Peptidic Inhibitors

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# Conformational Control of Integrin-Subtype Selectivity in *iso*DGR Peptide Motifs: A Biological Switch**

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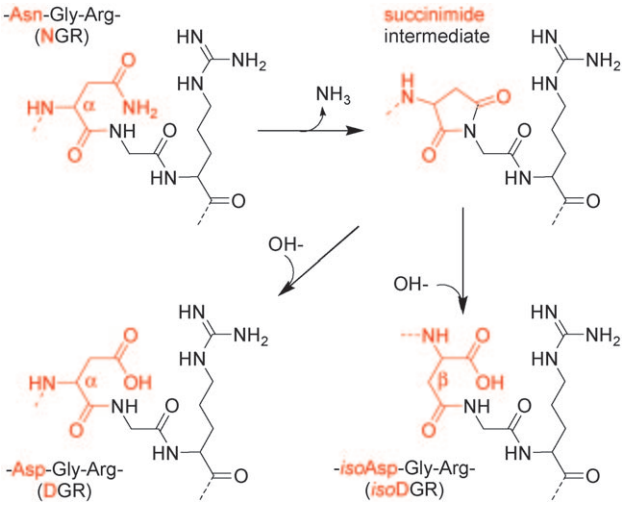
The rearrangement of asparagine into isoaspartate is a well-known (unwanted) side reaction in peptide synthesis,[1] which usually results in structures that lose biological activity (Scheme 1).[2] Isoaspartate formation occurs also in vivo, potentially leading to a loss of protein function. Therefore, this process has been proposed to be a biochemical clock that limits protein lifetimes.[4] In contrast, Curnis et al. have recently shown that deamidation of the Asn-Gly-Arg (NGR) motif in the extracellular matrix (ECM) protein fibronectin (FN) into *iso*DGR results in a gain of protein function by creating a new adhesion binding site for integrins.[5,6] Here, based on the *iso*DGR motif we present highly active head-to-tail-cyclized pentapeptides selective for the closely related αvβ3 and α5β1 integrins which have been identified by the study of spatial screening libraries[7] in vitro and in cellular assays.

Integrins are cell adhesion receptors that are involved in fundamental biological processes.[8] The peptide sequence Arg-Gly-Asp (RGD) is the most prominent motif to promote integrin-mediated cell adhesion to ECM proteins such as FN, vitronectin, and fibrinogen.[9] The RGD tripeptide in FN is recognized by at least four different integrins (α5β1, αvβx, α8β1, and αIIbβ3), leading to the assembly of an FN matrix around cells.[10] A mutation of the RGD sequence in the 10th type III repeat FN module (see Figure SI_1 in the Supporting Information) to RGE in mice abrogates integrin binding to the mutant motif.[6] Interestingly, despite this binding defect, FN containing the RGE mutant can still be assembled into FN fibrils[6] via αvβ3 integrin. Hence, it was suggested that the *iso*DGR motif, generated from the NGR sequences in the 5th type repeat I FN module (see Figure SI_1 in the Supporting Information), serves as novel integrin binding site.[6] However, this notion has recently been questioned in a report using recombinant FN with mutations in the NGR motif.[11] Even though the two key FN receptors αvβ3 and α5β1 share the same ligand-recognition motif, their function is not redundant. Upon FN binding they induce different cellular signals and behaviors, which is important for many physiological and pathophysiological conditions such as wound healing, angiogenesis, and cancer metastasis.[12]

To prove the hypothesis that deamidation of NGR into *iso*DGR generates de novo binding epitopes for the αvβ3 integrin, we studied constrained *iso*DGR peptides for their affinities for integrin subtypes. In addition, inspired by our previous findings that the conformation of the RGD sequence controls the selectivity between αvβ3 and the platelet integrin αIIbβ3,[13] we tested these peptides for selective binding to the closely related integrins αvβ3 and α5β1. For this purpose, we created different libraries of head-to-tail-cyclized pentapep-




***Scheme 1.*** The deamidation of NGR occurs via hydrolysis of a succinimide intermediate which leads to the formation of *iso*DGR or DGR, depending on the neighboring amino acid sequences, temperature, and ionic strength.[3]

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tides containing the *iso*DGR amino acid motif. New biologically active peptides comprising this sequence could serve as model compounds for the various NGR-containing modules of FN and other matrix components and may help to elucidate the structure–activity and the structure–selectivity relationships of the *iso*DGR sequence.

Our first approach was to synthesize a small library of peptides based on the retrosequence of the highly active integrin-binding cyclic peptide *c*(-RGDfV-),[14] for example, *c*(-Vf*iso*DGR-), by means of a D-amino acid scan. These peptides showed, in general, moderate to poor affinities for αvβ3 and no activity for α5β1 (compounds **I** to **V** in the Supporting Information). The results are comparable with those of a retrosequence approach published by Wermuth et al.[15] Hence, we created a second small library with sequences that mimic the GNGRG loops found in FN (modules I-5 and I-7; see Figure SI_I in the Supporting Information).[16] The *iso*DGR sequence was flanked by two glycines, and a D-amino acid scan was performed. In vitro testing showed no binding of the peptides to αvβ3 and α5β1 integrins (see peptide **1** in Table 1 and peptides **14** and **21** in the Supporting Information).

***Table 1:*** Inhibition of the binding of soluble α5β1 and αvβ3 integrin head groups to FN and vitronectin, respectively, by head-to-tail-cyclized *iso*DGR pentapeptides.[a]

| Cyclic peptide | $IC_{50}$ α5β1 [nM] | $IC_{50}$ αvβ3 [nM] |
|---|---|---|
| **1** c(G*iso*DGRG) | >2000 | 256 (±24) |
| **2** c(F*iso*DGRG) | >2000 | 633 (±524) |
| **3** c(f*iso*DGRG) | 838 (±160) | 377 (±272) |
| **4** c(G*iso*DGRF) | 816 (±345) | 168 (±63) |
| **5** c(G*iso*DGRf) | >2000 | 521 (±39) |
| **6** c(Hphe*iso*DGRG) | >2000 | >1000 |
| **7** c(hphe*iso*DGRG) | 83 (±21) | 410 (±107) |
| **8** c(G*iso*DGRHphe) | >1000 | 203 (±49) |
| **9** c(G*iso*DGRhphe) | 558 (±105) | 102 (±45) |
| **10** c(Phg*iso*DGRG) | 57 (±8) | 753 (±150) |
| **11** c(phg*iso*DGRG) | 19 (±4) | >1000 |
| **12** c(G*iso*DGRPhg) | >2000 | 467 (±162) |
| **13** c(G*iso*DGRphg) | 406 (±191) | 89 (±19) |
| Cilengitide | 15 (±3) | 0.54 (±0.15) |

[a] Inhibition constants were determined using a competitive solid-phase binding ELISA assay (see the Supporting Information for details). Hphe: L-homophenylalanine, hphe: D-homophenylalanine, Phg: L-phenylglycine, phg: D-phenylglycine. Cilengitide, *c*(-RGDf$^{Me}$V-), a superactive cyclic pentapeptide binding to αvβ3 integrin,[17] was included in the assay as a control compound.

In contrast, we have been successful with our third library which combines the two approaches explained above: the cyclic *iso*DGR peptides were flanked by one aromatic amino acid and one glycine. It is well known from the *c*(-RGDfV-) peptide that the aromatic moiety of phenylalanine is essential for αvβ3 integrin binding, whereas valine can be replaced by other amino acids, for example, lysine, without affecting receptor affinity.[18] We therefore synthesized cyclic *iso*DGR peptides containing L- and D-Phe as the aromatic residue (Table 1, peptides **2**–**5**). Since the structures of these peptides might differ from that observed for *c*(-RGDfV-), resulting in, for example, a different distance between the integrin binding pocket and the aromatic group, we decided to also modify the spatial position of the pharmacophoric phenyl group using homophenylalanine (Hphe; Table 1, peptides **6**–**9**) and phenylglycine (Phg; Table 1, peptides **10**–**13**).

Interestingly, the relative position of the *iso*DGR-flanking residues (aromatic amino acid and glycine) determines the affinity of the pentapeptides to either αvβ3 or α5β1. This effect was mainly observed when the aromatic residue was introduced as a D-phenylglycine, confirming the importance of the interaction of the aromatic group with the integrin binding pocket. In particular, it should be highlighted that peptide **11** has an activity for α5β1 comparable to that of the anticancer drug Cilengitide but is inactive for αvβ3 (see Table 1). To our knowledge, this is the first reported cyclic peptide based on a retro-RGD sequence with nanomolar activity and selectivity for α5β1. Only one natural peptide (*c*(CRRETAWAC)[19]) and some cyclic RGD peptides with ß-amino acids[20, 21]) have been described with similar activities and selectivities in cellular assays. Such a biological profile could help to clarify the molecular basis of the cancer-inhibitory effect of Cilengitide. Indeed, it is not fully understood whether the antitumor activity of Cilengitide is more ascribable to its αvβ3 or α5β1 inhibition. Moreover, the achieved results prove that head-to-tail-cyclized pentapeptides containing the *iso*DGR sequence are useful templates for targeting different integrin receptor subtypes.

To understand the selectivity profile of the most interesting peptides, we determined the three-dimensional structures of peptides **11** and **13** by solution-state NMR spectroscopy and molecular dynamics (MD) calculations[22,23] (Figure 1)

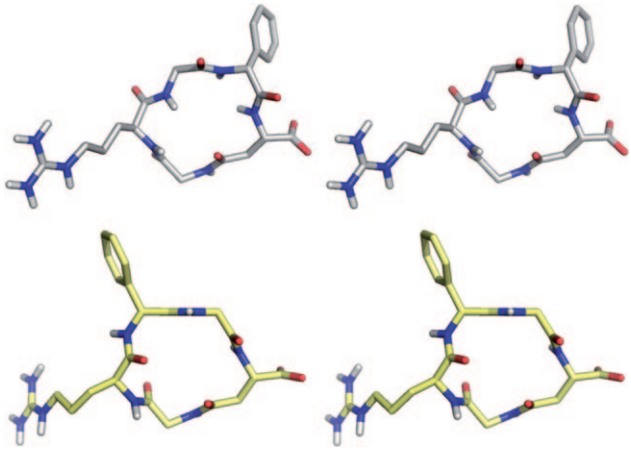

***Figure 1.*** Stereostructures of the cyclic pentapeptides **11** (top) and **13** (bottom) containing the *iso*DGR motif which bind to different integrin receptor subtypes. Peptide **11** shows nanomolar affinity towards α5β1, whereas **13** binds to αvβ3 integrin.

and docked each to both the αvβ3 receptor (X-ray structure of the Cilengitide–αvβ3 complex[24]) and the α5β1 receptor (homology model[25]; see the Supporting Information). (A detailed description of the structure calculations is given in the Supporting Information.)

According to docking results, **11** binds to α5β1 receptor with the *iso*Asp carboxylate group coordinating the metal ion

  

at the MIDAS (metal-ion-dependent adhesion site), and the Arg guanidinium moiety establishing a bidentate salt bridge with (α5)-Asp227 and a hydrogen bond with the backbone CO of (α5)-Ala222 (Figure 2). An additional hydrogen bond

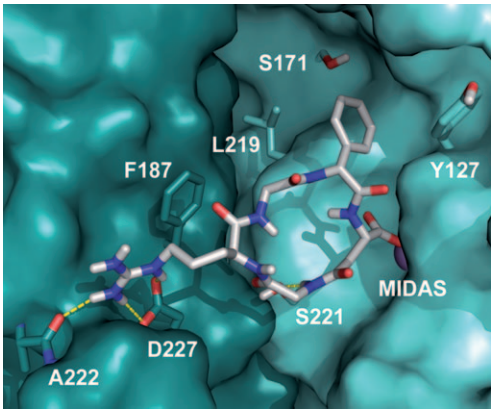


***Figure 2.*** Docked structure of **11** (white) in the α5β1 integrin binding pocket. The α5 and β1 subunits are represented by the dark and light turquoise surfaces, respectively. In both subunits, amino acid side chains relevant for ligand binding are shown as stick models. The metal ion in the MIDAS region is represented by a purple sphere. The one-letter code for the amino acids is used: A = Ala, D = Asp, F = Phe, G = Gly, L = Leu, S = Ser, Y = Tyr.

was detected between the peptide Gly NH group and the (β1)-Ser221 side chain. Interestingly, D-Phg is in the proximity of (β1)-Tyr127 (distance between the ring centroids 5.6 Å) and a π–π interaction is likely. Notably, D-Phg points towards the wide pocket below the SDL (specificity-determining loop), which consists of amino acids (β1)-Leu219, (β1)-Ser171, and (β1)-Gly217. The predicted binding mode could explain the observed high activity of **11** towards the α5β1 receptor. Predictably, this favorable binding mode of **11** cannot be found in the αvβ3 receptor (see the Supporting Information).

Conversely, besides the interaction with the metal ion and with (αv)-Asp218 (Figure 3), the lowest energy conformation of **13** bound to αvβ3 places D-Phg between the (αv)-Tyr178

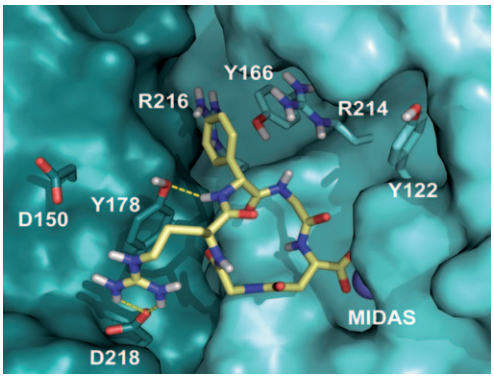


***Figure 3.*** Docked structure of **13** (pale yellow) in the αvβ3 integrin binding pocket. The αv and β3 subunits are represented by the dark and light turquoise surfaces, respectively.

and (β3)-Tyr166 aromatic side chains. In particular, the hydroxy groups of both Tyr residues point towards the edge of the D-Phg ring, establishing favorable interactions while a hydrogen bond is formed between the D-Phg NH group and the (αv)-Tyr178 OH group.

An alternative binding conformation close in energy to the lowest energy solution places D-Phg in the same pocket but forming a cation–π interaction with the (β3)-Arg214 side chain (see Figure SI_5 in the Supporting Information). Noteworthy, the favorable interactions with (αv)-Tyr178, (β3)-Tyr166, and (β3)-Arg214, which are characteristic for αvβ3, seem to be the reason for the observed drop in α5β1 affinity (see the Supporting Information). A comparison of the binding modes of **11**, **13**, and Cilengitide in αvβ3 is also provided (see the Supporting Information).

Integrin-selective compounds may be used to target cells with a specific profile of integrins, as exemplified by the success of the αvβ3- and α5β1-selective drug Cilengitide.[26] In order to test the binding capacity and specificity of compounds **11** and **13** also on living cells, we pre-incubated mouse fibroblasts expressing either only αvβ3 or only α5β1 with increasing concentrations of **11** and **13** before incubating them on FN (see the Supporting Information for details). The reduction of cell binding to FN via αvβ3 or α5β1 is relative to the binding constants of the cyclic peptides to the respective integrins (Table 2). Note that because of the different expression level of integrins on the different cell lines (left and right column) only relative binding activities can be compared. However, the data correlate nicely with the competitive solid-phase binding ELISA assay (see Table 1). For **11** the $IC_{50}$ value for blocking α5β1 was approximately 20 times less than that for **13**, but similar to that for the reference Cilengitide. On the other hand, the $IC_{50}$ value for blocking αvβ3 was approximately three times less for **13** than for **11**. In summary, we could demonstrate that in agreement with the binding constants measured with purified soluble receptors the compounds **11** and **13** target α5β1 and αvβ3 selectively on cells.

***Table 2:*** Binding activities of head-to-tail-cyclized *iso*DGR pentapeptides to mouse fibroblasts with selective expression of either α5β1 or αvβ3 integrin heterodimers.[a]

| Cyclic peptide | $IC_{50}$ α5β1 [μM] | $IC_{50}$ αvβ3 [μM] |
|---|---|---|
| **11** c(phg*iso*DGRG) | 4.2 | 1.0 |
| **13** c(G*iso*DGRphg) | 87 | 0.35 |
| Cilengitide | 2.0 | 0.009 |

[a] Inhibitory concentrations for cell adhesion to FN of the head-to-tail-cyclized *iso*DGR pentapeptides were tested using a cellular FN adhesion blocking assay (see the Supporting Information for details). $IC_{50}$ values cannot be compared for the different receptors (middle and right columns) owing to the different extent of integrin expression for each cell line.

Peptides containing the NGR or *iso*DGR motif are being used similar to RGD peptides[14b, 17, 27] to inhibit or target and visualize tumor neovasculature,[28] which may be an important future tool for drug delivery and tumor therapy. It was observed that a free α-amino group next to the *iso*DGR

  

lowers integrin affinity, whereas acetylation of this group increased the affinity but caused loss of specificity.[29] In our case we worked with head-to-tail cyclic *iso*DGR peptides without free α-amino groups, which allows a direct comparison of the stereochemistry and chemistry of the flanking residues. The relative rigidity of the small cyclic peptides gives evidence that the aromatic substituents in the flanking amino acids are important for selectivity of these peptides for αvβ3 and α5β1 integrins. Furthermore, the orientation of the crucial residues has been investigated and evidence for stereochemical control of activity is given. Hence, our findings are of interest for the rational design of *iso*DGR drug conjugates as well as for fusion proteins. Furthermore, these results are essential for the design of proteins using the NGR–*iso*DGR rearrangement for the controlled switch of binding affinities for different integrin subtypes in in vivo studies.

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**Keywords:** cyclic pentapeptides · integrin ligands · *iso*DGR sequence · NMR spectroscopy · peptides

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