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Peptidomimetics

Cyclic *iso*DGR and RGD Peptidomimetics Containing Bifunctional Diketopiperazine Scaffolds are Integrin Antagonists

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Abstract: The *cyclo*[DKP-*iso*DGR] peptidomimetics **2**–**5**, containing bifunctional diketopiperazine (DKP) scaffolds that differ in the configuration of the two DKP stereocenters and in the substitution at the DKP nitrogen atoms, were prepared and examined in vitro in competitive binding assays with purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. IC_{50} values ranged from low nanomolar (ligand **3**) to submicromolar with $\alpha_v\beta_3$ integrin. The biological activities of ligands *cyclo*[DKP3-RGD] **1** and *cyclo*[DKP3-*iso*DGR] **3**, bearing the same bifunctional DKP scaffold and showing similar $\alpha_v\beta_3$ integrin binding values, were compared in terms of their cellular effects in human U373 glioblastoma cells. Compounds **1** and **3** displayed overlapping inhibitory effects on the FAK/Akt integrin activated transduction pathway and on integrin-mediated cell infiltration processes, and qualify therefore, despite the different RGD and *iso*DGR sequences, as integrin antagonists. Both compounds induced apoptosis in glioma cells after 72 hour treatment.

Introduction

Integrins are a large family of cell adhesion receptors composed of two noncovalently bound α and β transmembrane glycoproteins.[1] Integrins recognize their ligands at the extracellular α/β subunit interface containing the metal ion-dependent adhesion site (MIDAS), through tripeptide sequences bearing an acidic residue that are generally present in flexible loop regions.[2] Several integrins, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$, bind the tripeptide sequence Arg-Gly-Asp (RGD) in endogenous ligands, and the context of the ligand RGD sequence (flanking residues, three-dimensional presentation) and individual features of the integrin binding pockets determine the recognition specificity. RGD-binding integrins play an important role in biological and pathological processes, promoting endothelial cell attachment on the surrounding extracellular matrix, cell migration, cell to cell interaction, and intracellular signal transduction. In particular, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins are involved in angiogenesis, tumor progression, and metastasis, whereas the platelet $\alpha_{IIb}\beta_3$ receptor is central to hemostasis and contributes to thrombosis.[3]

Nowadays, a wealth of linear or cyclic peptidic and peptidomimetic integrin ligands have been developed and a few potent compounds are in different stages of clinical trials as anticancer drugs, or in clinical use for antithrombotic therapy.[3,4]

We have recently reported a new class of cyclic peptidomimetic RGD-integrin ligands containing a bifunctional diketopiperazine scaffold (DKP1-DKP7, Figure 1) formally derived from 2,3-diaminopropionic acid and aspartic acid and differing in the configuration of the two DKP stereocenters and in the substitution at the DKP nitrogen atoms.[5] The RGD-peptidomimet-

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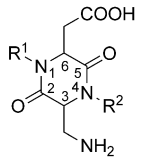
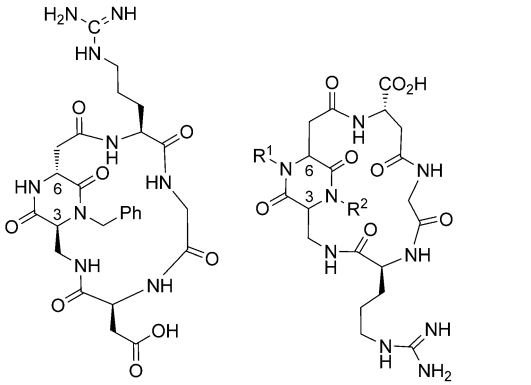


DKP1 = 3*S*, 6*S*, R^1 = H, R^2 = CH_2Ph
DKP2 = 3*R*, 6*S*, R^1 = H, R^2 = CH_2Ph
DKP3 = 3*S*, 6*R*, R^1 = H, R^2 = CH_2Ph
DKP4 = 3*R*, 6*S*, R^1 = CH_2Ph, R^2 = H
DKP5 = 3*R*, 6*S*, R^1 = CH_2Ph, R^2 = CH_2Ph
DKP6 = 3*S*, 6*R*, R^1 = CH_2Ph, R^2 = H
DKP7 = 3*S*, 6*R*, R^1 = CH_2Ph, R^2 = CH_2Ph

Figure 1. Bifunctional DKP scaffolds.

ics derived from *trans*-DKP scaffolds inhibited biotinylated vitronectin binding to the purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors with nanomolar IC_{50} values.[5c,d,6] This high affinity was rationalized in terms of well-defined preferred conformations featuring intramolecular hydrogen-bonded turn motifs and an extended arrangement of the RGD sequence, which are preserved in the interaction of these ligands with the receptors (docking studies).[5c,d]

These results were further supported by cell adhesion experiments on ECV304 bladder cancer cells, which are known to overexpress integrin $\alpha_v\beta_3$ on their membrane.[7] The binding epitope of some of these ligands in their interaction with living ECV304 bladder cancer cells and human platelets (expressing integrin $\alpha_{IIb}\beta_3$) was investigated by tr-NOE and saturation transfer difference (STD) NMR experiments, and the results were consistent with those obtained by docking studies.[7] In addition, *cyclo*[DKP3-RGD] **1** (Figure 2) was shown to effectively



1 (3*S*, 6*R*)

2 = 3*R*, 6*S*, R^1 = H, R^2 = CH_2Ph
3 = 3*S*, 6*R*, R^1 = H, R^2 = CH_2Ph
4 = 3*R*, 6*S*, R^1 = CH_2Ph, R^2 = CH_2Ph
5 = 3*S*, 6*R*, R^1 = CH_2Ph, R^2 = CH_2Ph

Figure 2. Cyclic RGD and *iso*-DGR integrin ligands containing the bifunctional diketopiperazine scaffolds.

inhibit the angiogenic processes (reduction of capillary network formation) in HUVEC, possibly through mechanisms involving reduced Akt phosphorylation and disruption of integrin-mediated adhesion, without affecting their viability and proliferation.[8]

Recently, biochemical studies have shown that the Asn-Gly-Arg (NGR) motif of the extracellular matrix protein fibronectin can spontaneously transform into the *iso*Asp-Gly-Arg (*iso*DGR) sequence by a post-translational modification.[9] This asparagine/*iso*aspartate rearrangement, which involves attack of the protein backbone NH to the primary amide side chain of asparagine and ring opening of the succinimide, is a well-known side reaction normally leading to loss of biological activity.[10] In contrast, in this case, the result is a gain of protein function and the creation of a new adhesion binding site for integrins.[11] The *iso*DGR sequence, as was demonstrated by subsequent biochemical, spectroscopic, and computational investigations, can fit into the RGD-binding pocket of $\alpha_v\beta_3$ integrin, establishing the same electrostatic clamp as well as additional polar interactions.[12] Based on these observations, a few conformationally constrained cyclopeptides containing the *iso*DGR sequence were synthesized.[13] However, these ligands showed a moderate affinity for $\alpha_v\beta_3$ integrin in competitive binding assays.[13b]

Results and Discussion

We have recently prepared two cyclic *iso*DGR peptidomimetics (**2** and **3**; Figure 2) containing the bifunctional diketopiperazine scaffolds DKP2 and DKP3 (Figure 1), and investigated their conformation in solution and their ability to compete with biotinylated vitronectin for binding to purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors.[14] Conformational studies (NMR, molecular modeling and docking calculations) revealed that the *cyclo*[DKP-*iso*DGR] ligands adopt extended conformations in solution and in the binding site of integrin $\alpha_v\beta_3$, thus behaving similarly to the *cyclo*[DKP-RGD] ligands.[14] In the present paper, we have extended the *cyclo*[DKP-*iso*DGR] integrin ligand collection by adding two more members (**4** and **5**; Figure 2), containing the dibenzylated diketopiperazine scaffolds DKP5 and DKP7 (Figure 1).

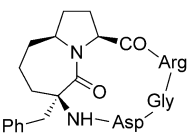
The *cyclo*[DKP-*iso*DGR] ligands **2**–**5** were conveniently prepared with a mixed solid-phase/solution-phase methodology on SASRIN resin, by following the strategy depicted in Scheme 1. The DKP-scaffolds are normally obtained as *N*-Boc protected amino acids,[5] and, as such, they are unsuitable for solid-phase synthetic applications. To avoid an exchange of the nitrogen protecting group, with a deprotection–reprotection sequence, DKP azido-acids **6**–**9** were used (Scheme 2).

Compounds **6**–**9** were obtained from the azido esters **10** and **11**, which are intermediates in the synthetic scheme usually employed for the DKP synthesis.[5] Azido esters **12** and **13** were synthesized by deprotonation with KHMDS and alkylation with benzyl bromide of compounds **10** and **11**, respectively (Scheme 2). Azido esters **10**–**13** were deallylated ($[Pd(PPh_3)_4]$ cat., *N*-methyl aniline) to afford the carboxylic acid derivatives **6**–**9** ready for coupling to the solid support.

A detailed description of the solid-phase/solution-phase synthesis of *cyclo*[DKP-*iso*DGR] ligands **2**–**5** (Scheme 1) is reported in the Supporting Information.

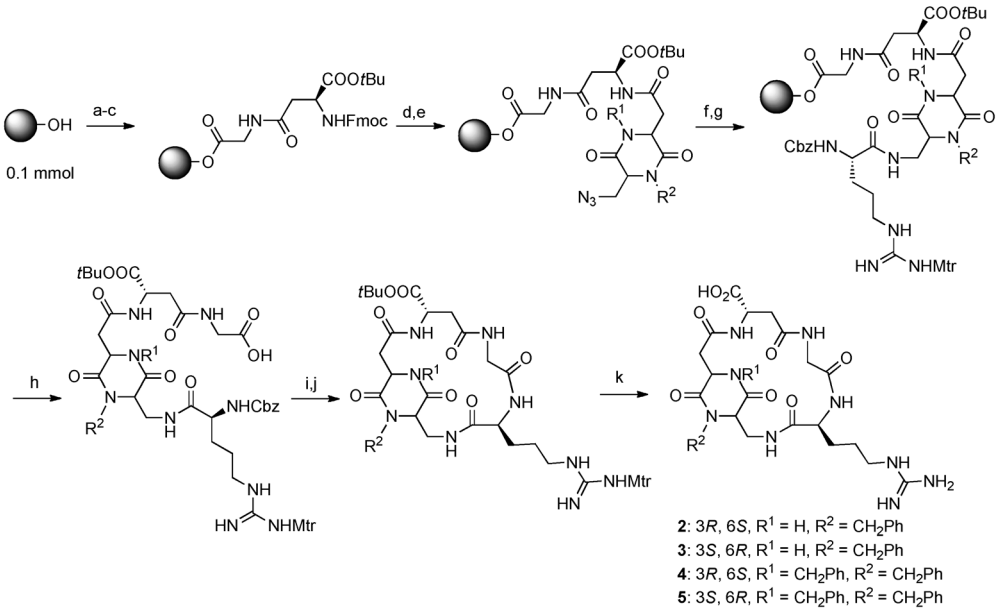
Initially, cyclic *iso*DGR peptidomimetics **2**–**5** were examined in vitro in competitive binding assays with biotinylated vitronectin for binding to purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors. The results are reported in Table 1, in which we have also included the values of *cyclo*[DKP3-RGD] ligand **1**, of *cyclo*[RGDf(*N*-Me)V] (Cilengitide), and of the cyclic peptidomimetic *cyclo*[1a-RGD] **14** (Figure 3), previously investigated as an integrin antagonist.[15]

Compared with the corresponding RGD ligands,[5d] the *iso*DGR compounds showed a much more pronounced dependence of integrin binding affinity on the structure of the DKP scaffold: the IC_{50} values of ligands **2**–**5** (Table 1, entries 2–5) ranged from low nanomolar to



Cyclo[1a-RGD] **14**

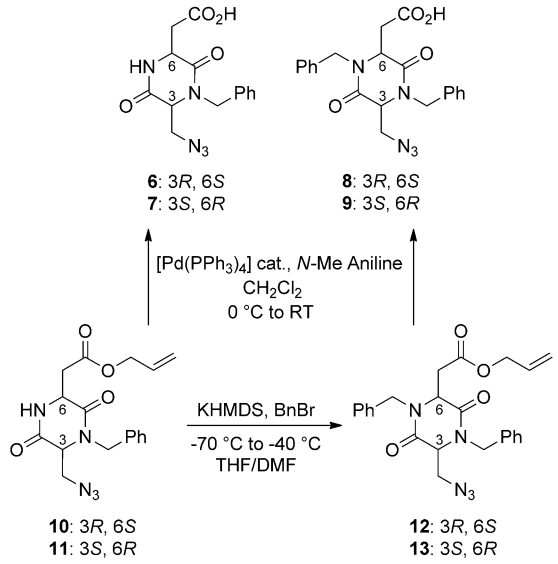
Figure 3. The integrin antagonist *cyclo*[1a-RGD] **14**.



Scheme 1. Reagents and conditions: a) Fmoc-Gly-OH, DIC, DMAP (cat.), DMF; b) 2% piperidine and 2% DBU in DMF; c) Fmoc-Asp(OH)-O*t*Bu, DIC, HOAt, DMF; d) 2% piperidine and 2% DBU in DMF; e) **6**–**9**, DIC, HOAt, DMF; f) Me_3P, dioxane/water 4:1, 3×20 min; g) Cbz-Arg(Mtr)-OH, DIC, HOAt, DMF; h) 1% TFA in CH_2Cl_2, RT, 10×5 min; i) H_2, 10% Pd/C, THF/water 1:1; j) HATU, HOAt, DIPEA, DMF (1.4 mM); k) TFA/thioanisole/EDT/anisole 90:5:3:2.

azine scaffold and showing similar $\alpha_v\beta_3$ integrin binding values, were investigated and compared by evaluating several cellular functional effects in human U373 glioblastoma cells that overexpress $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins.

Integrin ligands containing the RGD sequence have been shown to exert antiangiogenic activity in tumors expressing high levels of $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins, such as human glioblastoma.[17] The role of these ligands is to prevent the interaction of integrins with their endogenous extracellular matrix (ECM) ligands. The binding of ECM ligands to integrins activates integrin receptor clustering and formation of an activated complex between the cytosolic tyrosine kinase SRC, which is



Scheme 2. Diketopiperazine azido acids intermediates **6**–**9**.

Table 1. Inhibition of biotinylated vitronectin binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors.

Entry	Ligand	Structure	$\alpha_v\beta_3$ IC_{50} [nM][a]	$\alpha_v\beta_5$ IC_{50} [nM][a]
1	**1**	*cyclo*[DKP3-RGD]	4.5 ± 1.1	149 ± 25
2	**2**	*cyclo*[DKP2-*iso*DGR]	46.7 ± 18.2	220 ± 84
3	**3**	*cyclo*[DKP3-*iso*DGR]	9.2 ± 1.1	312 ± 21
4	**4**	*cyclo*[DKP5-*iso*DGR]	490 ± 77	9100 ± 800
5	**5**	*cyclo*[DKP7-*iso*DGR]	255 ± 140	5100 ± 400
6	Cilengitide	*cyclo*[RGDf(*N*-Me)V]	0.6 ± 0.1[b]	11.7 ± 1.5[b]
7	**14**	*cyclo*[1a-RGD]	3.3 ± 1.4	266.5 ± 18.4

[a] IC_{50} values were calculated as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding. Screening assays were performed by incubating the immobilized integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ with increasing concentrations (10^{-12}–10^{-5} M) of the *iso*DGR ligands in the presence of biotinylated vitronectin (1 mg mL^{-1}), and measuring the concentration of bound vitronectin in the presence of the competitive ligands, see ref. [14]. [b] Calculated as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding, see ref. [16].

submicromolar with $\alpha_v\beta_3$ integrin. In particular, ligand **3** (entry 3) showed an IC_{50} value similar to that known for high-affinity RGD ligands such as the cyclic peptidomimetic *cyclo*[DKP3-RGD] **1** (entry 1), the cyclopentapeptide Cilengitide[16] (entry 6), and the cyclic peptidomimetic *cyclo*[1a-RGD] **14** (entry 7).[15] The other *iso*DGR ligands, and in particular **4** and **5**, are weaker binders and are comparable to other *iso*DGR compounds recently published by Kessler and co-workers.[13b]

The biological properties of one RGD ligand, namely *cyclo*[DKP3-*RGD*] **1**, and the highest affinity *iso*DGR ligand *cyclo*[DKP3-*iso*DGR] **3**, bearing the same bifunctional diketopiperazine scaffold, were investigated in human U373 glioblastoma cells that overexpress $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins, which are constitutively bound to the integrin β cytoplasmic tail, and the focal adhesion kinase (FAK).[18] FAK is at the intersection of several signaling pathways that promote cancer growth and metastasis, such as the activation of downstream ERK- and Akt-dependent signaling pathways, which are responsible for cell survival. Several integrin ligands are currently under investigation for their antiangiogenic and anticancer activity, administered either alone or in combination with other therapeutic agents such as temozolomide.[19] Among these, Cilengitide has recently failed a Phase III clinical trial, called CENTRIC, for the treatment of patients with newly-diagnosed glioblastoma and

methylated O6-methylguanine-DNA methyltransferase (MGMT) gene promoter status.[20] In this study, Cilengitide was administered as an angiogenesis inhibitor, in combination with temozolomide and radiotherapy. However, Cilengitide is involved in other clinical trials (Phase II) both alone and in combination with other drugs and monoclonal antibodies.[21]

Notwithstanding this failure, integrins remain a potential target for the treatment of several tumors. These receptors, in fact, besides their role in the neo-angiogenic processes, have been shown to actively contribute to tumor proliferation and metastatic behavior, in both in vivo and in vitro studies.[17] For these reasons, integrin antagonists are intensively investigated to unravel the cellular signaling pathways in which they are involved.

As described in the introduction, we have recently studied the activity of ligand *cyclo*[DKP3-RGD] **1** as an angiogenesis inhibitor in human umbilical vein endothelial cells (HUVEC), and observed a significant inhibition of Akt phosphorylation.[8] Similarly, a marked inhibition of FAK phosphorylation was reported in experiments with *cyclo*[1a-RGD] **14**[15] on glioblastoma cell lines, opening an interesting outlook for the anticancer activity of these compounds. Considering the comparable binding values of *cyclo*[1a-RGD] **14**, *cyclo*[DKP3-RGD] **1**, and *cyclo*[DKP3-*iso*DGR] **3**, and the structural correlation of the latter two ligands, we investigated the activity of compounds **1** and **3** on U373 human glioblastoma cells, with particular attention focused on their ability to affect cell viability and cell infiltration as well as their effect on FAK and Akt phosphorylation.[22] The U373 cell line has been previously shown to express $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrin receptors and the expression pattern of these receptors does not vary following treatment with integrin antagonists.[15]

Preliminary cell viability experiments gave no significant results in short time treatments: in fact, the MTS cell viability assay showed a decrease of cell viability only after 72 h treatment (not shown). These results are not surprising because it has been previously shown that RGD integrin antagonists do not exert direct cytotoxic effects on cancer cells.[6,8,15]

To ascertain whether the cell viability decrease was due to apoptotic cell death, ELISA nucleosome assays were performed by treating the cells with 1 and 25 μM concentrations of ligands **1** and **3** and for increasing times. A significant increase of nucleosome content was observed when cells were treated with a 25 μM solution of either **1** or **3** for 72 h (Figure 4), indicating that the cells were undergoing apoptotic death.

It is known that RGD-binding integrin inhibitors exert an inhibitory effect on cell migration and cell infiltration processes and this is probably the most interesting effect of RGD-binding integrin antagonists.[23,24] To test the cellular effects of **1** and **3**, infiltration assays were performed by using U373 glioblastoma cell lines and fetal bovine serum (FBS) as chemo-attractant. The compounds were added to the medium at increasing concentrations when cells were plated in serum-free medium on the insert membrane covered by maxgel; after 8 h incubation, the trans-well membranes were removed, stained by DAPI (a fluorescent blue dye) and cells were counted. A marked decrease of infiltrated cells was observed for both compounds at

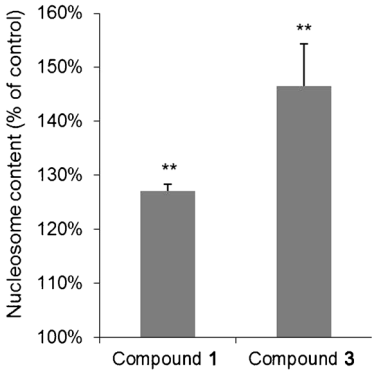


Figure 4. Compounds **1** and **3** induce apoptosis in U373 cells. A significant increase of nucleosome content was observed when cells were treated with compounds **1** (25 μM) or **3** (25 μM) for 72 h. Data are expressed as percent of controls (100%). **Calculated probability value $p < 0.005$.

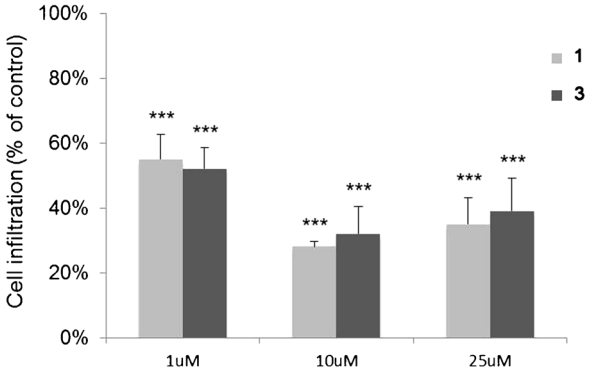


Figure 5. Compounds **1** and **3** inhibit cell infiltration induced by serum. Cell infiltration tests were performed by using increasing concentrations (1, 10, 25 μM) of compounds **1** and **3**. After 8 h incubation, the number of cells counted on the lower side of the membrane was significantly diminished in treated cells compared with those of the controls. Data are expressed as percent of controls (100%). ***$p < 0.001$.

10 μM concentration, remaining substantially unchanged at 25 μM (Figure 5). These data are consistent with previously reported data on *cyclo*[1a-RGD] **14**.[15]

FAK is a cytoplasmic tyrosine kinase that promotes cell motility, survival, and proliferation through different mechanisms. FAK associates with receptors at the plasma membrane and with distinct protein complexes in the nucleus. The most well-characterized mechanism that promotes FAK activation involves integrin receptor clustering upon the binding of cells to extracellular matrix proteins. Integrin-induced FAK clustering and activation appear to be the key step in tumor cell growth, tumor progression, and metastasis spreading.[18] This leads to the formation of an activated FAK-SRC complex that, in turn, activates the Akt signaling cascade. Akt is an intracellular kinase that is directly activated by integrin-mediated FAK-SRC activation. It is involved in survival signal cascade and represents a suitable intracellular integrin-linked effector to evaluate the activation of RGD-binding integrin transduction pathway.

To investigate whether the binding of the two compounds affected integrin-induced FAK and Akt phosphorylation, U373 cells were treated with the two ligands **1** and **3** (25 μM) for increasing times and western blot analysis was performed on the cell extracts. A decrease of both FAK and Akt phosphorylation was observed when the cells were treated with the two compounds for 8 and 24 h. The decrease of the phosphorylated signal was particularly evident after 24 h treatment (Figure 6, Figure 7). Taken together, these data show that both

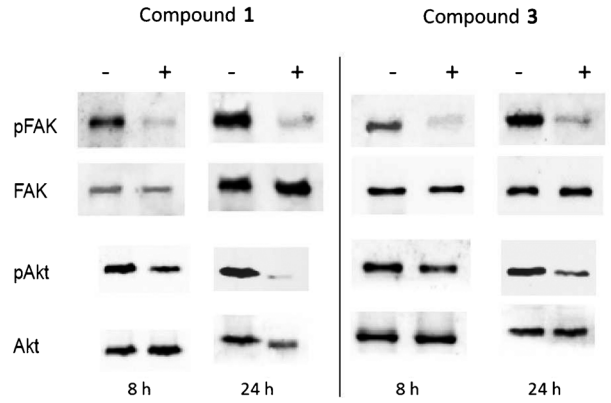


Figure 6. Compounds **1** and **3** inhibit FAK and Akt phosphorylation. Cells were treated with a 25 μM solution of compounds **1** or **3** (+) for 8 and 24 h; 30 μg of protein extracts for each sample were analyzed by western blot. Both compounds strongly reduced the phosphorylated signal after 24 h treatment compared with controls (−).

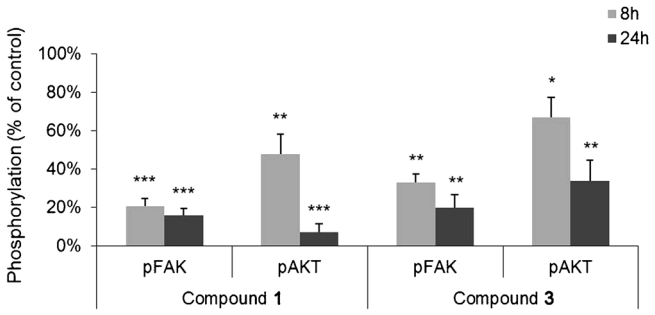


Figure 7. Inhibition of FAK and Akt phosphorylation by compounds **1** and **3** after 8 and 24 h: densitometric analysis. *$p < 0.5$; **$p < 0.005$; ***$p < 0.001$.

compounds exert a marked inhibitory effect on tumor cells infiltration processes and suggest that this effect could be mediated by inhibition of the FAK and Akt signaling cascade.

A few studies have investigated the cellular effects and the underlying molecular mechanisms exerted by $\alpha_v\beta_3$ integrin ligands in in vitro models of human glioblastoma.[15] Our work expands these investigations, examining the effects on U373 human glioblastoma cells of both RGD and *iso*DGR peptidomimetic $\alpha_v\beta_3$ integrin ligands. In particular, our results for *cyclo*[DKP3-RGD] **1** are consistent with previously reported data on other small molecule RGD ligands such as *cyclo*[1a-RGD] **14**[15] at comparable concentrations, and are indicative of an integrin antagonist activity. Similar results were obtained for *cyclo*[DKP3-RGD] **1** in human endothelial cells in which *cyclo*[DKP3-RGD] **1** was able to effectively inhibit the angiogenic process (reduction of capillary network formation) already at nanomolar concentrations, and to inhibit Akt phosphorylation at micromolar (1 μM) concentration.[8]

Whereas the cellular effects of compounds **1** and **3** appear to be consistent with their profile of integrin antagonist, like *cyclo*[1a-RGD] **14**, the comparison with Cilengitide activity profile appears more controversial. In fact, previous studies have demonstrated that, under certain experimental conditions (in particular at nanomolar concentrations), Cilengitide induces agonist-like activities and adverse paradoxical integrin activation effects, and have raised concerns about the efficacy of RGD compounds as true integrin antagonists.[12c,25] Specifically, Cilengitide was shown to promote angiogenesis in HUVEC at nanomolar concentrations.[25] Furthermore, in the same cellular model, Cilengitide was reported to induce FAK phosphorylation but at micromolar concentrations (10 μM).[26] On the other hand, in a previous paper, Cilengitide was reported to induce anoikis and to inhibit FAK phosphorylation in glioblastoma and endothelial cells in the micromolar concentration range (1–80 μM).[27] In light of the cited literature, Cilengitide cellular effects appear to be strictly dependent on the experimental conditions and on the cellular model. For these reasons, a comparison of compounds **1** and **3** with Cilengitide is not appropriate because the cited data on Cilengitide[25,26,27] have been obtained under different experimental settings and using different cell types.

As far as *cyclo*[DKP3-*iso*DGR] **3** is concerned, to our knowledge, this is the first time that an *iso*DGR ligand has been studied and found to be active in the inhibition of FAK/Akt integrin activated transduction pathway and on integrin-mediated cell infiltration processes. These results, which are suggestive of an antagonist activity, are consistent with previously reported conclusions for other *iso*DGR-containing cyclopeptides.[12c]

Conclusion

Four *cyclo*[DKP-*iso*DGR] integrin ligands (**2**–**5**) were synthesized and their ability to bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins was studied. Differences in the scaffold substitution were shown to induce greater variability compared with the analogous *cyclo*[DKP-RGD] compounds. At least one low-nanomolar ligand was identified, *cyclo*[DKP3-*iso*DGR] **3**, which is, to our knowledge, the most potent *iso*DGR $\alpha_v\beta_3$ integrin ligand. To better investigate the *iso*DGR in comparison to the RGD motif, we evaluated the activity of *cyclo*[DKP3-RGD] **1** and *cyclo*[DKP3-*iso*DGR] **3**, bearing the same bifunctional diketopiperazine scaffold and showing similar $\alpha_v\beta_3$ integrin binding values, with respect to several cellular effects in human U373 glioblastoma cells. Compounds **1** and **3** display overlapping inhibitory effects on the FAK/Akt integrin activated transduction pathway and on integrin-mediated cell infiltration processes and qualify therefore, despite the different RGD and *iso*DGR sequences, as integrin antagonists. In addition, they induce apoptosis in glioma cells after 72 h treatments. These findings appear to be consistent with the biological effect exerted by other integrin antagonists

(e.g., *cyclo*[1a-RGD] **14**) and suggest that compounds **1** and **3** are good candidates for further in vitro and in vivo studies.

Experimental Section

The synthesis of intermediates **6** and **7**,[5d] starting from protected (*S* or *R*)-serine and (*S* or *R*)-aspartic acid derivatives, and of the final compounds **2** and **3** was conducted by following published procedures.[14]

General procedure for diketopiperazine N-benzylation: In a flame-dried flask, KHMDS (1.1 equiv) was dissolved in anhydrous toluene under nitrogen atmosphere to give a 0.5 M solution. The solution was cooled to −70 °C and the *N*-monobenzylated diketopiperazine **10** or **11** (1 equiv), previously dissolved in anhydrous THF, was added dropwise. The mixture was stirred under these conditions for 30 min, then benzyl bromide (5 equiv) and DMF were sequentially added. The reaction was allowed to reach −40 °C and stirred under these conditions for 5 h. After this time, the reaction mixture was diluted with a saturated solution of NH_4Cl and extracted three times with EtOAc. Organic layers were combined, washed with brine, and dried over Na_2SO_4. Volatiles were removed under reduced pressure and the crude material was purified by flash chromatography on silica gel, affording the desired product as a transparent oil.

General procedure for diketopiperazine allyl ester deprotection: Diketopiperazine allyl esters **10**–**13** (1 equiv) were dissolved in CH_2Cl_2 under a nitrogen atmosphere, and the mixture was cooled to 0 °C. [$Pd(PPh_3)_4$] (0.3 equiv) and freshly distilled *N*-methyl aniline (1.2 equiv) were added in sequence and the mixture was then warmed to RT. After stirring for 1 h, the mixture was diluted with EtOAc and extracted four times with aqueous $NaHCO_3$. The combined aqueous phases were acidified to pH 2 with 1 M $KHSO_4$ and then extracted four times with CH_2Cl_2. The resulting organic phase was dried over Na_2SO_4 and the solvent was evaporated to afford the desired product as a slightly yellow solid.

Cell culture

The U373 human glioblastoma cell line was purchased from Istituto Zootecnico Regione Lombardia (Brescia, Italy) and grown as described.[15] The reagents used for the cell cultures were from Gibco - life technologies.

Infiltration assay

Cells were plated in serum-free DMEM on transwells (Costar) coated by Maxgel (Sigma–Aldrich). In the bottom of the wells, 500 μL DMEM containing 10% FBS were placed as chemoattractant. The infiltration assays were carried out for 8 h in the presence of either **1** or **3** (1, 10, and 25 μM) in a cell culture incubator. After removing the Maxgel, the cells migrated on the lower face of the membrane were stained by DAPI (Sigma–Aldrich) and counted with a fluorescence microscope. The experiments were run in duplicate and at least ten fields were counted for each membrane.

Western blot analysis

Cells grown in 60 mm dishes were treated for the indicated time with 25 μM **1** or **3**. Afterwards, the cells were rinsed twice in ice-cold PBS and 200 μL of cell lysis buffer were added to the dishes [composition: 50 mM Tris-HCl pH 7.4, 1% v/v NP40, 0.25% w/v sodium deoxycholate, 1 mM phenylmethylsulfonyl-fluoride (PMSF), 1 mM Na_3VO_4, 1 mM EDTA, 30 mM sodium pyrophosphate, 1 mM NaF, 1 μg mL^{-1} leupeptin, 1 μg mL^{-1} pepstatin A, 1 μg mL^{-1} aprotinin, and 1 μg mL^{-1} microcystin]. After scraping, the cells were sonicated for 10 s, centrifuged at 12,000 g for 5 min at 4 °C and the amount of protein in the supernatant was measured by using the BCA protein Assay Kit (Pierce). For western blot analysis, 30 μg proteins were separated by 10% SDS-PAGE at 150 V for 2 h and blotted onto 0.22 μm nitrocellulose membranes at 50 mA for 16 h. The membranes were first blocked for 2 h in Tris buffered saline solution (TBST composition: Tris 10 mM, NaCl 150 mM, 0.1% Tween 20) plus 2% low-fat dry milk and 2% albumin (TBSTM) and then incubated with the appropriate antibody diluted 1:1000 in TBSTM, for 16 h at 4 °C under gentle agitation. The membranes were rinsed three times in TBST and then incubated for 2 h at 21 °C with a goat anti-rabbit IgG horseradish-peroxidase conjugate secondary antibody (Upstate Biotechnology), diluted 1:10,000 in TBSTM. The membranes were rinsed three times in TTBS and the luminescent signal was detected by the ECL plus Western Blotting Detection System (Amersham). Each experiment was repeated at least three times.

ELISA apoptosis assay

For the relative quantification of apoptosis, a sandwich immunoassay was performed to detect nucleosomes (Cell death detection ELISA, Roche diagnostics). Cells were plated in a 12 multiwell in the growth medium and treated with 25 μM **1** or **3** for increasing times. At the end of the incubation time, the assay was performed by following the manufacturer's instructions. Finally, the samples were read in a multiwell reader at 405 nm. Five wells were used for each experiment and each experiment was repeated three times.

Statistics

Differences between groups (one-way analysis of variance with Tukey's post hoc) and differences between pairs of data (unpaired two-tailed Student's *t* test) were analyzed by using Prism (v5.0d; GraphPad Software).

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