Stereoisomerism and Biological Activity of the Selective and Superactive $\alpha_v\beta_3$ Integrin Inhibitor cyclo(-RGDfV-) and Its Retro-Inverso Peptide

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Abstract: The cyclic pentapeptide cyclo(-Arg-Gly-Asp-D-Phe-Val-) is a highly potent and selective inhibitor for the $\alpha_v\beta_3$ integrin and is a prospective anticancer drug by acting to inhibit angiogenesis and by inducing apoptosis in vascular cells. The cyclic retro-inverso peptides as well as the inverso and the cyclic retro peptide analogs of the four cyclic Arg-Gly-Asp-Phe-Val peptides with one amino acid in the D configuration and the corresponding all-L peptide have been synthesized. The inhibitory activities of 18 compounds have been tested on the isolated integrin $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ receptors. The conformations of the cyclic retro-inverso pentapeptides were investigated by NMR spectroscopy using NOEs, ROEs, and coupling constants and were determined by distance geometry (DG) calculations. The structures were compared to their parent analogs, and the relationship between their conformation and the biological activity is discussed. Due to the reversal of the peptide bonds in the retro-inverso peptides, the hydrogen bond pattern is shifted and the spatial structure differs from its parent compound structure. These conformational changes result in a dramatic decrease of activity in comparison to the high-active parent peptides. On the other hand the retro-inverso peptide analog of the poorly active parent peptide cyclo(-D-Arg-Gly-Asp-Phe-Val-) was found to be highly active and selective for the $\alpha_v\beta_3$ receptor. Furthermore, the almost perfect similarity of the side chain orientation between the highly active peptide cyclo(-Arg-Gly-Asp-D-Phe-Val-) and the inactive retro-inverso peptide cyclo-(-Val-D-Phe-D-Asp-Gly-D-Arg-) indicates a distinct interaction of at least one peptide bond of the backbone with the $\alpha_v\beta_3$ receptor.

Introduction

Integrins of different types play a major role in cell−cell and cell−matrix interactions.[1−3] They interact with the cytoskeleton and are involved in signal transduction processes. In this respect, the observation that selective inhibition of the $\alpha_v\beta_3$ integrin prevents angiogenesis and induces apoptosis arouses hope for a new way of cancer treatment by starving and killing the tumor.[4] Selective inhibition of the $\alpha_v\beta_3$ receptor was achieved with antibodies as well as with our selective superpotent cyclic pentapeptide cyclo(-RGDfV-)[5,6] which was found in a "spatial screening" procedure,[7] using cyclo(-D-Ala-L-Ala_4-) as template. Many derivatives of that peptide have been investigated in the last five years in our group.[5,6,8] Recently, the biological importance of $\alpha_v\beta_3$ integrin inhibitors gained interest in other groups as well.[9]

While exploring the required spatial orientation of the pharmacophoric groups, we performed a systematic study of all cyclic retro-inverso isomers, retro isomers and inverso isomers of the pentapeptide sequence RGDFV containing one amino acid in the D-configuration. This sequence was chosen because we have shown that cyclo(-RGDfV-) and cyclo-(-RGDFv-) are not only very potent inhibitors of the $\alpha_v\beta_3$ integrin but also highly selective. Their inhibitory activity on the $\alpha_{IIb}\beta_3$ receptor is low. It is obvious that the appropriate spacing and the orientation of the arginyl and the aspartyl side chains required to inhibit the $\alpha_v\beta_3$ integrin receptor are better matched or can be more easily reached in these cyclic pentapeptides than in the less active cyclic hexapeptides or linear

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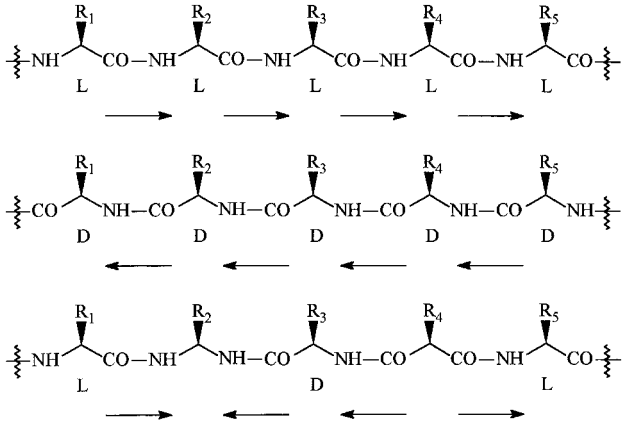

Figure 1. Parent peptide (top), its retro-inverso analog (middle), and a partial retro-inverso analog (bottom). The arrows indicate the direction of the amide bond. The orientation of the side chains is similar in all three peptide structures.

peptides.[5] At this point we emphasize that even the small-ring cyclic peptides have a certain degree of flexibility. In particular, the amide bond in the γ-turn can easily flip about the adjacent Φ and Ψ angles.[10] Restriction by cyclization may prevent the molecule from reaching its bioactive conformation (mismatched structure, inactive peptide) or facilitate it (superactive peptide in the matched case). In any case, due to the mutual fitting of both receptor and substrate by double induced fit,[11] the most stable conformation in solution may differ from the bioactive conformation.

The Retro-Inverso Concept. The structural influence of amino acid chirality and direction of the sequence has been investigated in numerous publications. For example, it is well known that a D-amino acid induces turns, especially a βII′-turn with the D-residue in the $i + 1$ position. This is routinely used in D-amino acid scans for analyzing structure/activity relationships of new bioactive peptides. In cyclic peptides this phenomenon can be exploited for conformational design. For example, in a cyclic hexapeptide of the general structure cyclo(-D-Pro-$A_1A_2A_3A_4A_5$-) where A_n is any L-amino acid, the sequence D-Pro-A_1 is always found in the βII′-turn. This turn induces another β-turn around A_3 and A_4 at the opposite side of the ring. This has been exploited in forcing a particular amino acid sequence into a distinct β-turn arrangement.[12]

A systematic exploration of chirality and sequence in cyclic peptides has already been performed by Shemyakin, Ovchinnikov, and Ivanov in 1969.[13] They postulated that a total *inversion of chirality* should be combined with *inversion of the sequence* ("retro sequence") to achieve an orientation of the side chains (Figure 1) similar to the parent peptide (P). Many of these so-called retro-inverso peptides (RI) were synthesized and tested biologically, and several reviews in this field appeared.[14−18] Retro-inverso analogs of linear peptides have an opposite C- and N-terminal alignment with the parent peptide. If these end groups are important for receptor binding, the concept does not work. Therefore, normally only a part of the sequence is exchanged, and for that suitable linkers are necessary (Figure 1, bottom).[19] In cyclic peptides, because of the absence of C- and N-terminals, such a problem does not exist when a total reversal of peptide bonds is done. In this context "cycloenantiomer" and "cyclodiastereomer" are two other frequently used terms, coined by Prelog and Gerlach,[20] and they should be briefly explained. In the first case reversal of the sequence leads to a mirror image of the molecule (e.g., cyclo(-XxyY-) is enantiomeric to cyclo(-YyxX-) or cyclo(-XxYy-) is enantiomeric to cyclo(-yYxX-)). The retro-inverso isomer then is the parent isomer itself. In a cyclodiastereomer the reversal of the sequence leads to a diastereomer of the original molecule (e.g., cyclo(-XXYy-) is diastereomeric to cyclo(-yYXX-) and cyclo(-XYxy-) is diastereomeric to cyclo(-yxYX-)). None of these stereochemical terms can be applied to any of the compounds of this work.

The advantage of retro-inverso peptides is high metabolic stability, because peptide bonds adjacent to D-amino acids normally are stable to enzymatic cleavage. However, in reality the biological activity of retro-inverso peptides mostly is modified compared to that of their parent compounds. This may be caused by two effects: (i) The spatial structure may not be retained, because the concept shown above neglects conformational features such as the different hydrogen-bonding pattern of the inverted peptide formed in the backbone or from the backbone to the side chains. (ii) Interactions of amide groups of the parent peptide's backbone with the receptor are important. Due to the inverted orientation in the retro-inverso analog the specific peptide bonds that interact with the receptor do not fit.

In the present study we will demonstrate that the latter is the case with the inhibitors of the $\alpha_v\beta_3$ receptor. We will also show that the backbone conformations and hence the biological activity of the parent peptides and their retro-inverso analogs strongly differ. These results could only be obtained by a systematic study of the structure/activity relationship of stereoisomers and retro peptides.

The peptides studied in this paper are outlined in Table 1.

General Methods

Synthesis of the Cyclic Peptides. All linear peptides were synthesized by standard Fmoc solid-phase strategy using an *o*-chlorotrityl chloride resin.[21] Arginine was protected by the (4-methoxy-2,3,6-trimethylphenyl)sulfonyl (Mtr) group, and aspartic acid was protected as a *tert*-butyl ester. Glycine was always chosen as the C-terminal amino acid in order to avoid racemization problems in the cyclization step. The other amino acids (1.5 equiv each) were sequentially coupled with 1.7 equiv of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetra-fluoroborate (TBTU)[22] and 1-hydroxybenzo-

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Table 1. Cyclic Pentapeptides of the Sequence RGDFV: Five Parent Peptides (P) with One D-Residue (Lower Case Letter) and Their Retro (R), Inverso (I), and Retro-Inverso Isomers (RI)

parent	inverso	retro	retro-inverso
cyclo(-RGDFv-) (**P1**)	cyclo(-rGdfV-) (**I1**)	cyclo(-vFDGR-) (**R1**)	cyclo(-VfdGr-) (**RI1**)
cyclo(-RGDfV-) (**P2**)	cyclo(-rGdFv-) (**I2**)	cyclo(-VfDGR-) (**R2**)	cyclo(-vFdGr-) (**RI2**)
cyclo(-RGdFV-) (**P3**)	cyclo(-rGDfv-) (**I3**)	cyclo(-VFdGR-) (**R3**)	cyclo(-vfDGr-) (**RI3**)
cyclo(-rGDFV-) (**P4**)	cyclo(-RGdfv-) (**I4**)	cyclo(-VFDGr-) (**R4**)	cyclo(-vfdGR-) (**RI4**)
cyclo(-RGDFV-) (**P5**)			cyclo(-vfdGr-) (**RI5**)

triazole (HOBt) in 1-methyl-2-pyrrolidinone (NMP) as the solvent. *N*,*N*-Diisopropylethylamine (DIEA) was used to adjust the pH to 8. Due to HOBt and HBF_4 formation, the pH drops while the reaction proceeds, which leads to reduced nucleophilicity of the amino group. Therefore, in the case of insufficient couplings, monitored with the Kaiser test,[23] additional base was added, but the pH was not allowed to exceed a value of 8.5. The coupling times ranged from 10 to 40 min. The *o*-chlorotrityl linker offers the possibility of cleaving off the linear peptide with a mild acetic acid/2,2,2-trifluoroethane (TFE) mixture in dichloromethane (DCM) without affecting the side chain protecting groups.

The head-to-tail cyclization was performed with diphenylphosphoric acid azide (DPPA),[24] applying the solid base method using $NaHCO_3$ in NMP.[25] Precipitating the crude product in water allows the complete removal of all cyclization reagents. Generally, the cyclic peptides obtained this way are pure and show only one peak in reversed phase high-performance liquid chromatography (RP-HPLC). Finally the side chain protecting groups were removed with trifluoroacetic acid (TFA) and thioanisol, thiocresol, and water as scavengers. The crude products were purified by RP-HPLC. Because of the arginine residue, the peptide TFA salts were obtained. The peptides were characterized by fast atom bombardment mass spectroscopy (FAB-MS) and various NMR techniques.

NMR Spectroscopy.[26] TOCSYs with short (20 ms) and long (80 ms) mixing times were used for proton assignment and HMQC and HMQC-TOCSY for carbon assignment. Sequential assignment of proton resonances was achieved by NOESY (500 MHz) and HMBC, which also proves the cyclization. $^3J_{HH}$ coupling constants were extracted from 1D or PE-COSY spectra. The NOE effects were quantified by NOESY experiments at 500 MHz with a mixing time of 150 ms.

All compounds examined showed only one conformation on the NMR time scale, as was expected for no secondary amines (e.g., proline) were incorporated. The inverso compounds and retro-inverso compounds showed exactly the same ^1H and ^{13}C spectra as their parent and retro compounds, respectively.

Inhibition Assays. The quantity of peptide necessary to block 50% of the ligand binding (IC_{50}) was measured using purified human $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins and biotinylated human plasma vitronectin and fibrinogen in an ELISA-like assay.[27] To compensate for interassay variability, the IC_{50} values are shown as a ratio, normalized to the inhibition of the standard linear peptide GRGDSPK (IC_{50}(test)/IC_{50}(GRGDSPK)). The mean IC_{50} for GRGDSPK inhibition of ligand−integrin interaction was 2 μM. The specificity of the assay was indicated by the specific binding of vitronectin to $\alpha_v\beta_3$ but not to $\alpha_{IIb}\beta_3$ integrin, and the lack of cross-reactivity of the preparations with various integrin specific antibodies.

Structure Determination. The conformations of the parent peptides P1, P2, and P4 were investigated in DMSO-d_6 by molecular dynamics simulation in a solvent box using NOE constraints in the GROMOS force field and have been published previously.[5] The structures of the retro-inverso peptides P3 and P5 were obtained by distance geometry (DG) calculations[28] using a modified version[29,30] of the program DISGEO,[31] to include *J* coupling restraints.[32] For each cyclic peptide 50 structures were randomly embedded in 4 dimensions using the metric matrix method. The resulting structures were optimized in 400 steps with a conjugate gradient algorithm and subsequently were subject to distance driven dynamics (DDD) procedure with the application of SHAKE[33] at 300 K for 50 ps with a 20 fs integration step.[34] The potential for distance violations of NOE restraints as well as the holonomic boundaries (*b*) was $V_{dist} = k_1(d^2 - b^2)^2$, which is harder than a fourth power potential (x^4) and therefore cannot be assigned a physical meaning. By calculating the three highest eigenvalues of the metric matrices of the 4-dimensional structures, they were embedded in the third dimension and again optimized and shaken (50 ps at 300 K and 50 ps at 1 K). To refine the structures during the last SHAKE procedure, a set of Φ and χ_1 angle restraints, obtained from $^3J_{NH-H_\alpha}$ and $^3J_{H_\alpha-H_\beta}$ coupling constants (distance and angle driven dynamics, DADD, $V_J = k_2(J_{exp} - J_{calc})^2$) and a hydrogen bond potential for amide protons with small temperature gradients ($-\Delta\delta/\Delta T < 2.5$ ppb/K) was added.[35] In this short range potential ($V = k_2(d_{NH-OC})^{-4}$, inner cutoff at 2.2 Å) the protons were allowed to form hydrogen bonds to all possible acceptors. Because NHO and HOC angles were not considered, the potentials to the carbonyls *i* and *i* - 1 were set to zero to avoid artificial forces within the amide bond ($d_{NH-OC} = 3.1$ Å) or within one amino acid (maximum $d_{NH-OC} = 4.55$ Å). Geminal protons were diastereotopically assigned, if possible. For the assignment of Asp H_β and Phe H_β the standard procedure with NOEs and coupling constants was used.[36] Because the structures contained only one glycine, the method of floating chiralities[37] was not necessary for the diastereotopic assignment of the Gly H_α protons. Two separate calculations with both possible assignments for H_α^{proR} and H_α^{proS} were performed. For RI1,

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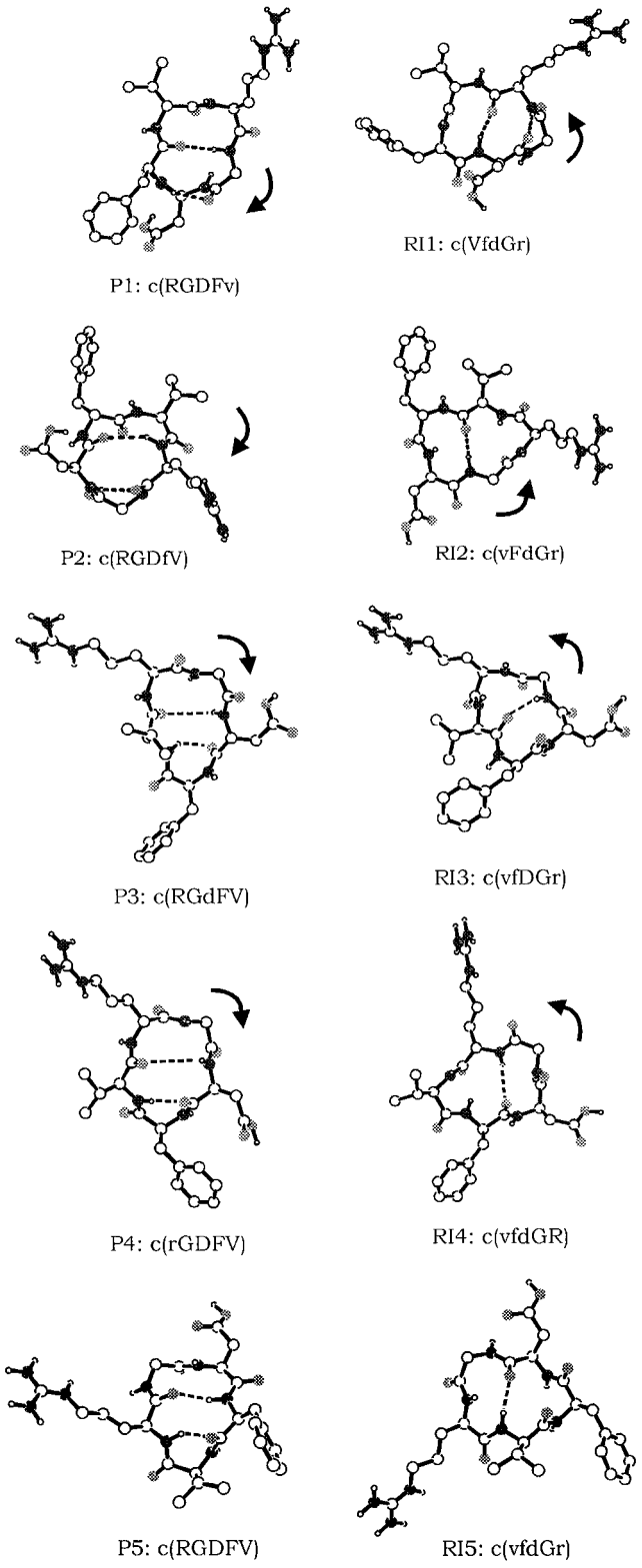

Figure 2. Topological comparison of the conformations of the parent peptides P1, P2, P3, P4, and P5 and their retro-inverso analogs. The five parent peptides are shown in the general representation of pentapeptides with the β-turn up and the γ-turn down. The retro-inverso structures are positioned in such a way that the conformation of the RGD sequence can be best compared to the parent structures and the conformational difference in this region can be easily recognized. The direction of the peptide bonds is indicated by arrows. Note that the direction of the amide bond in the RI peptides is reversed by looking at the peptide from "below" with respect to the normal view.

RI2, RI3, RI4, and P3, 50 randomly embedded structures led to a single set of structures with low total error. The homochiral structures P5 and RI5 suggested a higher flexibility at the amide bonds about the "γ-turn".[9]

Table 2. IC_{50} Values: Cyclic Peptide Inhibition of Vitronectin Binding to the Isolated $\alpha_v\beta_3$ Integrin and of Fibrinogen Binding to Integrin $\alpha_{IIb}\beta_3$[a]

$\alpha_v\beta_3$	P	RI	I	R
(**P1**) c(RGDFv)	0.025	5	>10	>10
(**P2**) c(RGDfV)	0.006	3.5	0.08	>10
(**P3**) c(RGdFV)	>10	>10	0.02	2
(**P4**) c(rGDFV)	>10	0.004	>10	>10
(**P5**) c(RGDFV)	0.3	>10	nd	nd

$\alpha_{IIb}\beta_3$	P	RI	I	R
(**P1**) c(RGDFv)	1.1	>10	>10	>10
(**P2**) c(RGDfV)	5.0	>10	>10	>10
(**P3**) c(RGdFV)	>10	>10	>10	>10
(**P4**) c(rGDFV)	>10	>10	>10	>10
(**P5**) c(RGDFV)	>10	>10	nd	nd

[a] All values are given as ratios to a standard: IC_{50}/IC_{50}[GRGDSPK]. Lower case letters indicate D-configured amino acids.

Results

Conformations. It is not necessary to analyze all 18 compounds, because parent and inverso as well as retro and retro-inverso peptides form 10 pairs of enantiomers. In general, the conformation of small cyclic peptides is strongly affected by the array of chirality in the ring. The functionalities at the side chains contribute less to the backbone conformation. We may therefore compare the structures with cyclo(-D-Ala-Ala_4-). However, the Gly residue which is always present may function as a structure-inducing D residue[38] as well, and it is necessary to study the conformations of 10 peptides in detail. Hence, the following 10 peptides were investigated: P1, RI1, P2, RI2, P3, RI3, P4, RI4, P5, RI5.

In all retro-inverso peptides with one L residue, two amide protons with low-temperature shift gradients were found. However, in three cases (RI2, RI3, and RI4), only one hydrogen bond was formed. This shows that the chosen d^{-4} potential for hydrogen bonding assures short-range interactions in the structure-refinement calculation, and that hydrogen bonds are not forced artificially in the calculation. Instead of forming a γ-turn, the two amide protons i and $i + 1$ of the turn (D-Arg NH/D-Val NH of RI2, D-Arg NH/D-Val NH of RI3, and D-Val NH/D-Phe NH of RI4) point in the same direction. Such a conformation is often involved in the γ-turn of the cyclic pentapeptide.[10,42] The Φ and Ψ values ($\Phi = 62$, $\Psi = 33$) for D-Arg in RI2, ($\Phi = 75$, $\Psi = 45$) for D-Arg in RI3, and ($\Phi = 80$, $\Psi = 11$) for D-Val in RI4 are situated in the left-handed α-helical region of the Ramachandran plot (α_D: $\Phi_{standard} = 68.6$, $\Psi_{Standard} = 17.5$, as defined by McAllister et al.[39]).[40] This conformation has a lower energy than the C_{7ax} (γ_t-turn) conformation. Glycine and aspartic acid are, with 7.4% and 1.7%, respectively, the amino acids with the highest probability for such a conformation in proteins (all others <1%), shown in an analysis of 73 protein structures by McAllister et al.[36]

An explanation for the low-temperature dependence of the D-Val (RI2 and RI3) and D-Phe (RI4) amide protons might be their internal orientation in the peptide (Figure 2), which shields them from the solvent molecules.

(38) L residue for inverso and retro-inverso peptides.

(39) McAllister, M. A.; Perczel, A.; Császár, P.; Viviani, W.; Rivail, J.; Csizmadia, I. G. *J. Mol. Struct.: THEOCHEM* **1993**, *288*, 161−179.

(40) Note that a D residue is discussed. One would have to change all angle signs, left/right and D/L for applying this to the retro structures with an L residue.

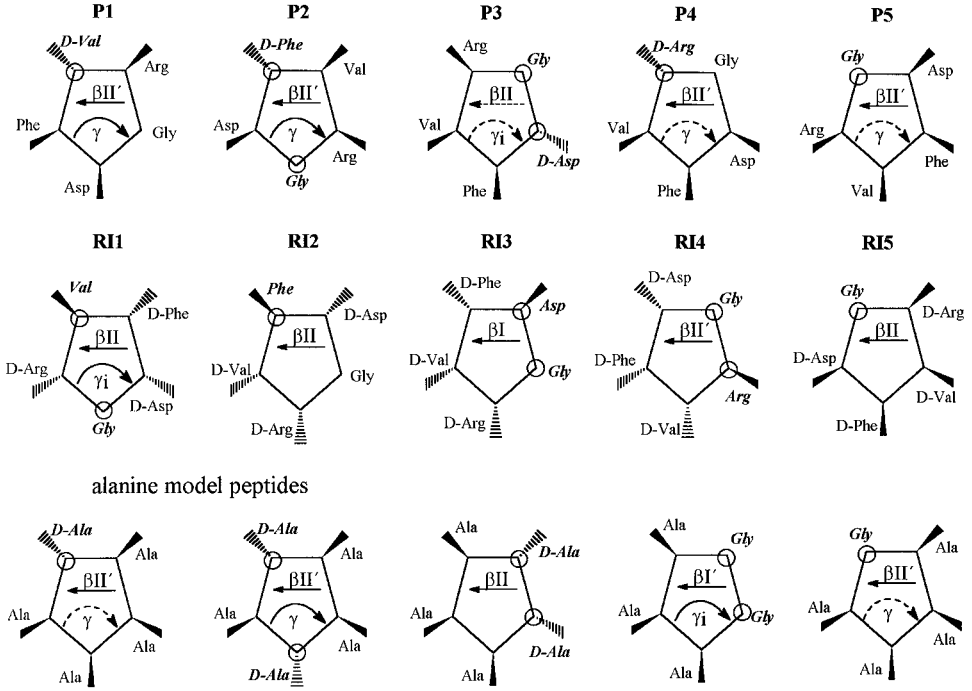

Figure 3. Schematic overview of all examined conformations of the cyclic RGD pentapeptides in comparison to cyclopentaalanine model peptides. The structure-inducing residues are stressed by a circle and are written in italic bold letters. The arrows with broken lines indicate hydrogen bonds that are less pronounced. c(GAAAA) has the same conformation as c(aAAAA), and c(GAAGA) has the same conformation as c(aAAaA) (not shown).

Besides Arg, all side chain conformations show preferred χ_1 angles in the retro-inverso peptides. It is remarkable that Phe prefers an orientation onto the Val residue. This results in $\chi_1 = +60°$ for the RI1, RI3, and RI4 where the Phe residue is in the D configuration but $\chi_1 = -60°$ for RI2 and RI5 (L-Phe). The orientation of the aromatic residue, evidenced also by a characteristic high-field shift in the corresponding Val methyl group resonances, can be explained by hydrophobic clustering.[41] In Figure 2 the five parent peptides are compared with their retro-inverso analogues. Of course, the conformations of the inverso peptides correspond to the parent and those of the retro-peptides to the retro inverso structures.

Biological Activity. The inhibition of integrin binding is summarized in Table 2.

Only 2 out of the 18 pentapeptides have inhibitory activity on the $\alpha_{IIb}\beta_3$ receptor approaching that of the linear reference peptide. Those contain the unaltered parent sequence RGD in the L configuration, and chirality is only varied in the other amino acids F and V. All others are inactive at the sensitivity of the applied test (10 μm). By contrast, there are distinct differences in the inhibition of the $\alpha_v\beta_3$ receptor. One of the retro-inverso peptides is the most potent compound in the series, and two of the inverso peptides are also 1−2 orders of magnitude more active than the linear reference peptide.

Discussion

All peptides exist in the expected β-turn conformation with (P1, P2, P3, P4, P5, RI1) or without (RI2, RI3, RI4, RI5) a γ-turn when compared to the alanine model peptides:[42] P1 = c(*v*RGDF),[43] P4 = c(*r*GDFV), P5 = c(***G***DFVR), and RI5 = c(***G***RVFD) show the conformations of c(***a***AAAA) and c(***G***AAAA); P2 = c(***f***VR***G***D) shows the conformation of c(***a***AA***a***A) and c(***G***AA***G***A); P3 = c(R***Gd***FV) has the conformation of c(A***aa***AA); RI1 = c(***V***fd***G***r) has the inverted conformation of c(***a***AA***a***A) and c(***G***AA***G***A); RI2 = c(***F***dGrv) shows one of the inverted conformations of c(***a***AAAA);[9] RI3 = c(f***DG***rv) is comparable to c(A***GG***AA), and RI4 = c(d***GR***vf) shows the inverted conformation of c(A***aa***AA). A schematic overview of the conformations and the corresponding alanine model peptides is given in Figure 3.

The parent peptide P2 cyclo(-Arg-Gly-Asp-D-Phe-Val-) has the well-known βII′, γ-conformation with the D residue in the $i + 1$ position of the β-turn. Inverting all chiralities of the amino acids results in the enantiomeric peptide with inverted turns (mirror plane in the plane of the paper, Figure 4). Hence, the inverso peptide exhibits a βII, γ_i-conformation, with dihedral angles in the mirror image of its parent peptide. In such an inverted peptide the L-amino acid occupies the position of the former D residue and forms the βII turn. If we now compare the retro-inverso peptide, we realize that the residues on both sides of a line through the D-Phe-α carbon and the middle of the Arg−Gly bond change their sites. The former residue in $i + 2$ is now in the i position and vice versa. In the case of RI2, the residue Asp of P2 is shifted to the former position of Val.

Because the retro-inverso isomer contains only one L-amino acid besides Gly (which can act as a D- or L-amino acid), the expected βII-conformation about Phe-D-Asp was found. The missing γ_i-turn about D-Arg may be a result of the higher flexibility around the Gly residue. It is known that the backbone conformation of cyclic peptides is determined by steric effects rather than by forming H-bonds. The different topology explains the dramatic decrease in activity in comparison with

(41) (a) Wiley, R. A.; Rich, D. H. *Med. Res. Rev.* **1993**, *13*, 327−384. (b) Konat, R. K.; Grdadolnik, S. G.; Schmitt, W.; Kessler, H. *Peptides 1994*, Proceedings of the 23rd European Peptide Symposium, Braga, Portugal, Sept 4−10, 1994; Maia, H. L. S., Ed.; ESCOM: Leiden, 1995; pp 521−522.

(42) Kurz, M. Thesis, Technical University Munich, 1991 (see Supporting Information).

(43) For a better comparison with the model peptides the residues are sorted in such a way that the amino acid in the $i + 1$ position of the β-turn comes first. Structure-inducing residues are stressed by italic bold letters.

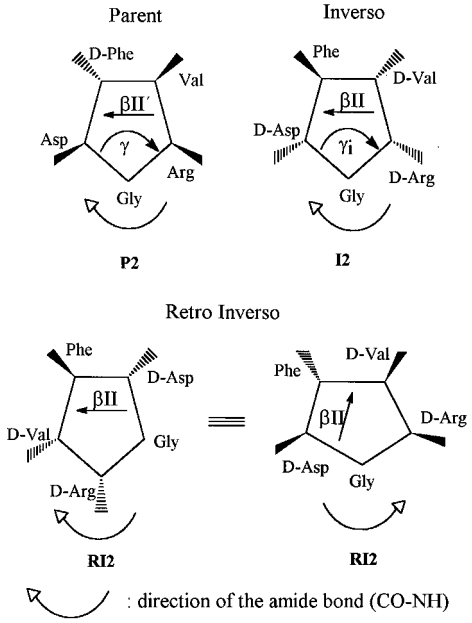

Figure 4. A schematic illustration of the topology of a cyclic parent pentapeptide with one D-amino acid and its inverso and its retro-inverso isomers: the case of cyclo(-Arg-Gly-Asp-D-Phe-Val-).

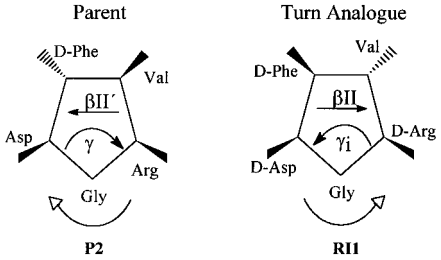

Figure 5. A schematic illustration of P2 and its retro enantiomer turn analog RI1.

the very potent parent peptides (Table 2). On the other hand, the retro-inverso peptide RI4 (cyclo(-D-Val-D-Phe-D-Asp-Gly-Arg-)) of the poorly active P4 (cyclo(-D-Arg-Gly-Asp-Phe-Val-)) was found to be highly active on the $\alpha_v\beta_3$ receptor. To our knowledge this is the only active RGD peptide with a D-Asp residue.

To our surprise the analogue RI1 cyclo(-Val-D-Phe-D-Asp-Gly-D-Arg-), which has almost exactly the same side chain topology as the biologically active parent peptide P2 (Figures 5 and 6), showed no activity on the $\alpha_v\beta_3$ integrin receptor. As the only difference between these two compounds is the direction of the amide bonds, this indicates that an interaction of the RGD backbone with the $\alpha_v\beta_3$ receptor occurs. We do not know which peptide bond reversal is responsible for the low affinity of RI1. Our attempts to remove one amide bond by introducing a reduced peptide bond ($\Psi[CH_2NH]$) or thioamides ($\Psi[CSNH]$) were accompanied by a dramatic change in backbone conformation.[44] Therefore, it was not clear whether the reduced activities induced by these modifications were due to the lack of the amide bond or to the overall change in conformation. On the other hand, the results here give clear evidence that at least one of the peptide bonds is important for binding to the $\alpha_v\beta_3$ integrin receptor.

Following the general design principles for cyclic peptides developed previously,[10,7b] we found the expected structures with

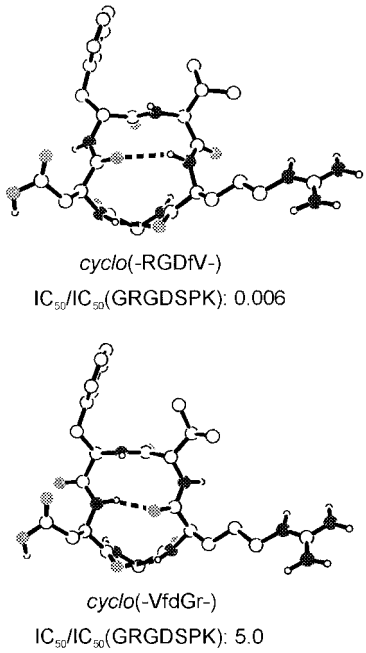

Figure 6. Comparison of the conformations of P2 and RI1. An almost identical side chain topology is found although the amide bonds are reversed.

the L residue (Gly for RI5) in the $i + 1$ position of a βII-turn, apart from RI2, where Gly acts as an additional L residue (change L to D for applying this to the retro structures). Here Gly and L-Arg are situated in the $i + 2$ and $i + 3$ positions of a βII′-turn. But due to the reversed sequence, a cyclic retro-inverso peptide does not mimic the side chain topology of its parent peptide, when the secondary structure is taken into account. Freidinger and Veber[45] have already shown in a theoretical study that, from a geometrical point of view, parent and retro-inverso peptides cannot be identical. The replacement of CONH with NHCO results in changed bond angles and bond lengths (e.g., Cα−C′ = 1.45 Å is changed to Cα−N = 1.53 Å), and the best fit for a cyclic hexapeptide results in an average Cα deviation of 0.35 Å for the peptide and its mimic (Cβ deviation: 0.2 Å), which is still quite good. In our case, the rms Cα deviations are 1.41 Å for P1/RI1 (Cβ: 2.02 Å), 1.06 Å for P2/RI2 (Cβ: 1.15 Å), 0.59 Å for P3/RI3 (Cβ: 1.51 Å), 0.61 Å for P4/RI4 (Cβ: 0.58 Å), and 0.96 Å for P5/ RI5 (Cβ: 1.30 Å). The main reason for this difference is not the changed donor−acceptor properties *per se*, because every donor is changed upon interaction with its acceptor and vice versa, therefore, in principle the same hydrogen bond pattern should be possible. (Disregarding the dissymmetry of hydrogen bonds, angle ∠NHO ≠ ∠HOC, which also may lead to conformational changes.) The reason is rather the changed energy hypersurface (Φ^*, Ψ^*). In Figure 7 the (Φ, Ψ) energy hypersurface of the model peptide Ac-L-Ala- NHMe (Figure 7, left)[46] is compared with its "retro-inverso" isomer Ac-Ψ[NHCO]-L-Ala-Ψ[NHCO]-Me, which is Ac-D-Ala-NHMe (Figure 7, right). The new energies for (Φ^*, Ψ^*) can be calculated by a transformation of the old coordinates (Φ, Ψ) with the matrix

$$M_3 = \begin{pmatrix} 0 & -1 \\ -1 & 0 \end{pmatrix}$$

or (Φ,Ψ) → ($-\Psi$, $-\Phi$)), which is merely a reflection of the

(44) Geyer, A.; Müller, G.; Kessler, H. *J. Am. Chem. Soc.* **1994**, *116*, 7735−7743.

(45) Freidinger, R. M.; Veber, D. F. *J. Am. Chem. Soc.* **1979**, *101*, 6129−6131.

(46) Kostrowicki, J.; Scheraga, H. A. *J. Phys. Chem.* **1992**, *96*, 7447.

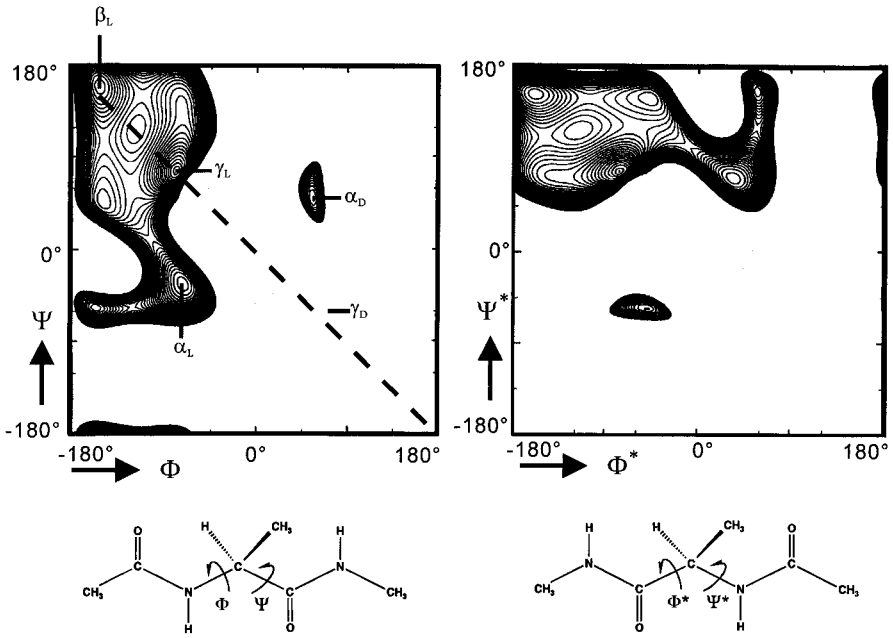

Figure 7. Comparison of the energy hypersurface of the model peptide Ac-Ala-NHMe (left) and its retro-inverso mimic (right). The original (Φ, Ψ) was calculated with ECEPP/2. Reprinted with permission from ref 46. Copyright 1992 American Chemical Society. β_L = β-sheet, α_L = right-handed α-helix, α_D = left-handed α-helix, γ_L = γ_t-turn, and γ_D = γ-turn.

hypersurface at the diagonal between the points (−180°, +180°) and (+180°, −180°) of the Ramachandran plot. The reversal of the peptide sequence is represented by (Φ, Ψ) → (Ψ, Φ), or

$$M_1 = \begin{pmatrix} 0 & 1 \\ 1 & 0 \end{pmatrix}$$

The inversion of the chiralities is represented by

$$M_1 = \begin{pmatrix} -1 & 0 \\ 0 & -1 \end{pmatrix}$$

With $M_1 = M_2 \times M_3$ one obtains the new energy hypersurface (Figure 7, right).

Taking a parent peptide with a low-energy conformation and building its retro-inverso analog with the same dihedral angles (i.e., Φ = Φ* and Ψ = Ψ*) means changing the energy hypersurface from Figure 7, left, to Figure 7, right, for each residue. Besides the points which are situated near the diagonal (e.g., γ_D (74.3, −59.5), γ_L (−84.5, 68.7), and β_L (−167.6, 169.9)) the energies change dramatically for other dihedral angles. A γ_L (−84.5, 68.7) conformation, for example (i.e., C_{7eq}), can exactly keep its energy by a slight rearrangement to Φ* = −68.7 and Ψ* = 84.5. This leads to the same C_{7eq} conformation as before. The effect is much stronger in other conformations such as α_L. As a consequence, a retro-inverso isomer is forced to rearrange its conformation with respect to the parent peptide.

Conclusion

With a conformational screening, it was possible to partially map the conformational space of the RGD sequence, and to obtain highly active and selective inhibitors for the $\alpha_v\beta_3$ integrin. The analysis of the structures of this work confirms that the conformation of cyclic pentapeptides is controlled by the array of chiralities in the ring. For that reason cyclic retro-inverso pentapeptides assume a different backbone conformation, and thus have another side chain topology compared to their parents. This and the changed amide bond directions lead to drastically changed inhibitory activities of the retro-inverso molecules. The

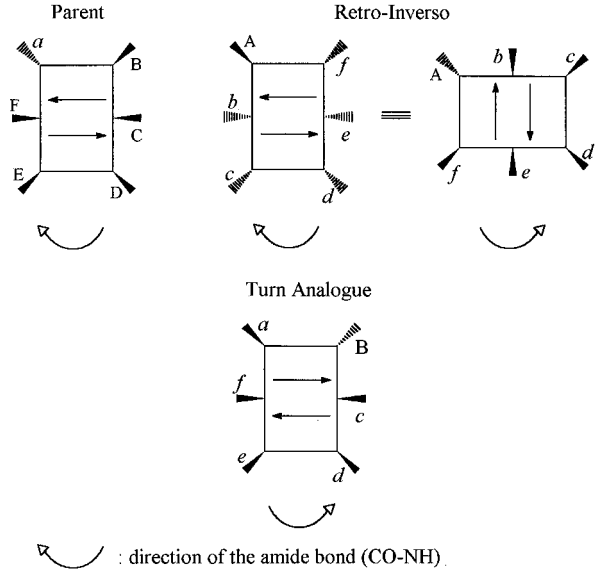

Figure 8. Cyclic hexapeptides and their retro-inverso analogs. In the postulated turn analog a possible recognition sequence CDEF is oriented the same way as in the parent, but with reversed amide bonds. D-configured residues are indicated by italic lower case letters.

retro-inverso analog of P4 proved to be a highly active inhibitor of the $\alpha_v\beta_3$ integrin. The excellent similarity of the side chain topology of active P2 and inactive RI1 strongly suggests an interaction of the RGD backbone with the receptor.

A topological difference of parent and retro-inverso peptides can be concluded for cyclic peptides with other ring sizes as well. For cyclic hexapeptides with one D-configured residue, for example, one expects the result schematically given in Figure 8. The D-configured residue is situated in the $i + 1$ position of the β-turn of the parent peptide. While residues A and D remain at the corner of the rectangle, the environment for all other residues is changed. B and E, for example, move to the side with an extended conformation in the retro-inverso peptide.

As for the pentapeptides, in the retro-inverso peptide a turn mimic can be generated by taking B as the L residue and

A as the D residue. If the residues CDEF were a recognition sequence in the biologically active hexapeptide c(aBCDEF) (Figure 8, parent), the importance of the amide bonds for binding to a receptor could be tested with the turn mimetic c(fedcBa).

Regarding the retro-inverso (RI) mimics of larger bulkier peptides,[47] one can draw further conclusions: if the considered (part of the) protein predominantly contains structural elements, which do not change their energy very much in the transformation of the Ramachandran map discussed above (e.g., β-sheet, γ-turns), the RI peptide could exhibit a conformation similar to that of the parent peptide. It could be stabilized by similar side chain−side chain interactions. However, if an α-helix (right-handed) is in the parent molecule, a different conformation for the RI peptide is expected, for an all-D peptide cannot exhibit a right-handed helix (like natural proteins, which never show left-handed helices).[48]

Acknowledgment. This contribution is dedicated to Gerhard Quinkert on the occasion of his 70th birthday. Financial support by the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

Supporting Information Available: Experimental details of synthesis, NMR spectroscopy, ligand−receptor inhibition assays, ^{1}H and ^{13}C resonances, $^{3}J_{HH}$ coupling constants, temperature gradients of chemical shift of amide protons, measured and calculated distances, dihedral angles of the structures, and comparison of the conformations with alanine model peptides as templates (19 pages). See any current masthead page of ordering and Internet access instructions.

JA961908L

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(48) Banerjee, A.; Raghothama, S. R.; Karle, I. L.; Balaram, P. *Biopolymers* **1996**, *39*, 279−285.