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Integrin $\alpha_5\beta_1$ Ligands: Biological Evaluation and Conformational Analysis

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Integrins are heterodimeric glycoprotein receptors that are involved in many physiological and pathological processes. They mediate cell–cell and cell–matrix adhesion. Integrin $\alpha_5\beta_1$ is the classical fibronectin receptor. Together with integrin $\alpha_4\beta_1$, it has an important function in the migration of activated lymphocytes during the immune response and is also involved in cancer, diabetes and inflammatory diseases like rheumatoid arthritis.[1] The tripeptide sequence -Arg-Gly-Asp- (RGD) present in the extracellular-matrix protein, fibronectin, is essential for binding to integrin $\alpha_5\beta_1$. This sequence is found in the tenth type III repeat of fibronectin, which is part of the major cell-binding domain.[1]

Small molecules that are able to interfere with the fibronectin–$\alpha_5\beta_1$ binding event might be of interest in the therapy of cancer and inflammatory diseases.[2] Selective $\alpha_5\beta_1$ ligands have been proposed as lead structures for anticancer agents.[3] Synthetic peptides containing the RGD sequence emerged as an excellent starting point for the identification, synthesis and development of selective integrin ligands, as was shown, for example, for integrin $\alpha_v\beta_3$.[2,4] As the three-dimensional structure of integrin $\alpha_5\beta_1$ is not yet available, cyclic peptides are optimally suited to explore the structural requirements with respect to the three-dimensional arrangement of pharmacophoric groups (e.g., the guanidino group of Arg and the carboxylate of Asp) on a rational basis. For this approach the term "spatial screening" was coined by Kessler et al.[4] In order to analyze the integrin $\alpha_5\beta_1$–fibronectin interaction in the frame of the spatial screening procedure, it is necessary to induce different conformations within the RGD sequence.[4] We synthesized a library of cyclic RGD peptides that contain β-amino acids as

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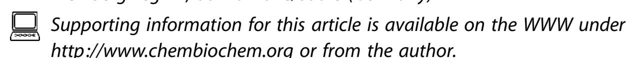
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building blocks with predictable conformational bias. Some of these peptides display high affinity towards integrin $\alpha_v\beta_3$ together with good selectivity between integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$.[5,6] Here we report on our finding that two cyclic peptides containing the RGD sequence and a β-amino acid display considerable affinity towards integrin $\alpha_5\beta_1$.

There are several relevant methods for analyzing the inhibitory properties of small-molecule integrin ligands, for example, cell-adhesion assays and microtiter plate-based integrin-binding assays.[7,8] Cell adhesion assays not only allow for a fast screening of the substances but also keep the experimental conditions close to those of in vivo systems. K562 cells are reputed to present $\alpha_5\beta_1$ as the predominant or exclusive integrin.[9] Therefore, this cell line seems to be especially suited for investigations of $\alpha_5\beta_1$–ligand interactions. Microtiter plate-based integrin-binding assays as classical in vitro assays provide more detailed information about the ligand-binding affinity, but require the purified receptor.[10] In addition to performing these two types of assay, we investigated the feasibility of analyzing integrin–peptide interactions with competition experiments in solution by using surface plasmon resonance (SPR). We analyzed the receptor–ligand interaction in vitro with enriched integrin, which was obtained in the form of a membrane extract of cells exclusively presenting one specific integrin type, for example, K562 cells. In contrast to the microtiter plate-based integrin binding assay, no purified integrin is required for this experiment. This type of assay would not only combine the advantages of the cell-adhesion assay and of the integrin-binding assay, but would also allow for the evaluation of kinetic parameters.

Conventional cell-adhesion assays (Figures 1 and 2) were performed in parallel to validate the results of the SPR analysis.[8,9,11] The peptide concentrations required to reduce specific binding by 50% (IC_{50}) were determined in a concentration series (Figure 2, Table 1).

The cyclic hexapeptide **3** and the cyclic pentapeptide **2** displayed the highest biological activity in this assay. They nearly

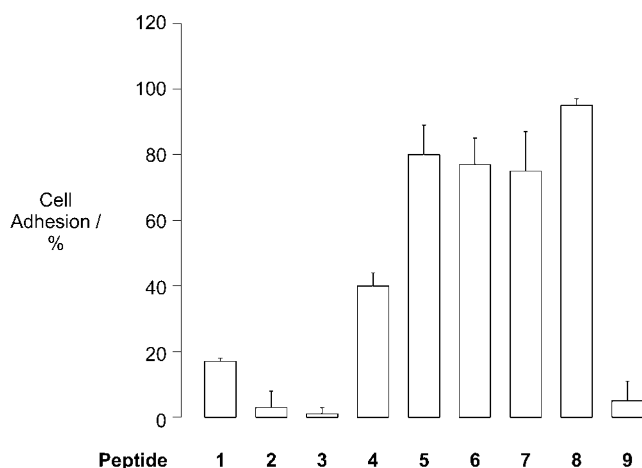

Figure 1. Influence of cyclic peptides on K562 cells in a cell-adhesion assay with immobilized fibronectin. The peptides were tested at a concentration of 70 μg mL^{-1}. The data represent the mean values with standard deviation for the tests, each of which was performed in triplicate.

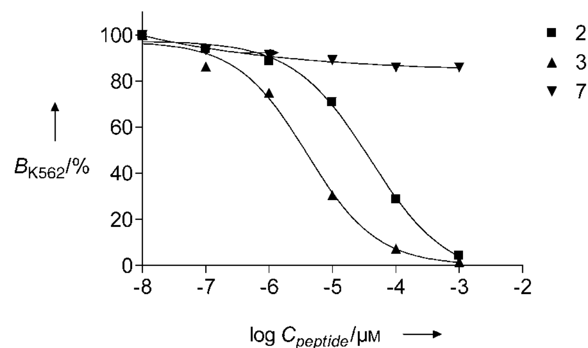

Figure 2. Effect of cyclic peptides on the attachment of K562 cells to immobilized human plasma fibronectin. $C_{peptide}$, peptide concentration; B_{K562}, binding of K562 cells to immobilized fibronectin.

Table 1. *IC_{50} values of $\alpha_5\beta_1$ ligands as determined from the cell-adhesion assay.*

Peptide	No.	IC_{50} [μM]
c-(-Arg-Gly-Asp-D-Phe-Val-)	1	279 ± 121
c-(-Arg-Gly-Asp-D-Phe-β-Leu-)	2	3 ± 1
c-(-Arg-Gly-Asp-D-Phe-Val-β-Ala-)	3	2 ± 1
c-(-Arg-Gly-Asp-D-β-Phe-Val-Gly-)	4	$>1\times10^3$
c-(-Arg-Gly-Asp-D-Phe-Val-Gly-)	5	$>1\times10^3$
c-(-Arg-Gly-Asp-D-Phe-β-Leu-Gly-)	6	$>1\times10^3$
c-(-Arg-Gly-Asp-D-β-HPhe-)	7	$>1\times10^3$
c-(-Arg-Gly-Asp-D-Phe-β-Leu-Ala-)	8	$>1\times10^3$
H-Gly-Ala-c-(Cys[S-S]-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys[S-S])-Gly-Ala-O(CH$_2$CH$_2$O)$_2$CH$_2$CH$_2$-NH$_2$	9	0.6 ± 0.2

completely inhibited the attachment of K562 cells to fibronectin at a concentration of 70 μg mL^{-1}. In spite of its high affinity towards $\alpha_5\beta_1$, peptide **2** is a nonselective ligand because it also binds tightly to integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$.[5,6] Interestingly, peptide **7** displays no affinity towards $\alpha_5\beta_1$, but is a nanomolar ligand for $\alpha_v\beta_3$.[5] The IC_{50} values of peptides **2** and **3** are highly comparable with the IC_{50} value of the reference peptide **9**, which is based on the sequence of the $\alpha_5\beta_1$-selective cyclic disulfide H-Gly-Ala-c-(Cys[S-S]-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys[S-S])-Gly-Ala-OH. This peptide was discovered by phage display, does not contain an RGD sequence and was until now regarded as the small-molecule ligand with the highest affinity to $\alpha_5\beta_1$.[12] Peptide **9**, a C-terminally modified analogue of this peptide, has been recently employed successfully by us for the isolation and affinity purification of integrin $\alpha_5\beta_1$.[13]

The inhibitory activity of peptide **1**, known to efficiently inhibit $\alpha_v\beta_3$-mediated cell adhesion to fibronectin, is about 10^2 times lower than that of **2** or **3**.[14,15] Peptide **4** only partly inhibits the adhesion of the cells (60% reduction), and almost no biological activity is displayed by peptides **5**–**8** (Figure 1). In these cases, the RGD sequence is most probably not presented in an appropriate conformation to interact with integrin $\alpha_5\beta_1$.

SPR has emerged as a powerful technique for the evaluation of protein–protein interactions.[16,17] The technique gives information on sensitivity and specificity like an ELISA, but also provides, in principle, the opportunity for kinetic studies on a dy-

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namic system. SPR has already been used for the investigation of different integrin–ligand interactions, like the binding between integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ and collagen type I, or the interaction between a recombinant chimeric epidermal growth factor-like module and integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$.[18,19] Here we describe for the first time the application of SPR to the analysis of the interaction between fibronectin and integrin $\alpha_5\beta_1$. SPR usually relies on the application of purified interaction partners. Integrin-containing cell-membrane extracts were used in our studies to evaluate the inhibitory properties of the peptides with SPR.

During the enrichment process of the membrane extract preparation, the $\alpha_5\beta_1$ protein expression of K562 and the $\alpha_5\beta_1$ membrane extract were analyzed by SDS-PAGE and visualized by silver staining or Western blotting with anti-α_5 antibody. The $\alpha_5\beta_1$ integrin concentrations of the extracts were determined according to the method of Bhown and Bennett.[20] For the SPR experiments, fibronectin was immobilized on a *N*-hydroxysuccinimide (NHS)-activated CM5 sensor chip. The $\alpha_5\beta_1$-containing membrane extract was injected as the soluble analyte in different concentrations, and the resulting SPR response between fibronectin and $\alpha_5\beta_1$ was found to be concentration dependent (Figure 3). This protocol allows the evaluation of

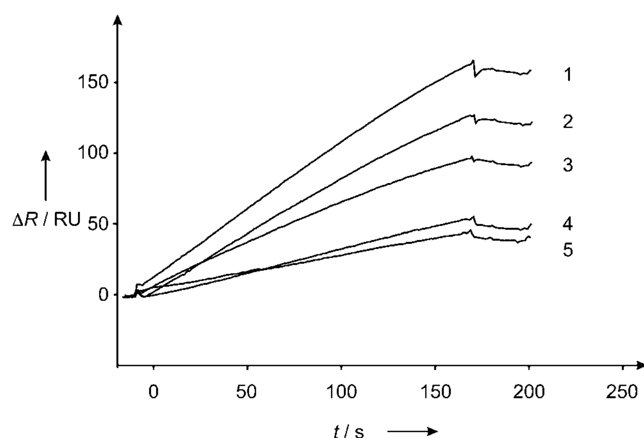

Figure 3. *SPR sensorgrams for $\alpha_5\beta_1$–fibronectin interaction with different integrin concentrations as obtained from the K562 membrane extract. The integrin at 6.4 $\mu g\,mL^{-1}$ (1), 4.6 $\mu g\,mL^{-1}$ (2), 3.2 $\mu g\,mL^{-1}$ (3), 2.3 $\mu g\,mL^{-1}$ (4) and 1.6 $\mu g\,mL^{-1}$ (5) was injected over the fibronectin-derivatized sensor chip (flow rate: 20 $\mu L\,min^{-1}$, contact time: 180 s, 25 °C).*

the association (K_A) and dissociation (K_D) constants for this interaction. K_D (1.5×10^{-8} M) and K_A (6.7×10^{7} M), as determined by SPR, indicate a high-affinity binding between $\alpha_5\beta_1$ and immobilized fibronectin.

Analysis of the SPR experiments revealed that high concentrations of Triton X-100 or octyl-β-D-glucopyranoside prevent the interaction between $\alpha_5\beta_1$ and the peptides. Therefore, the membrane extract was diluted with the extraction buffer without any detergent.

SPR competition experiments were also performed in solution. The membrane extract was incubated with different concentrations of peptide **3** to allow for complex formation prior to injection. The resulting sensorgrams (Figure 4) prove

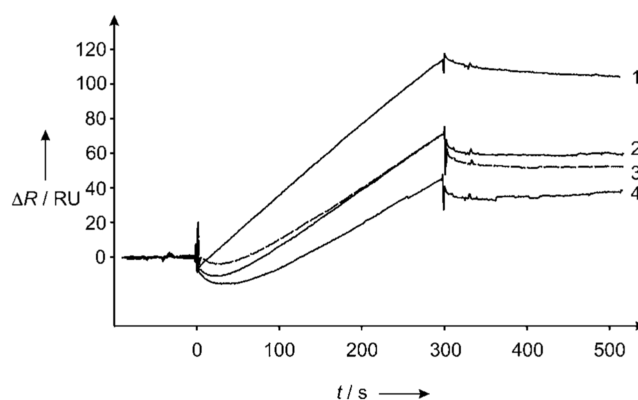

Figure 4. *SPR competition experiments in solution with K562 membrane extract and peptide **3** on the fibronectin-derivatized sensor chip. The membrane extract was incubated for 30 min with different concentrations of **3**. Data are shown for membrane extract alone (1) and after incubation with 349 μM (2), 465 μM (3) and 930 μM (4) of peptide **3**, (flow rate: 20 $\mu L\,min^{-1}$, contact time: 300 s, 25 °C).*

the inhibition of the interaction between fibronectin and $\alpha_5\beta_1$ in a concentration-dependent manner. As expected, the relative response decreased with increasing peptide concentrations. This indicates that binding is proportional to the concentration of the protein with free binding sites. The binding data generated were used to calculate the concentration of peptide **3** (6×10^{-4} M) that results in an attenuation of integrin binding to immobilized fibronectin to 50 % (B_{50} value).[21] Hence, in principle, SPR studies are feasible for the evaluation of peptide–integrin affinity. However, the detrimental influence of detergents on the interaction has to be taken into account and will prohibit broad application of this type of interaction screening.

SPR technology has also been demonstrated to be, in principle, useful for monitoring cell adsorption to ligand-coated surfaces.[22] SPR experiments were performed with whole K562 cells and surface-bound fibronectin (Figure 5). The specificity of the K562–fibronectin interaction was verified by competition

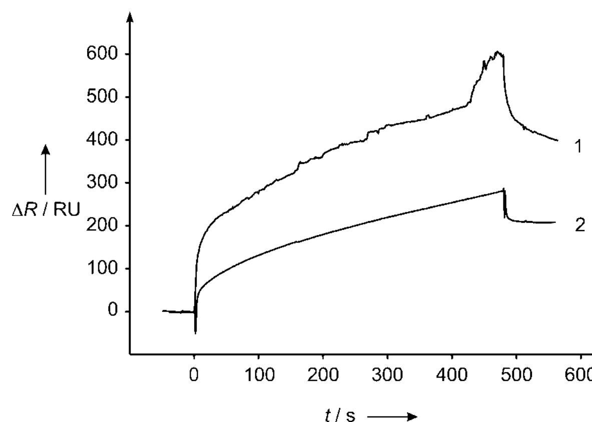

Figure 5. *SPR sensorgrams for whole K562 cells (1) and membrane extract of K562 cells (2). Cells and membrane extracts were injected over a sensor-chip surface with immobilized fibronectin (flow rate: 20 $\mu L\,min^{-1}$, contact time: 500 s, 25 °C).*

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experiments with fibronectin or peptide in solution (results not shown). However, the observed sensorgrams (Figure 5) indicate limited reproducibility in the analysis of interactions when intact K562 cells are used. Superior results were obtained with the membrane extracts of K562 cells, as described above.

Additional microtiter plate-based integrin-binding assays were performed. The results of this series of assays reflect the same tendency as the results of the cell-adhesion assay and the SPR studies (Table 2, Figure 6).

Table 2. *IC_{50} values determined from the integrin binding assay.*

Peptide	No.	IC_{50} [μM]
c-(-Arg-Gly-Asp-D-Phe-Val-)	1	11.0
c-(-Arg-Gly-Asp-D-Phe-β-Leu-)	2	2.0
c-(-Arg-Gly-Asp-D-Phe-Val-β-Ala-)	3	0.6
c-(-Arg-Gly-Asp-D-Phe-Val-Gly-)	5	51.0
c-(-Arg-Gly-Asp-D-β-HPhe-)	7	130.0
H-Gly-Ala-c-(Cys[S-S]-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys[S-S])-Gly-Ala-$O(CH_2CH_2O)_2CH_2CH_2$-NH_2	9	0.1

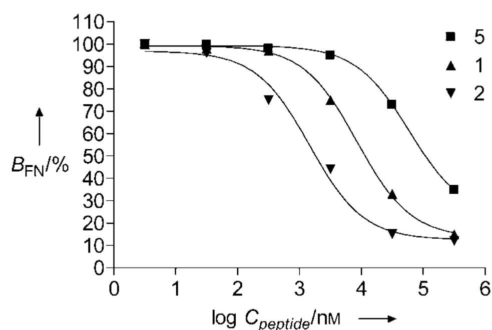

Figure 6. *Dose-response curves for peptides **1**, **2** and **5**, as determined with the microtiter plate-based integrin-binding assay. $C_{peptide}$, peptide concentration; B_{FN}, binding of biotinylated fibronectin to immobilized integrin $\alpha_5\beta_1$.*

Conformation analysis by NMR and molecular-dynamics (MD) simulations were performed for the most active representative, peptide **3**. Thirty-two distance restraints from a ROESY spectrum were used in the procedure, which comprised distance geometry, restrained MD calculations and free MD simulations.

As can be seen from the free MD simulations, the dominant solution structure of peptide **3**, *c*-(-Arg-Gly-Asp-D-Phe-Val-β-Ala-), presented in Figure 7[23] is characterized by a βII turn with Asp and D-Phe in the $i+1$ and $i+2$ positions. This is supported by a strong NOE between Asp H^{α} and D-Phe H^N and a medium NOE between D-Phe H^N and Val H^N. The βII turn is overlapped by a II-αRS turn, again with Asp in the $i+1$ position. The turns are stabilized by a bifurcated hydrogen bond between the amide protons of Val and β-Ala and the carbonyl oxygen of Gly. A distorted γ^i turn around Arg can also be observed. Remarkably, D-Phe is found in the $i+2$ position of the βII turn. While providing a reasonable model for the preferred solution structure of *c*-(-Arg-Gly-Asp-D-Phe-Val-β-Ala-), the MD

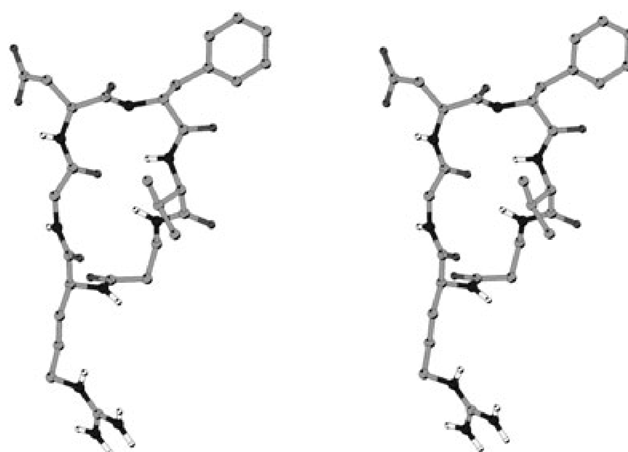

Figure 7. *Dominant solution structure of peptide **3**.*

trajectories also show that the molecule retains a significant degree of intrinsic flexibility. The highest flexibility is observed around β-Ala, as illustrated by the reversible flip of the peptide bond between Val and β-Ala. The main feature of the peptide is that the pharmacophoric RGD sequence is presented in a somewhat elongated conformation with an average distance between Arg C^{β} and Asp C^{β} of 930 pm.

The peptide *c*-(Mpa[S-S]-Arg-Gly-Asp-Asp-Val-Cys[S-S])-NH_2,[24] a heterodetic cyclopeptide with a disulfide bridge, has been reported to display affinity to $\alpha_5\beta_1$ ($IC_{50}=1.2$ μM).[25] The free and receptor-bound conformations (as obtained from trNOESY experiments) of this peptide are characterized by either a βI turn (free) or a βII′ turn (bound) with Gly^2-Asp^3 in the $i+1$ and $i+2$ positions. The authors claim that the accessibility of a conformation with a small average distance between Arg C^{β} and Asp C^{β} (560 pm for the bound peptide) is required for binding to $\alpha_5\beta_1$. Presumably, peptide **3** would not match these criteria, as the minimum value of this distance (600 pm) is only observed for short periods during the 10 ns trajectory. Further investigations on the receptor-bound conformation of **3** are currently in progress.

In summary, we report on the biological evaluation and conformational analysis of the peptide *c*-(-Arg-Gly-Asp-D-Phe-Val-β-Ala-), a ligand of integrin $\alpha_5\beta_1$. It emerged from a series of cyclic RGD peptides that were tested in a cell-adhesion assay, surface plasmon resonance studies and an integrin-binding assay.

Peptide **3** is shown to be a low-micromolar inhibitor of the $\alpha_5\beta_1$–fibronectin interaction. The solution conformation of this peptide was determined, but the receptor-bound conformation is still the subject of further investigations.

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Keywords: cell adhesion · conformation analysis · integrins · peptides · surface plasmon resonance

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