Novel and Selective $\alpha_v\beta_3$ Receptor Peptide Antagonist: Design, Synthesis, and Biological Behavior

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Among RGD-dependent integrins, the $\alpha_v\beta_3$ receptor has recently received increasing attention as a therapeutic target because of its critical role in tumor-induced angiogenesis and metastasis formation. Here, we describe a new peptide antagonist of $\alpha_v\beta_3$ receptor, designed on the basis of the crystal structure of integrin $\alpha_v\beta_3$ in complex with c(RGDf[NMe]V) and the NMR structure of echistatin. Cell adhesion assays have demonstrated that the peptide is a potent and selective antagonist of the $\alpha_v\beta_3$ receptor.

Introduction

Integrins are members of a family of heterodimeric transmembrane cell-surface receptors that play a crucial role in cell−cell and cell−matrix adhesion processes.[2] These receptors consist of an α- and a β-subunit that noncovalently associate in defined combinations.[3] Most of them recognize the Arg-Gly-Asp (RGD) triad found in many extracellular matrix proteins (i.e., vitronectin)[4] and snake venom disintegrins.[5−7] Even if different integrins recognize different proteins containing the RGD sequence, several studies have demonstrated that the amino acid residues flanking the RGD sequence of high-affinity ligands modulate their specificity of interaction with integrin complexes. Despite numerous studies reported in the literature, ligand selectivity toward different integrin subtypes is still a challenging problem mainly because most of the 3D structures of integrin subtypes remain unknown.[8]

An extensively studied member of this receptor class is integrin $\alpha_v\beta_3$. This integrin is strongly expressed in activated endothelial and melanoma cells. In contrast, it is weakly expressed in quiescent blood vessels and pre-neoplastic melanomas.[9] Along with $\alpha_v\beta_5$ integrin, $\alpha_v\beta_3$ is reported to be involved in physiological processes including angiogenesis and tissue repair as well as pathological conditions, such as tumor-induced angiogenesis[10,11] and tumor cell migration and invasion.[12] Despite the fact that both integrins promote cell attachment to vitronectin and participate in the same processes, they are reported to be structurally designed to respond to different signaling events. Previous studies provided evidence that bFGF-induced angiogenesis is mediated by $\alpha_v\beta_3$, whereas VEGF-induced angiogenesis is mediated by $\alpha_v\beta_5$.[13] Melanoma cells expressing $\alpha_v\beta_3$ migrate in vitro and metastasize in vivo without the need for exogenous cytokine stimulation.[14] Conversely, tumor cells expressing $\alpha_v\beta_5$ integrin require a tyrosine kinase receptor-mediated signaling event for motility on vitronectin and in vivo dissemination.[15] Although $\alpha_v\beta_5$ is widely expressed by many malignant tumor cells, $\alpha_v\beta_3$ has a relatively limited cellular distribution compared with that of $\alpha_v\beta_5$.[16,17] Therefore, to target $\alpha_v\beta_3$-mediated processes for diagnostic or therapeutic purposes, the development of new compounds that can discriminate between $\alpha_v\beta_3$ and $\alpha_v\beta_5$ is required.

To date, various therapeutic candidates, including antibodies,[18] small molecules[19,20] peptidomimetics,[21,22] and cyclic peptides,[23,24] have been clinically evaluated and shown to successfully modulate $\alpha_v\beta_3$-mediated processes. So far, the pentapeptide cyclo(-Arg-Gly-Asp-D-Phe-NMeVal-), referred to as c(RGDf-[NMe]V),[25] is one of the most active $\alpha_v\beta_3$ antagonists reported in the literature.[24,25] Previous studies have demonstrated a higher affinity of this peptide for integrin $\alpha_v\beta_3$ compared to $\alpha_v\beta_5$ and have reported the inhibition of $\alpha_v\beta_3$-mediated cell adhesion with IC_{50} values in the micromolar range when assayed in different tumor cell lines.[26]

The crystal structures of the extracellular segment of integrin $\alpha_v\beta_3$ in its unligated state and in complex with c(RGDf[NMe]V) and the docking studies on $\alpha_v\beta_3$ integrin ligands have shown that the main interactions are between the positively charged arginine and the α-subunit and between the anionic aspartic acid and the β-subunit[27−29] and that selectivity between different subunits is achieved by the RGD sequence conformation. Previous studies also reported that echistatin, the smallest (49 residues) of the viper (*Echis carinatus*) disintegrins, is a potent antagonist of the integrins $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$[30] and that the amino acids adjacent to the RGD motif together with the 41−49 *C*-terminal residues appear to be critical for the selective recognition of integrins. Mutation and photoaffinity cross-linking experiments and NMR conformational analysis combined with docking studies[31,32] have provided evidence that the *C*-terminal region of echistatin binds to a site within the β_3 subunit of the $\alpha_v\beta_3$ receptor, which is distinct from the sites that bind residues flanking the RGD triad in small peptide ligands.

Starting from this structural information, we have attempted to design and synthesize a novel and selective peptidomimetic $\alpha_v\beta_3$ receptor antagonist, referred to as RGDechi. The peptide was evaluated for its ability to inhibit cell adhesion to vitronectin in human erythroleukemia K562 cells overexpressing $\alpha_v\beta_3$ ($K\alpha_v\beta_3$) and $\alpha_v\beta_5$ ($K\alpha_v\beta_5$) receptors.

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Results and Discussion

Design. RGDechi is a bifunctional chimeric molecule containing a cyclic RGD motif and a sequence corresponding to the echistatin *C*-terminal tail connected by a linker.

As a starting point for the rational design, we used as template the crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with c(RGDf[NMe]V)[28,29] and the NMR structure of echistatin.[32]

We have built a theoretical model of the complex between the extracellular segment of integrin $\alpha_v\beta_3$ and echistatin using the following protocol: (a) the RGD sequence of echistatin was superimposed onto the RGD sequence of c(RGDf[NMe]V) in complex with integrin; (b) the cyclic peptide c(RGDf[NMe]V) was removed from the model; (c) an energy minimization on the echistatin/receptor complex was carried out by keeping all backbone atoms fixed to refine the spatial position of side chains; and (d) a full energy minimization was employed to refine the relative position of the ligand with respect to the receptor. The conformational and structural features of the obtained model are in good agreement with those reported in the literature. In particular, the *C*-terminal moiety (Arg^{41}-Thr^{49}) and the Met^{28}-Asp^{30} sequence interact mainly with the β_3 subunit, as reported in photoaffinity cross-linking experiments.[31] Furthermore, the residues from Met^{28} to Asp^{30} seem to contact the β_3 loop residues from Tyr^{122} to Lys^{125}. The *C*-terminal moiety adopts a conformation that gives rise to hydrophobic and hydrophilic interactions with the β_3 subunit and with a kink at position 47 due to the Pro residue that allows the moiety to accommodate on the β_3 subunit surface without unfavorable interactions. Then, the analysis underlines the importance of the ^{41}RNPHKGPAT^{49} residues for the selectivity of echistatin toward the $\alpha_v\beta_3$ receptor. Finally, the RGD loop shows an interaction pattern with the receptor similar to that found for c(RGDf[NMe]V).

On the superimposed models of echistatin/receptor and c(RGDf[NMe]V)/receptor complexes, we selected the Met^{28}-Asp^{30} and the Arg^{41}-Thr^{49} sequences of the echistatin and the RGD cyclic peptide with the aim of covalently binding these three modules.

The two echistatin fragments were covalently linked using an appropriate spacer. We tested, using computational procedures, several different dipeptides, and the most favorable linker was the Pro-Gly sequence, both from a structural and minimum energy point of view. In particular, the Pro residue introduces a kink in the structure, required to link the two modules of the molecule; the Gly residue gives the appropriate flexibility to the junction of the two modules, avoiding the high-energy structure.

The D-Phe residue of c(RGDf[NMe]V) was replaced with a D-Glu residue, and the γ- carboxyl of Glu was covalently linked via an amide bond to the αNH group of Met^{28}. Finally, the [NMe]Val residue was substituted by a Lys residue to further label the peptide for tumor-imaging studies and therapy. The final sequence of RGDechi is shown in Figure 1.

The final model of the RGDechi/receptor complex was minimized to refine the complex structure and to verify if we had introduced, in the design phases, any interaction regions of high energy. The minimized model, presented in Figure 2, keeps all predicted interactions.

To evaluate the activity of the bifunctional molecule RGDechi, peptides echiL, echi6−19, and echi11−19 (Figure 1) were designed. Echi6−19 and echi11−19 encompass the 6−19 and 11−19 RGDechi sequence, respectively, and echiL corresponds to the linear precursor of RGDechi.

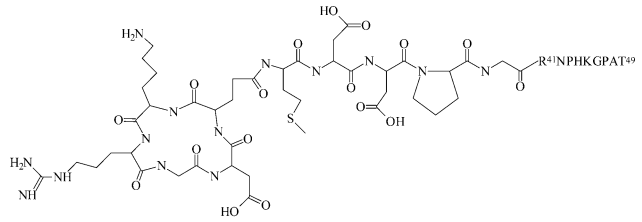

Figure 1. Schematic representation of the synthesized molecules.

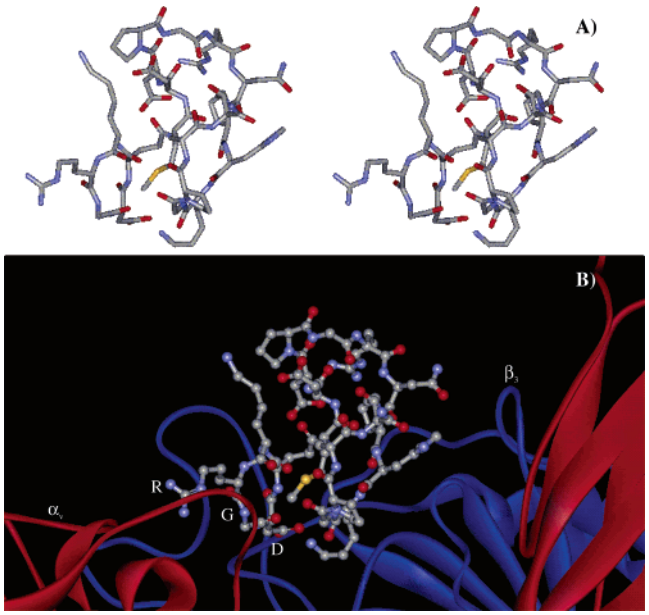

Figure 2. (A) Stereo drawing of the molecular model of RGDechi, obtained after the energy-minimization procedure. (B) Theoretical model of the complex RGDechi/$\alpha_v\beta_3$ receptor (the receptor is represented by solid ribbons).

Synthesis. All peptides were synthesized by the solid-phase method using Fmoc chemistry. All amino acids were coupled according to the HBTU/HOBt/DIPEA procedure.[33] Final deprotection and cleavage from the resin were achieved with TFA and scavengers. During the RGDechi synthesis, before the Fmoc deprotection of Lys^1, α-carboxyl-selective deprotection of the D-Glu^5 residue from the allyl group was carried out by the treatment of the peptidyl resin with $PhSiH_3/Pd(PPh_3)_4$/DCM.[34] Before the final cyclization, the resin was split into two parts, to obtain echiL and to continue the synthesis of RGDechi. The cyclization between the αNH group of Lys^1 and the αCO group of D-Glu^5 was performed with PyBop/HOBt/DIPEA[35] in DMF.

The purity and identity of the peptides were confirmed by analytical RP-HPLC and MALDI-TOF mass spectrometry. The overall yields of RGDechi, echiL, echi6−19, and echi11−19, purified by preparative RP-HPLC, were 24, 30, 54, and 58%, respectively.

Cell Adhesion and Competitive Assays. All synthesized peptides were tested for their ability to inhibit cell adhesion to vitronectin. Human erythroleukemia K562 cells overexpressing $\alpha_v\beta_3$ (K$\alpha_v\beta_3$) were incubated with increasing concentrations of the tested peptide and then allowed to adhere to vitronectin-coated plates. Both c(RGDfV),[21,24] which is a c(RGDf[NMe]V) analogue with comparable biological activity, and RGDechi were able to inhibit adhesion of K$\alpha_v\beta_3$ cells to vitronectin. Figure 3 shows representative inhibition curves obtained by

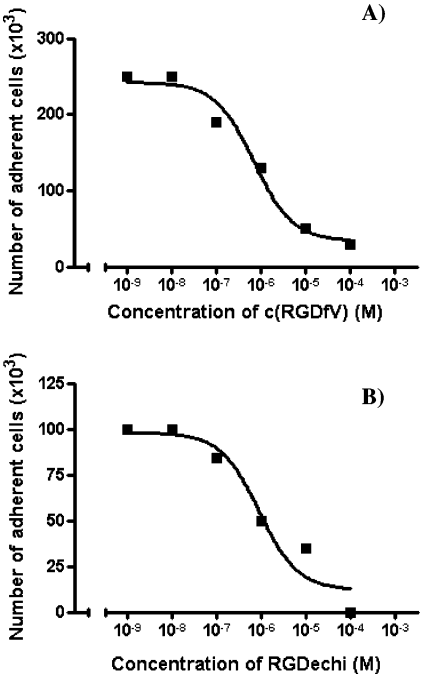

Figure 3. Representative inhibition curves obtained from adhesion assays performed in K$\alpha_v\beta_3$ cells. (A) Cells were preincubated with increasing concentrations of c(RGDfV) (IC_{50} = 0.68 μM) and (B) RGDechi (IC_{50} = 0.88 μM) for 1 h at 4 °C and then seeded on vitronectin-coated plates. The cells were allowed to adhere for 1 h at 37 °C and counted.

incubating K$\alpha_v\beta_3$ with c(RGDfV) and RGDechi, respectively. The IC_{50} value for c(RGDfV) ranged between 0.64 and 3.48 μM, whereas the IC_{50} of RGDechi ranged between 0.79 and 7.59 μM. The RGDechi fragments were tested for their ability to inhibit K$\alpha_v\beta_3$ cell adhesion. Incubation with 10 μM of selected amino acid sequences, such as echi11−19, echi6−19, and echiL, failed to inhibit cell adhesion, which remained 97.5, 99.0, and 89.5% compared to that of untreated control cells.

To test the selectivity of the binding of the novel peptide RGDechi, $\alpha_v\beta_5$-overexpressing cells (K$\alpha_v\beta_5$) were used in the adhesion assay. In Figure 4A, representative inhibition curves are reported. Although c(RGDfV) was able to efficiently inhibit the adhesion of cells to vitronectin, RGDechi did not show any significant inhibitory effect on K$\alpha_v\beta_5$ cell adhesion, indicating a lack of cross-reactivity with $\alpha_v\beta_5$. In parallel experiments, $\alpha_{IIb}\beta_3$-overexpressing K562 cells (K$\alpha_{IIb}\beta_3$) were preincubated with LM609 monoclonal antibody and then allowed to adhere to fibrinogen in the presence or absence of the selected peptide. Figure 4B shows that, although c(RGDfV) was able to efficiently inhibit the adhesion of cells to fibrinogen, RGDechi did not show any significant inhibitory effect on $\alpha_{IIb}\beta_3$-overexpressing cells.

Consistent results were obtained from competition binding experiments indicating that the novel peptide RGDechi efficiently competes with a c(RGDf[NMe]V) analogue labeled with ^{125}I [c(RGDyV)] for the binding to $\alpha_v\beta_3$-overexpressing cells and not to $\alpha_v\beta_5$-overexpressing clones (data not shown).

Conclusion

The present study describes a novel and selective ligand for $\alpha_v\beta_3$ integrin containing a cyclic RGD motif and two echistatin *C*-terminal moieties covalently linked using a Pro-Gly spacer sequence. The rationale behind this investigation was that the conjugation of echistatin sequences with a cyclic RGD motif in the RGDechi peptide would have enhanced the ability of the

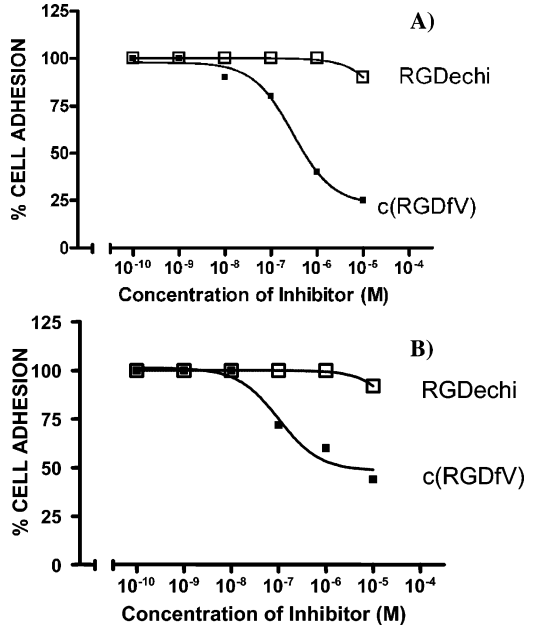

Figure 4. Selectivity of RGDechi for $\alpha_v\beta_3$. (A) Representative inhibition curves obtained from adhesion assays performed in K$\alpha_v\beta_5$ cells. The cells were preincubated with increasing concentrations of c(RGDfV) (■) and RGDechi (□) for 1 h at 4 °C, and adhesion was determined as described in Figure 3. The results are expressed as the percentage of adherent cells considering the untreated control sample as 100%. (B) Representative inhibition curves obtained from adhesion assays performed in K$\alpha_{IIb}\beta_3$ cells. The cells were preincubated with anti-$\alpha_v\beta_3$-blocking LM609 monoclonal antibody and subjected to the adhesion assay on fibrinogen (10 μg/mL).

RGD motif to efficiently inhibit the adhesion of $\alpha_v\beta_3$-overexpressing cells to vitronectin, thus supporting a functional synergy hypothesis between the RGD loop and the *C*-terminal sequence of the peptide. Moreover, RGDechi did not show any significant inhibitory effect on K$\alpha_v\beta_5$ cell adhesion, indicating a lack of cross-reactivity with $\alpha_v\beta_5$-overexpressing cells used in the adhesion assay.

In conclusion, our findings indicate that the RGDechi chimeric peptide is a novel and selective ligand for $\alpha_v\beta_3$ integrin. The design of new molecules, based on the lead compound presented here, is currently ongoing with the aim of developing novel anticancer drugs and/or a new class of diagnostic noninvasive tracers as suitable tools for $\alpha_v\beta_3$ -targeted therapy and imaging.

Experimental Section

Synthesis. All peptides were synthesized on a ABI433A automated peptide synthesizer using the Fmoc solid-phase strategy (0.25 mmol). The synthesis was carried out with Novasyn TGA resin (substitution 0.29 mmol g^{-1}), using all standard amino acids except for Fmoc-D-Glu-OAll to insert the D-Glu[5] residue in the peptide sequence by its carboxyl side chain. The first amino acid was bound to the resin by treatment with Fmoc-Thr(*t*Bu)-OH (5 equiv)/MSNT (5 equiv)/MeIm (3.75 equiv) in DCM for 3 h. The Fmoc deprotection step was performed with 30% piperidine in DMF for 10 min and active ester-coupling reactions were carried out under a 4-fold excess of amino acid and HBTU (4 equiv)/HOBt (4 equiv)/DIPEA (8 equiv) in DMF.[33] Each coupling was repeated twice for 1 h followed by a capping step (5 min) performed with acetic anhydride/DIPEA/DMF (2.6:4.8:92.6 v/v/v).

After the Arg[11] coupling reaction, an aliquote of the peptidyl resin was removed to yield the echi11−19 peptide. At the end of the Met[6] coupling, another part of the resin was removed to obtain echi6−19 peptide. During the RGDechi synthesis, before the Fmoc deprotection of Lys[1], selective α-carboxyl deprotection of the

D-Glu[5] residue from the allyl group was carried out by treatment of the peptidyl resin with $PhSiH_3$ (24 equiv)/$Pd(PPh_3)_4$ (0.25 equiv) in DCM. Before the final cyclization the resin was divided in two parts to obtain echiL and to continue the synthesis of RGDechi. The cyclization between αNH of Lys[1] and αCO of D-Glu[5] was performed with PyBop (1.5 equiv)/HOBt (1.5 equiv)/DIPEA (2 equiv) in DMF.

The peptides were cleaved off the resin and deprotected using a mixture of TFA/H_2O/EDT/TIS (94:2.5:2.5:1 v/v/v/v). The resins were then filtered, and the peptides were precipitated using cold anhydrous diethyl ether.

The crude products were purified by preparative RP-HPLC on the Shimadzu LC-8A system, equipped with an UV−Vis detector SPD-10A using a Phenomenex C18 column (21 × 250 mm; 15 μm; 300 Å) and a linear gradient of H_2O (0.1% TFA)/CH_3CN (0.1% TFA) from 5 to 70% of CH_3CN (0.1% TFA) in 30 min at flow rate of 20 mL/min. The purified peptides were characterized using MALDI-TOF spectrometry on a MALDI-TOF Voyager-DE (Perseptive Biosystem) spectrometer, which gave the expected molecular ion peaks $[M - H]^+$ of 2061.2, 978.1, 1493.6, and 2079.2 for RGDechi, echi11−19, echi 8−19, and echiL, respectively.

The peptides c(RGDfV) and c(RGDyV) were synthesized as reported in the literature.[36]

Model Building. The INSIGHT/DISCOVER package (Accelrys, Inc., San Diego, CA) was used to build all structures and to perform energy minimizations at pH 7.0 using the CVFF force field.[37] All calculations and graphical analyses were run on a Silicon Graphics Octene2 workstation. Energy minimizations were carried out using the conjugate gradient algorithm. These procedures were stopped when the maximum derivative was ≤0.01 kcal/mol. The template used was the X-ray diffraction structure of the $\alpha_v\beta_3$ integrin in complex with the c(RGDf[NMe]V) (pdb entry code: 1L5G). The starting structure for the echistatin was that obtained from NMR analysis (pdb entry code: 1RO3).

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Supporting Information Available: Cell adhesion and competitive assay experimental details. This material is available free of charge via the Internet at http://pubs.acs.org

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(1) (a) Dr. A. Del Gatto, A. and Dr. L. Zaccaro contributed equally to this work; b) In addition to those in the IUPAC−IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14−42), the following symbols and abbreviations are used herein: DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EDT, 1,2-ethanedithiol; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MeIm, 1-methyl imidazole; MSNT, 1-(mesitylene-2-sulphonyl)-3-nitro-1H-1,2,4-triazole; OAll, allyloxy; PyBop, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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