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Inhibition of cancer cell adhesion by heterochiral Pro-containing RGD mimetics

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Abstract—In this paper, we describe the synthesis of a selected library of heterochiral D-Pro-containing RGD-peptidomimetics (RpD) and we investigate the biological activity as inhibitors of fibronectin adhesion to SK-MEL-24 tumor cells. In particular, peptides **4** and **8** showed an IC_{50} in the 10^{-8} M range. Despite the linear structure, the peptides tend to adopt a folded conformation in a polar environment.

Most of the physiological functions are regulated by interactions between peptides or proteins and receptors or other proteins. Very often, such ligands exert their biological activity[1] by means of relatively small regions of their folded surfaces, therefore their actions can be reproduced by much smaller drugs that retain these localized bioactive surfaces. A prototypic example is constituted by integrins[2] and their native ECM ligands, fibronectin, vitronectin, fibrinogen, laminin, von Willebrand factor, etc. For instance, $\alpha_v\beta_3$ integrins, expressed in various cell types (endothelial cells, melanoma, osteoclast, smooth muscle cells, etc.), recognize their ligands by interacting with a very short tripeptidic-binding motif, RGD. Therefore, a number of integrin antagonists based on the RGD sequence have been designed as therapeutics for the treatment of angiogenesis,[3] tumors,[4] osteoporosis,[5] etc. However, for the structural similarities within the integrin family and between their respective ligands, α_v integrins, as well as integrins $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_{IIb}\beta_3$, recognize the same RGD sequence. As a consequence, the selectivity issue constitutes a major concern in the design of potential therapeutic agents. For this reason, RGD mimetics[6–8] have been quite often equipped with some kind of conformational biases, such as rigid cores, cyclic structures, turn-inducers, etc.

Since low molecular weight and high bioavailability are necessary features to pursue for the development of a drug,[6,8] the search for effective integrin antagonists moved from large, cyclic peptides to smaller, structurally simpler, and possibly achiral peptidomimetics[6] or non-peptide analogues,[8] leading to the identification of numerous small-sized selective antagonists that entered clinical stages.[1,6,8] Accordingly, we decided to investigate the possibility to design small, simple di- or tri-peptidic RGD-analogues having an intrinsic tendency to adopt well-defined secondary conformations compatible with the requisites for ligand–receptor interaction. Extensive SAR, molecular modeling, and docking studies[6,9–11] have shown that a comparatively short distance between guanidino and carboxylic groups allowed selective binding to $\alpha_v\beta_3$ over other RGD-binding integrins and, in several cases, a γ-turn conformation centered on Gly[6,12,13] has been found to favor the bioactive conformation.

Herein we describe the solid-phase synthesis and the pharmacological characterization of a minilibrary of short RGD mimetics containing the heterochiral sequence Xaa-D-Pro-Yaa, where Xaa is Arg or Arg-mimetic and Yaa is an Asp derivative. Heterochiral Pro-containing sequences are considered privileged

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inductors of folded conformations characterized by β- or γ-turn like structures.[14–17] Besides, it has been shown that 10- and 7-membered H-bonded rings form competitively with each other, depending on the solvent,[18] on the prevalence of a *trans* over *cis* conformation of the amide bond preceding Pro,[19] and/or on the nature and steric hindrance of the amino acids preceding and following Pro.[20,21]

We synthesized by standard SPPS[22] a preliminary RpD library (Fig. 1) with different additional lipophilic flanking groups[23] at the N- and C-termini, which very often proved to be crucial for high affinity and selectivity.[6,8] To introduce such groups, Asp was N-capped with different acyl or tosyl derivatives, and cleavage of the peptides from the resin was performed by aminolysis with different amines (see also Scheme 1).[24]

To evaluate the potential activity as $\alpha_v\beta_3$ integrin inhibitors, we tested their ability to inhibit the adhesion of an $\alpha_v\beta_3$ integrin-expressing cell line, SK-MEL-24, (human malignant melanoma, metastasis to node) to fibronectin.[25] Fibronectin was immobilized on each well of 96-assay plates. SK-MEL-24 cells were pre-incubated with various concentrations of peptides (30 min, rt), and aliquots of this suspension were added to fibronectin-coated wells (in quadruplicate). The number of adherent cells was quantified by fluorometry. The activity of potential antagonists was determined by the number of cells adhered as compared to the control.

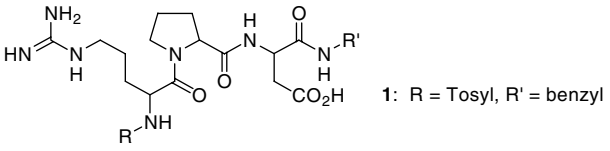
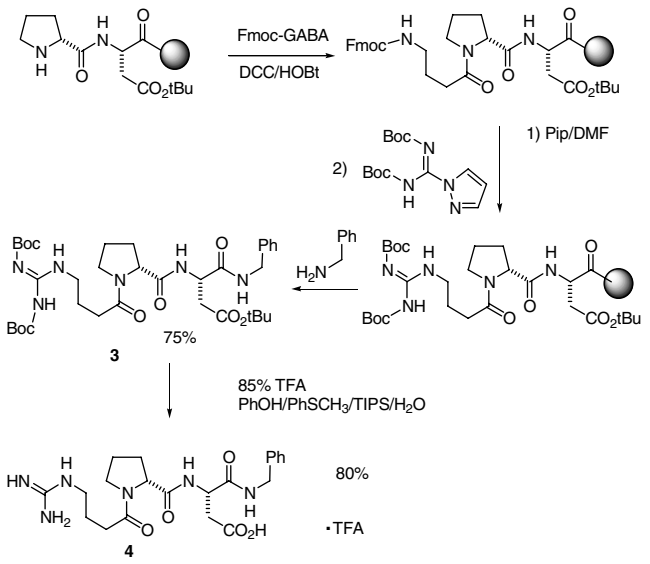


Figure 1. Structure of the RpD peptides.



Scheme 1. Synthesis of **4** by SPPS followed by aminolysis.

Among the peptides, the only active compound was **1**, which showed an IC_{50} of 1.5×10^{-7} M (Table 1). The diastereomeric peptide **2**, containing L-Pro, showed a 66-fold lower activity (Table 1).[26] The comparatively higher activity induced by the presence of a benzyl group close to Asp with respect to alkyl substituents is not unexpected. Benzyl carbamates, phenyl-sulfonamides or other isosteric groups adjacent to the acid have been beneficially introduced in integrin antagonists.[6,8,13] On the other hand, the presence of aromatic substituents in proximity of Arg gave contrasting results, leading to the generally accepted opinion that these groups are not strictly necessary for good receptor affinity.[6,8,13]

Based on these assumptions, to improve biological activity we decided to diminish molecular size and weight by removing the N-tosyl moiety. Besides, we modified the distance between the C-terminal carboxylic acid and the N-terminal guanidino group. Therefore, we synthesized a second minilibrary of peptides based on the structure of **1**, according to the methodology reported in Scheme 1 for the peptidomimetic **4**.

The peptide has been easily obtained by SPPS, using a Wang resin, Fmoc-protected amino acids, and DCC/HOBt as coupling agents in 9/1 DCM/DMF.[22] The cleavage of Fmoc group was performed by means of 20% piperidine/DMF. Introduction of the guanidino group on GABA-D-Pro-Asp was performed by treatment with *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine,[27] and the fully protected peptide was cleaved from the resin by aminolysis with benzylamine (Scheme 1).[24]

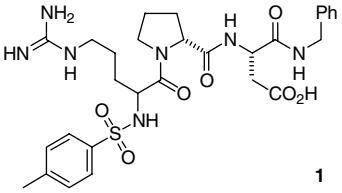
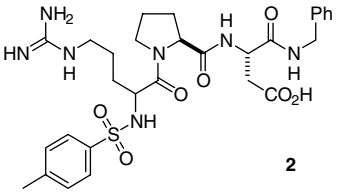
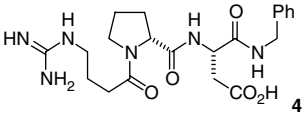
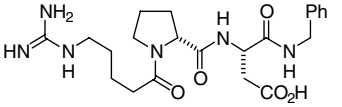
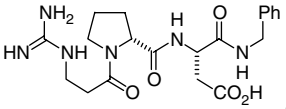
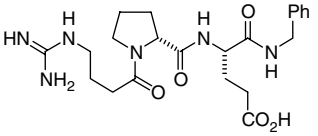
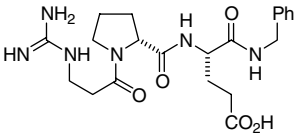
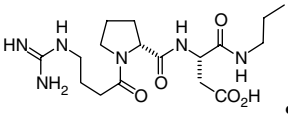
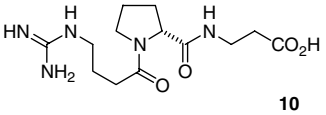
After purification of resulting **3** (75%) by flash chromatography over silica-gel (eluant EtOAc/MeOH 98:2), side chains were deprotected using a mixture of TFA and scavengers. Purification of **4** by semi-preparative RP-HPLC gave the peptide (80%), 96% pure by RP-HPLC/ES-MS analysis.

In this manner, we prepared the RpD analogues reported in Table 1. Their efficacy as $\alpha_v\beta_3$ integrin antagonists has been evaluated as above reported, by measuring their ability to inhibit fibronectin adhesion to SK-MEL-24 intact cells.

The synthesis of an analogue of **1** deprived of N-Ts group gave peptidomimetic **5**, but this modification was accompanied by a very strong activity decrease. Interestingly, much better results have been obtained for peptides **4** and **8**, which showed IC_{50} values of 2.6×10^{-8} and 3.5×10^{-8} M, comparable to that of the potent $\alpha_v\beta_3$ integrin antagonist AcDRGDS (Table 1, **11**).[28]

The comparison of **4** and **8** with the other peptides of Table 1 encouraged some considerations. First, while **4** and **8** have 11 bonds between the C-terminal carboxylic acid and the N-terminal guanidino group, peptides **5** and **7**, which possess 12 bonds, and **6**, which has 10 bonds between the same functionalities, show rather poor activities (Table 1). According to the literature, as a general rule for an $\alpha_v\beta_3$ antagonist optimal distance between the C-terminal carboxylic acid and the N-terminal guanidino

Table 1. Structures, analytical data, and inhibition of SK-MEL-24 cell adhesion to fibronectin, of compounds **1**–**11**

Compound	[M + 1] versus calculated[a]	Purity[b] (%)	IC_{50}[c], μM
 **1**	630.5/630.2	94	0.15 ± 0.02
 **2**	630.5/630.2	95	100 ± 10
 **4**	447.3/447.2	96	0.026 ± 0.002
 **5**	461.2/461.2	94	230 ± 18
 **6**	433.5/433.2	93	>100
 **7**	462.1/461.2	96	2.6 ± 0.3
 **8**	447.1/447.2	97	0.035 ± 0.004
 **9**	399.2/399.2	93	20 ± 2
 **10**	314.3/314.1	95	1.2
AcDRGDS **11**	—	—	0.020 ± 0.002

[a] Determined by ES-MS on a HP 1100 MSD.

[b] Determined by RP-HPLC on a HP Series 1100, with a HP Hypersil ODS column (4.6-μm particle size, 100 Å pore diameter, 250 mm), DAD 215.8 nm, under two distinct H_2O/CH_3CN gradient conditions, after purification by semi-preparative RP-HPLC.

[c] Values and SD are means of four experiments.

group seems to be roughly 12 C–C bonds, as in RGD. A few exceptions were described with 13[29]or 11 bonds.[30–33]

Second, the comparison of the activities of **4**, **8** with **9** and **10** confirms the beneficial role of the benzyl at the C-terminus.

Finally, it can be conjectured that the higher activity displayed by **4**, **8** in comparison to **1** could be also linked to the existence of a population of structures with a better defined secondary structure.[16] To provide evidence of the occurrence of a significant amount of folded conformations, we performed a preliminary ^{1}H-NMR and VT-NMR analysis of **12**, Ts-Arg(Mtr)-D-Pro-Asp(Ot-Bu)NH-Bzl, and **3** (Scheme 1), the fully protected[34] precursors of **1** and **4**, respectively, in a polar solvent (DMSO-d_6). It can be postulated that short peptides adopt in a polar environment different conformations; besides, Pro-containing peptides generally exist in the *trans* and *cis* configuration with respect to the Pro-omega bond.[14] Interestingly, while the ^{1}H-NMR of **12** revealed the presence of the two conformers in around 6:4 ratio, the ^{1}H-NMR of **3** showed a single set of resonances.[35] Furthermore, VT-NMR analysis of **3** is indicative of some folded population stabilized by a H-bond involving the NH-Bzl amide proton; for **12** only the NH-Bzl signal of the major *trans* conformer tends to be H-bonded. For **3** (ppb/°K), $\Delta\delta/\Delta t_{\text{NH-Bzl}} = -3.1$, versus $\Delta\delta/\Delta t_{\text{NH-Asp}} = -5.1$, in agreement with the literature for similar compounds characterized by the tendency to give a β-turn;[16] for **12**, $\Delta\delta/\Delta t_{\text{NH-Bzl-}trans} = -3.4$, $\Delta\delta/\Delta t_{\text{NH-Bzl-}cis} = -5.2$.

Albeit these observations are not conclusive, they support the speculation that **4** could be conformationally more homogeneous and well defined with respect to **1**. Apparently, the NH-Bzl proton of **4** seems to be involved in some type of β-turn. Several studies proposed that a β-turn on RGD leads to improved affinity toward $\alpha_{IIb}\beta_3$ integrins with respect to $\alpha_v\beta_3$, since the carboxylic acid and the N-terminal guanidino group adopt opposite orientations.[6,36,37] Nevertheless, heterochiral peptides with the sequence Xaa-Pro-D-Yaa behave differently and tend to fold in an inverse β-turn type II structure with the side chains of Xaa and D-Yaa oriented on the same side.[16] Accordingly, since GABA can behave as a D-amino acid, the enantiomeric sequence GABA-D-Pro-Asp can adopt a β-turn type II structure centered on D-Pro-L-Yaa with the guanidino group and Asp carboxylate side-chain oriented on the same side,[16] an overall conformation capable to satisfy the requisites for binding $\alpha_v\beta_3$ integrins. Further experiments are in progress to investigate in detail the conformational features of peptides **4** and **8**.

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Supplementary data

Full details on peptide synthesis and purification, and cell adhesion assays are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.073.

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