.95–3.82 (m, 2H), 3.73 (s, 3H), 3.38 (dd, 1H, J = 12.1 Hz, J = 2.8 Hz), 2.25 (m, 1H), 2.18–1.96 (m, 4H), 1.92–1.72 (m, 3H), 1.55 (m, 1H), 1.42 (s, 9H). **¹³C NMR** (100.6 MHz, $CDCl_3$) δ 173.1, 171.4, 158.3, 81.3, 65.3, 61.3, 59.6, 55.9, 53.0, 42.5, 34.2, 33.5, 31.8, 28.9, 28.4. **MS (ESI):** m/z calcd for $C_{17}H_{28}N_2O_6$: 356.19; found: 257.0 $[M - Boc + H]^+$.

(3S,6S,7R,9aS)-Methyl 6-((tert-butoxycarbonyl)amino)-7-(cyano-methyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate **33**. A 0.1 M solution of compound **32** (210 mg, 0.59 mmol, 1 eq.) in dry CH_2Cl_2 (6 mL) was prepared and stirred under nitrogen atmosphere; MsCl (101 mg, 0.88 mmol, 1.5 eq.) and dry TEA (246 µL, 1.76 mmol, 3 eq.) were added at 0 °C and the reaction mixture was then stirred for 2 h at room temperature. After reaction completion the solution was diluted in 20 mL of CH_2Cl_2, washed with a 5% citric acid aqueous solution (2 × 50 mL), a saturated solution of $NaHCO_3$ (2 × 50 mL) and brine (2 × 50 mL). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The crude mesylate (0.83 g, theoretical 1.91 mmol, quantitative yield) was used without any purification.

A 2 N solution of the crude mesylate in dry DMF (1 mL) was prepared and $nBu_4^+CN^-$ (770 mg, 2.87 mmol, 1.5 eq.) was added. The solution was stirred at 80 °C in a microwave synthesizer for 2 h. The solvent was removed at reduced pressure and the crude product was purified by flash chromatography (n-hexane–EtOAc 4 : 6 as eluent). The desired pure product **33** (0.59 g, 1.61 mmol) was obtained as a white powder (yield 84%).

¹H NMR (400 MHz, $CDCl_3$) δ 5.83 (bd, 1H), 4.60 (dd, 1H, J = 7.9, J = 4.3 Hz), 4.26 (m, 1H, J = 10.0 Hz, J = 7.6 Hz), 3.93 (dd, 1H, J = 15.3 Hz, 7.6 Hz), 3.76 (s, 3H), 2.76 (dd, 1H, J = 4.5 Hz, J = 17.0 Hz), 2.42–2.23 (m, 3H), 2.08 (m, 2H), 1.96 (m, 1H), 1.91–1.62 (m, 4H), 1.42 (s, 9H). **¹³C NMR** (100.6 MHz, $CDCl_3$) δ 172.4, 169.6, 80.4, 60.6, 58.8, 56.3, 52.6, 37.8, 34.1, 33.5, 32.9, 28.4, 27.9, 21.0. **MS (ESI):** m/z calcd for $C_{18}H_{27}N_3O_5$: 365.20; found: 366.2 $[M + H]^+$.

(3S,6S,7R,9aS)-Methyl 6-amino-7-(cyanomethyl)-5-oxoocta-hydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate **34**. Compound **33** (317 mg, 0.87 mmol, 1 eq.) was dissolved in 2.8 mL of dry MeOH under nitrogen atmosphere at room temperature. Acetyl chloride (930 µL, 13.03 mmol, 15 eq.) was added one pot and

under vigorous stirring. After 5 minutes a white solid started to precipitate. The solvent was then removed at reduced pressure, yielding 258 mg of desired pure compound **34** as a white solid (0.87 mmol, quantitative yield).

¹H NMR (400 MHz, $CDCl_3$) δ 4.58 (dd, 1H, J = 4.2 Hz, J = 8.3 Hz), 4.19 (d, 1H, J = 10.2 Hz), 4.01 (m, 1H), 3.72 (s, 3H), 2.80 (dd, 1H, J = 17.0 Hz, J = 3.8 Hz), 2.61 (dd, 1H, J = 17.0 Hz, J = 9.0 Hz), 2.32 (m, 1H), 2.26–2.14 (m, 2H), 2.11–2.02 (m, 1H), 2.01–1.92 (m, 2H), 1.92–1.74 (m, 3H). **¹³C NMR** (100.6 MHz, $CDCl_3$) δ 170.1, 120.8, 70.4, 64.7, 64.2, 63.0, 62.0, 59.5, 55.5, 43.7, 40.7, 38.0, 37.4, 36.6, 36.1, 35.6, 35.3, 31.2, 31.1, 24.2. **MS (ESI):** m/z calcd for $C_{13}H_{19}N_3O_3$: 265.14; found: 266.27 $[M + H]^+$.

(3S,6S,7R,9aS)-Methyl 6-((R)-2-((tert-butoxycarbonyl)(methyl)-amino)butanamido)-7-(cyanomethyl)-5-oxooctahydro-1H-pyrrolo-[1,2-a]azepine-3-carboxylate **35**. A solution of N-methyl-N-Boc-aminobutyric acid (270 mg, 1.24 mmol, 1.4 eq.), EDC·HCl (240.3 mg, 1.25 mmol, 1.4 eq.) and HOBt (181 mg, 1.34 mmol, 1.5 eq.) in 2.5 mL of dry CH_2Cl_2 was prepared under nitrogen atmosphere, then 300 µL of dry DIPEA (1.68 mmol, 1.9 eq.) were added. The solution was left under stirring for 10 minutes. A solution of compound **34** (258 mg, 0.87 mmol, 1 eq.) and 320 µL of dry DIPEA (1.79 mmol, 2.1 eq.) in 6.2 mL of dry CH_2Cl_2 was then added dropwise, and the reaction mixture was left under stirring at rt for 22 h. The reaction mixture was diluted with CH_2Cl_2 and washed with 20 ml of a citric acid 5% solution, 20 mL of a $NaHCO_3$ saturated solution and 20 mL of brine. The organic phase was then dried with Na_2SO_4, filtered and the solvent was removed at reduced pressure. The crude product was purified by flash chromato-graphy (n-hexane–EtOAc 4 : 6 as eluent), yielding 330 mg of desired pure compound **35** as a white solid (0.71 mmol, 82% yield).

¹H NMR (400 MHz, $CDCl_3$) δ 7.14 (bd, 1H), 4.59 (dd, 1H, J = 3.9 Hz, J = 7.3 Hz), 4.54 (m, 1H), 4.35 (m, 1H), 3.94 (m, 1H), 3.76 (s, 3H), 2.85 (s, 3H), 2.64 (m, 1H), 2.38 (m, 1H), 2.34–2.26 (m, 2H), 2.08 (m, 2H), 2.01 (m, 1H), 1.97–1.68 (m, 6H), 1.48 (s, 9H), 0.91 (t, 3H, J = 6.9 Hz). **¹³C NMR** (100.6 MHz, $CDCl_3$) δ 173.1, 169.8, 79.8, 60.4, 58.7, 54.4, 52.5, 37.4, 33.8, 33.3, 32.6, 28.2, 27.6, 21.4, 10.5. **MS (ESI):** m/z calcd for $C_{23}H_{36}N_4O_6$: 464.26; found: 465.54 $[M + H]^+$.

(3S,6S,7R,9aS)-Methyl 7-(2-aminoethyl)-6-((R)-2-((tert-butoxy-carbonyl)(methyl)amino)butanamido)-5-oxooctahydro-1H-pyrrolo-[1,2-a]azepine-3-carboxylate **36**. Hydrogenation of the nitrile **35** was performed using the H-Cube™ continuous-flow hydrogen-ation reactor. Compound **35** (130 mg, 0.28 mmol, 1 eq.) was dissolved in 50 mL of 90 : 10 EtOH–H_2O and 1 M aqueous citric acid (0.28 mmol) was added. The reaction mixture was flowed through a Ni/Ra cartridge (hydrogen pressure: 60 bar; T = 60 °C; flow 0.5 mL min⁻¹). The reaction was completed after 24 hours. The solvent was removed at reduced pressure. The crude desired compound **36** (0.13 g, 0.277 mmol, quanti-tative yield) was used for the next step without any purification.

MS (ESI): m/z calcd for $C_{23}H_{40}N_4O_6$: 468.29; found: 469.40 $[M + H]^+$.

(3S,6S,7R,9aS)-Methyl 7-(2-benzamidoethyl)-6-((R)-2-((tert-butoxycarbonyl)(methyl)amino)butanamido)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate 37. Compound **36** (234 mg, 0.50 mmol, 1 eq.) was dissolved under inert atmosphere in 5.0 mL of dry CH_2Cl_2, then 350 μL of dry TEA were added (2.50 mmol, 5 eq.). Benzoyl chloride (70 μL, 0.60 mmol, 1.2 eq.) was slowly added under vigorous stirring. The solution turned from yellow to dark red. After 2 h additional TEA (50 μL, 0.37 mmol, 0.7 eq.) and benzoyl chloride (50 μL, 0.43 mmol, 0.86 eq.) were added. After 3 h the reaction mixture was diluted with CH_2Cl_2 and washed with 30 mL of a 5% citric acid solution, 30 mL of a saturated $NaHCO_3$ solution and 30 mL of brine. The organic phase was then dried with Na_2SO_4, filtered and the solvent removed under reduced pressure. A brownish oil residue was purified by flash chromatography (*n*-hexane–EtOAc 1:9), yielding 264 mg of pure desired compound **37** as a white solid (0.46 mmol, 92% yield).

¹H NMR (400 MHz, [D₆]acetone) δ 7.90 (m, 2H), 7.70 (bs, 1H), 7.54–7.42 (m, 3H), 7.16 (bs, 1H), 4.58–4.50 (m, 2H), 4.47 (dd, 1H, $J = 8.5$ Hz, $J = 3.9$ Hz), 4.06 (m, 1H), 3.65 (s, 3H), 3.41 (m, 2H), 2.80 (m, 3H), 2.31 (m, 1H), 2.25–2.09 (m, 2H), 2.01–1.84 (m, 5H), 1.73 (m, 1H), 1.70–1.56 (m, 4H), 1.52 (m, 1H), 1.42 (s, 9H), 0.87 (t, 3H, $J = 7.4$ Hz). **¹³C NMR** (100.6 MHz, [D₆]Acetone) δ 173.2, 171.4, 170.8, 167.0, 135.8, 131.3, 128.7, 127.6, 79.5, 60.2, 58.1, 55.0, 51.4, 37.4, 37.3, 33.0, 32.5, 31.7, 27.5, 27.3, 21.3, 10.0. **MS (ESI):** *m/z* calcd for $C_{30}H_{44}N_4O_7$: 572.32; found: 573.8 [M + H⁺].

(3S,6S,7R,9aS)-7-(2-Benzamidoethyl)-6-((R)-2-((tert-butoxy-carbonyl)(methyl)amino)butanamido)-5-oxooctahydro-1H-pyrrolo-[1,2-a]azepine-3-carboxylic acid 30. A 0.1 M solution of compound **37** (35 mg, 0.061 mmol, 1 eq.) in 1 mL of THF–H_2O 3/1 was prepared at 0 °C, and 2 N aqueous LiOH (92 μL, 0.183 mmol, 3 eq.) was slowly added. The reaction mixture was stirred at 0 °C for 30 minutes, then at room temperature for 5 h. The reaction was then quenched with 1 mL of 1 M aqueous $KHSO_4$ and the resulting mixture was extracted with CH_2Cl_2 (3 × 5 mL). The combined organic phases were dried over Na_2SO_4 and evaporated at reduced pressure. The crude desired product **30** (0.03 g, 0.054 mmol, yield 88%) was used for the next step without any purification.

¹H NMR (400 MHz, CDCl₃) δ 7.83 (m, 2H), 7.68 (bd, 1H), 7.48–7.32 (m, 4H), 4.76 (m, 1H), 4.57 (m, 1H), 4.51 (m, 1H), 3.99 (m, 1H), 3.41 (m, 2H), 2.94 (bs, 3H), 2.26 (m, 1H), 2.14–1.99 (m, 4H), 1.91–1.71 (m, 5H), 1.65–1.51 (m, 3H), 1.37 (s, 9H), 0.94 (t, 3H, $J = 6.0$ Hz). **¹³C NMR** (100.6 MHz, CDCl₃) δ 174.2, 173.0, 127.6, 135.3, 131.5, 128.8, 128.7, 128.1, 127.7, 81.1, 60.7, 60.1, 58.6, 54.7, 37.2, 36.7, 32.8, 32.7, 32.5, 31.4, 30.7, 29.5, 28.1, 27.4, 22.7, 10.8. **MS (ESI):** *m/z* calcd for $C_{29}H_{42}N_4O_7$: 558.31; found: 559.7 [M + H⁺].

11-Aminoundecanoic acid methyl ester 40. A 0.5 M solution of 11-aminoundecanoic acid **39** (5.09 g, 25.3 mmol, 1 eq.) in MeOH (50 mL) was prepared and $SOCl_2$ (2.93 mL, 40.4 mmol, 1.6 eq.) was slowly added. The reaction mixture was stirred at room temperature for 2 h, then refluxed at 80 °C for additional 2 h. The solvent was then evaporated at reduced pressure and the solid residue was washed with 10 mL of dry Et_2O, then

filtered. The desired compound **40** (5.3 g, 24.6 mmol, quantitative yield) was obtained as a white powder and used for the next step without any purification.

¹H NMR (400 MHz, CDCl₃) δ 8.25 (bs, 3H), 3.65 (s, 3H), 2.90 (m, 2H), 2.25 (t, 2H, $J = 7.6$ Hz), 1.70 (m, 2H), 1.60 (m, 2H), 1.40–1.15 (m, 12H).

(S)-Methyl 11-(2-((tert-butoxycarbonyl)amino-2-phenylaceta-mido)undecanoate 41. A solution of (S)-N-Boc-phenylglycine (573 mg, 2.28 mmol, 1 eq.), HOBt (369 mg, 2.74 mmol, 1.2 eq.), EDC·HCl (524 mg, 2.74 mmol, 1.2 eq.) and dry DIPEA (1.39 mL, 7.98 mmol, 3.5 eq.) in dry CH_2Cl_2 (12 mL) was stirred under nitrogen atmosphere for 15 minutes at 0 °C. Then a solution of compound **40** (491 mg, 2.28 mmol, 1 eq.) in dry CH_2Cl_2 (12 mL) was slowly added, and the reaction mixture was stirred at room temperature overnight. Then the reaction mixture was diluted in 50 mL of CH_2Cl_2 and washed with a 5% citric acid aqueous solution (2 × 50 mL), a saturated solution of $NaHCO_3$ (2 × 50 mL) and brine (2 × 50 mL). The combined organic phases were dried over Na_2SO_4 and evaporated at reduced pressure. The residue was purified by flash chromatography (*n*-hexane–EtOAc 6:4 as eluent), and the desired pure product **41** (0.74 g, 1.65 mmol) was obtained as a white foam (yield 72%).

¹H NMR (400 MHz, CDCl₃) δ 7.40–7.30 (m, 5H), 5.87 (bs, 1H), 5.76 (bs, 1H), 5.13 (bs, 1H), 3.69 (s, 3H), 3.23 (dd, 2H, $J = 7.0$ Hz, $J = 13.1$ Hz), 2.32 (t, 2H, $J = 7.5$ Hz), 1.63 (m, 2H), 1.43 (m, 9H), 1.38–1.10 (m, 14H). **¹³C NMR** (100.6 MHz, CDCl₃) δ 174.3, 169.9, 155.2, 138.8, 129.0, 128.3, 127.2, 80.0, 58.7, 51.4, 39.8, 34.1, 29.3, 29.2, 29.1, 28.3, 26.6, 24.9. **MS (ESI):** *m/z* calcd for $C_{25}H_{40}N_2O_5$: 448.29; found: 471.3 [M + Na⁺].

(S)-Methyl 11-(2-amino)-2-phenylacetamido)undecanoate 42. Compound **41** (298 mg, 0.66 mmol, 1 eq.) was dissolved in a 3 N methanolic HCl solution (11 mL 33 mmol, 50 eq.) and stirred under inert atmosphere at room temperature overnight. The reaction was then quenched with 10 mL of saturated $NaHCO_3$ and extracted with EtOAc (3 × 10 mL). The combined organic phases were dried over Na_2SO_4 and evaporated at reduced pressure. The desired compound **42** was obtained (0.22 g, 0.64 mmol, yield 97%) and used for the next step without any purification.

¹H NMR (400 MHz, CDCl₃) δ 7.42–7.26 (m, 5H), 7.03 (bs, 1H), 4.53 (s, 1H), 3.68 (s, 3H), 3.26 (ddd, 2H, $J = 13.1$ Hz, $J = 7.1$ Hz, $J = 2.2$ Hz), 2.31 (t, 2H, $J = 7.5$ Hz), 1.35–1.23 (m, 12H). **¹³C NMR** (100.6 MHz, CDCl₃) δ 175.0, 173.5, 141.7, 129.3, 128.4, 127.3, 60.0, 51.4, 39.2, 34.0, 29.4, 29.2, 29.1, 29.0, 26.7, 24.8. **MS (ESI):** *m/z* calcd for $C_{20}H_{32}N_2O_3$: 348.24; found: 349.6 [M + H⁺].

Methyl 11-((S)-2-((3S,6S,7R,9aS)-7-(2-benzamidoethyl)-6-((R)-2-((tert-butoxycarbonyl)(methyl)amino)butanamido)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxamido)-2-phenylacetamido)-undecanoate 43. Compound **30** (45.0 mg, 0.081 mmol, 1 eq.), HATU (47.0 mg, 0.124 mmol, 1.4 eq.), HOAt (24.3 mg, 0.178 mmol, 2 eq.) and dry DIPEA (50 μL, 0.28 mmol, 3 eq.) were dissolved in dry DMF (0.6 mL) under nitrogen atmosphere at room temperature. The solution was left under stirring for 10 minutes. Then a solution of compound **42**

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(46.8 mg, 0.122 mmol, 1.4 eq.) and dry DIPEA (100 μL, 0.56 mmol, 6 eq.) in dry DMF (0.6 mL) was prepared under nitrogen atmosphere, and was added dropwise. The resulting reaction mixture was left under stirring at rt for 43 hours. Then, the solvent was removed under reduced pressure, the crude was dissolved in EtOAc (90 mL) and washed with a 5% citric acid aqueous solution (25 mL) and a saturated $NaHCO_3$ solution (25 mL). The organic phase was then dried with Na_2SO_4, filtered and the solvent was removed at reduced pressure. The crude product (100 mg) was purified by flash chromatography (EtOAc 100% as eluant), and the desired pure product **43** (44.1 mg, 0.050 mmol, yield 61%) was obtained as a white solid.

^{1}H NMR (400 MHz, [D_6]DMSO, ≈1 : 1 mixture of two conformers) δ 8.61 (bd, 0.5H), 8.48 (d, 0.5H, J = 8 Hz), 8.33 (m, 1H), 8.21 (m, 0.5H), 7.88–7.72 (m, 3H), 7.68 (m, 0.5H), 7.52–7.21 (m, 8H), 5.36 (d, 0.5H, J = 6 Hz), 5.34 (d, 0.5H, J = 5.9 Hz), 4.59 (dd, 1H, J = 7.4 Hz, 3.7 Hz), 4.52 (m, 1H), 4.40 (m, 1H), 4.00–3.90 (m, 1H), 3.58 (s, 3H), 3.23 (m, 2H), 3.04 (m, 2H), 2.70 (s, 1.5H), 2.69 (s, 1.5H), 2.28 (td, 2H, J = 7.4 Hz, 2.8 Hz), 2.14 (m, 1H), 2.05 (m, 1H), 1.94 (m, 1H), 1.86 (m, 1H), 1.80 (m, 1H), 1.79–1.70 (m, 2H), 1.68–1.57 (m, 2H), 1.57–1.38 (m, 6H), 1.35 (m, 2H), 1.34 (s, 9H), 1.26–1.14 (m, 12H), 0.81 (bt, 3H). ^{13}C NMR (100.6 MHz, [D_6]Acetone, ≈1 : 1 mixture of two conformers) δ 172.2, 170.2, 170.5, 170.1, 169.9, 167.0, 140.1, 139.5, 135.8, 131.3, 129.0, 128.8, 128.7, 128.6, 128.2, 128.1, 127.8, 127.7, 127.6, 61.2, 61.1, 58.3, 57.2, 57.0, 54.9, 39.1, 37.3, 34.1, 33.4, 33.1, 32.9, 32.7, 31.9, 27.6, 26.4, 26.3, 24.7, 10.0. **MS (ESI):** m/z calcd for $C_{49}H_{72}N_6O_9$: 889.13; found: 890.0 [M + H+].

11-((S)-2-((3S,6S,7R,9aS)-7-(2-Benzamidoethyl)-6-((R)-2-((tert-butoxycarbonyl)(methyl)amino)butanamido)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxamido)-2-phenylacetamido)undecanoic acid 38. Compound **43** (34.1 mg, 0.038 mmol, 1 eq.) was dissolved in THF–H_2O 3 : 1 (400 μL), and 2 N aqueous LiOH (60 μL, 0.122 mmol, 3 eq.) was slowly added. The reaction mixture was stirred at room temperature for 5 h. Then the reaction mixture was quenched with 1 M aqueous $KHSO_4$ (1 mL), diluted with distilled water (10 mL) and extracted sequentially with CH_2Cl_2 (3 × 15 mL) and EtOAc (3 × 15 mL). The combined organic phases were dried over Na_2SO_4 and evaporated at reduced pressure. The desired compound **38** (0.0315 g, 0.036 mmol, yield 95%) was obtained as a white solid and used for the next step without any purification. **MS (ESI):** m/z calcd for $C_{48}H_{70}N_6O_9$: 874.52; found: 875.8 [M + H+].

Methyl 11-((S)-2-((3S,6S,7R,9aS)-7-(2-benzamidoethyl)-6-((S)-2-(methylamino)butanamido)-5-oxooctahydro-1H-pyrrolo[1,2-a]-azepine-3-carboxamido)-2-phenylacetamido)undecanoate 44. Compound **43** (5.3 mg, 0.0060 mmol, 1 eq.) was dissolved under inert atmosphere in 3 N methanolic HCl (200 μL). The reaction mixture was left under vigorous stirring at room temperature for 4 hours, then the solvent was slowly removed under reduced pressure. The pure desired acid **44** (5.0 mg, 0.0060 mmol, quantitative yield) was obtained as a white solid.

^{1}H NMR (400 MHz, [D_6]acetone, ≈1 : 1 mixture of two conformers) δ 8.64 (m, 0.5H), 8.57 (m, 0.5H), 8.53 8 m, 0.5H), 8.45 (m, 0.5H), 8.21 (m, 0.5H), 8.16–8.03 (m, 2.5H), 7.89 (m, 0.5H),

7.67 (m, 0.5H), 7.50–7.18 (m, 8H), 5.53 (d, 0.5H, J = 7.7 Hz), 5.50 (d, 0.5H, J = 7.8 Hz), 4.76 (m, 1H), 4.69 (m, 1H), 4.20 (m, 1H), 4.10 (m, 0.5H), 4.03 (m, 0.5H), 3.61 (s, 3H), 3.44 (m, 2H), 3.15 (m, 2H), 2.78 (s, 3H), 2.28 (td, 2H, J = 7.4 Hz, J = 1.8 Hz), 2.22 (m, 1H), 2.19–2.09 (m, 2.5H), 2.05 (m, 0.5H), 2.02–1.94 (m, 2.5H), 1.91 (m, 0.5H), 1.90–1.83 (m, 2.5H), 1.76–1.62 (m, 2.5H), 1.57 (m, 2H), 1.43 (m, 2H), 1.33 (m, 1H), 1.31–1.19 (m, 12H), 1.03 (m, 3H). ^{13}C NMR (100.6 MHz, [D_6]acetone, ≈1 : 1 mixture of two conformers) δ 130.4, 128.0, 127.6, 127.3, 127.2, 126.8, 126.7, 61.0, 60.4, 57.3, 56.1, 54.6, 49.6, 38.1, 36.4, 36.1, 35.7, 35.6, 32.7, 32.6, 32.5, 32.4, 32.1, 31.9, 31.8, 31.7, 31.3, 29.3, 28.3, 28.2, 26.2, 26.1, 25.5, 25.4, 23.7, 21.1, 21.0, 7.1.

Compound 46. Compound **38** (31.5 mg, 0.036 mmol, 1 eq.), HATU (20.8 mg, 0.053 mmol, 1.5 eq.), HOAt (10.0 mg, 0.073 mmol, 2 eq.) and dry DIPEA (50 μL, 0.28 mmol, 7.8 eq.) were dissolved in dry DMF (200 μL) and stirred for 10 minutes at room temperature under nitrogen atmosphere. Then a solution of compound **45**[16] (40.8 mg, 0.051 mmol, 1.4 eq.) in dry DMF (300 μL) was added dropwise. The reaction mixture was left stirring at room temperature for 72 h. The solvent was thoroughly removed under reduced pressure, then the crude was re-dissolved in EtOAc (50 mL) and washed with a 5% citric acid solution (30 mL) and with a saturated $NaHCO_3$ solution (30 mL). The organic phase was then dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude residue was purified by flash chromatography (Biotage™, reverse phase, from 5% CH_3CN–95% H_2O to 100% CH_3CN as eluent), yielding pure **46** as a white solid (18.1 mg, 0.011 mmol, 30% yield).

^{1}H NMR (400 MHz, [D_6]acetone, mixture of two conformers) δ 8.51–8.30 (m, 1H), 8.16–8.06 (m, 1H), 7.92–7.44 (m, 4H), 7.59 (bs, 1H), 7.51–7.17 (m, 9H), 6.96 (m, 1H), 6.68 (s, 1H), 6.51 (bs, 1H), 5.44 (m, 1H), 4.68 (m, 1H), 4.59 (m, 1H), 4.57 (m, 1H), 4.53 (m, 1H), 4.30 (m, 1H), 4.26 (m, 1H), 4.20 (m, 1H), 4.14 (m, 1H), 4.09 (m, 0.5H), 4.00 (m, 0.5H), 3.84 (s, 3H), 3.47–3.25 (m, 3H), 3.23–3.06 (m, 8H), 2.99 (m, 1H), 2.80 (s, 3H), 2.72 (m, 1H), 2.67 (s, 3H), 2.61 (s, 3H), 2.55 (m, 1H), 2.44–2.31 (m, 3H), 2.26 (m, 1H), 2.14 (m, 1H), 2.08 (m, 2H), 2.05 (m, 2H), 2.02–1.85 (m, 4H), 1.75–1.18 (m, 52H), 0.88 (m, 3H). ^{13}C NMR (75.4 MHz, [D_6]acetone, mixture of two conformers) δ 174.7, 172.2, 171.0, 170.8, 158.8, 157.3, 137.0, 135.9, 131.7, 129.3, 129.0, 128.5, 128.0, 124.7, 112.4, 80.9, 63.3, 62.0, 59.1, 57.8, 56.6, 55.8, 54.3, 52.0, 43.6, 41.1, 40.0, 38.1, 37.4, 36.8, 35.8, 34.2, 33.6, 32.8, 30.5, 30.3, 29.8, 29.3, 29.0, 28.5, 28.2, 27.3, 26.8, 26.3, 24.2, 22.4, 18.6, 14.3, 12.1, 11.1, 8.0. **MS (ESI):** m/z calcd for $C_{84}H_{123}N_{15}O_{18}S$: 1662.89; found: 1685.9 [M + Na+].

Cyclo-*RGD/SMAC mimetic conjugate 13.* Compound **46** (16.1 mg, 0.0097 mmol, 1 eq.) was dissolved in TFA–thioanisole–1,2-ethanedithiol–anisole 90 : 5 : 3 : 2 (2 mL) and stirred at room temperature for 2 h. After reaction completion, the solvent was evaporated under reduced pressure, then the crude was dissolved in H_2O (10 mL) and washed with iPr_2O (10 mL). The aqueous phase was evaporated under reduced pressure and the crude purified by HPLC (Waters Atlantis Prep T3 OBD 5 μm, 19 × 100 mm, column; solvents: (A) H_2O + 0.1% TFA, (B) CH_3CN + 0.1% TFA gradient from 90%A–10%B to

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30%A–70% B over 15 min.; flow rate 15 mL min^{-1}, $\lambda = 210$ nm, $R_t = 8.8$ min). The purified product was then freeze-dried to give the trifluoroacetate salt of the desired pure compound **13** as a white foam (8.4 mg, 0.0059 mmol, 57% yield).

^{1}H NMR (400 MHz, D$_2$O) δ: 7.66 (d, 2H), 7.52 (m, 1H), 7.42 (q, 2H, $J = 7.8$ Hz) 7.38–7.28 (m, 5H), 5.23 (m, 1H), 4.62 (dd, 1H, $J = 9.3$ Hz, $J = 7.3$ Hz), 4.55–4.40 (m, 3H), 4.33 (d, 1H, $J = 8.4$ Hz), 4.25–4.15 (m, 2H), 4.06 (m, 1H), 3.98–3.83 (m, 2H), 3.48–3.29 (m, 3H), 3.24–2.95 (m, 7H), 2.76–2.63 (m, 2H), 2.62 (s, 3H), 2.60 (s, 3H), 2.42–2.30 (m, 3H), 2.21 (m, 1H), 2.15–1.97 (m, 5H), 1.96–1.74 (m, 7H), 1.72–1.50 (m, 6H), 1.49–1.39 (m, 4H), 1.34 (m, 2H), 1.22–1.11 (m, 5H), 1.10–0.99 (m, 8H), 0.95 (t, 3H, $J = 7.5$ Hz), 0.90 (t, 3H, $J = 7.5$ Hz). **^{13}C NMR** (75.5 MHz, D$_2$O) δ: 178.3, 175.2, 174.6, 173.8, 172.3, 171.5, 171.0, 136.9, 132.9, 129.9, 129.6, 127.9, 127.8, 63.6, 62.9, 62.5, 59.0, 57.3, 57.1, 56.7, 53.7, 52.3, 43.2, 41.2, 40.9, 40.1, 38.3, 35.8, 33.6, 33.0, 32.3, 31.7, 30.9, 29.2, 29.1, 28.3, 27.7, 26.5, 26.1, 25.2, 24.3, 9.0. **HRMS (ESI):** m/z calcd for C$_{65}$H$_{95}$N$_{15}$O$_{13}$: 1294.7301; found: 1294.73243 [M + H$^+$].

Biology

General procedure for the fluorescence polarization-based BIR/IAP binding assay. The affinity of each tested compound for the BIR domains of XIAP and cIAP2 was evaluated as previously described.[36–38] Recombinant XIAP (60 nM final concentration) and cIAP2 (25 nM) BIR3, or XIAP BIR23 (3 nM) domains were diluted in the assay buffer containing 100 mM potassium phosphate, pH 7.5, 100 µg mL^{-1} bovine γ-globulin and 0.02% sodium azide. Serial dilutions of each tested compound (concentrations ranging from 2 µM to 0.2 nM) were added to displace a fluorescent probe (FITC-SMAC for BIR3 domains; SMAC-1F for BIR23) and fluorescence polarization was measured with the Ultra plate reader (Tecan), at excitation and emission wavelengths of 485 nm and 530 nm, respectively. All experiments were performed in black, flat-bottom 96 well plates (Greiner Bio-One). Three independent experiments were performed in duplicates. The analyses were performed using Graphpad Prism v.5.02.

General procedure for the solid-phase integrin receptor binding assay. Purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors (Millipore Corporation, Billerica, MA, USA) were diluted to 0.5 µg mL^{-1} in 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$_2$. An aliquot of diluted receptors (100 µL per well) was added to 96-well microtiter plates (NUNC MW 96F Maxisorp Straight) and incubated overnight at 4 °C. The plates were then incubated with blocking solution (coating buffer plus 1% bovine serum albumin) for an additional 2 h at room temperature to block nonspecific binding, followed by 3 h incubation at room temperature with various concentrations (10^{-5}–10^{-12} M) of test compounds in the presence of biotinylated vitronectin (1 µg mL^{-1}). Biotinylation was performed using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). After washing, the plates were incubated for 1 h at room temperature with biotinylated streptavidin–peroxidase complex (Amersham Biosciences, Uppsala, Sweden) followed by 30 min incubation

with 100 µL per well Substrate Reagent Solution (R&D Systems, Minneapolis, MN, USA) before stopping the reaction with the addition of 50 µL per well 2 N H$_2$SO$_4$. Absorbance at 415 nm was read in a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represents the average of triplicate wells; data analysis was carried out by non-linear regression analysis with GraphPad Prism software. Each experiment was repeated in triplicate.

General procedure for the measurement of cytotoxic activity. Human breast MDA-MB231 and ovarian IGROV-1 carcinoma cell lines were cultured *in vitro* with RPMI plus 10% fetal calf serum. For cytotoxic experiments, 10^4 cells were plated in 96 well plates and grown overnight before being treated with serial dilutions of the compounds (concentrations ranging from 25 µM to 10 nM). Cell viability was determined after 72 hours using the CellTiter-Glo (Promega) assay for ATP. Three independent experiments were performed in triplicates.

Acknowledgements

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