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Bicyclic RGD Peptides with Exquisite Selectivity for the Integrin $\alpha_v\beta_3$ Receptor Using a “Random Design” Approach

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INTRODUCTION

(Multi)cyclic peptides represent an important platform in drug development owing to their unique properties, such as conformational restriction and low toxicity. Peptides produced by nature, for example, romidepsin, vancomycin, and ciclosporin, and semisynthetic peptides such as dalbavancin are established peptide-based drugs. Over the past years, the bicyclic CLIPS-peptide platform, first described by our group, has attracted considerable interest by combining high target affinities and selectivities with appreciable proteolytic stabilities. It has been actively explored to provide a variety of (enzyme) inhibitors. For example, Heinis et al. used the technology in combination with phage-display library screening to identify a bicyclic peptide inhibitor (ACT$_3$DRFRNC$_{T3}$PADEAL$_{CT3}$G, T$_3$ = 1,3,5-trimesitylbenzene) displaying nanomolar affinity to plasma kallikrein ($K_i$ = 1.5 nM). Here, the consensus motif SDRFRN was identified in the first round of selection, followed by sequential optimization of the second loop. Notably, the activities of the linear peptides were at least 250-fold lower in comparison to the corresponding T$_3$-bicycles. The same group also reported a bicyclic peptide inhibitor (ACT$_3$SRYEVD$_{T3}$RGRGSA$_{CT3}$G) of urokinase-type plasminogen activator (uPA) with a $K_i$ of 53 nM and, most recently, an active bicyclic inhibitor (AC$_{T3}$HSRC$_{T3}$PQLPP$_{CT3}$G) of sortase A ($K_i$ = 1.1 $\mu$M) Luzzi et al. also explored the high-throughput potential of CLIPS phage-display libraries to identify potent bicyclic inhibitor (AC$_{T3}$PPC$_{T3}$LWQVLC$_{T3}$, $K_d$ = 10 nM) to TNF$\alpha$, one of the key mediators of several inflammatory disorders. As an alternative to this, Lian et al. developed a one-bead two-compound screening technology to identify double-digit nanomolar bicyclic peptide inhibitors for protein tyrosine phosphatase 1B (PTP1B), a type II diabetes target, with the target-binding sequence in the first loop and...
the cell-penetrating peptide FΦRRRQ (Φ: L-naphthylalanine) in the second loop. Recently, the same group reported a submicromolar bicyclic inhibitor for K-Ras, combining a cell-permeable peptide sequence in the first loop with the K-Ras binding motif AJFRaWID (J, D-Leu; Ψ, L-propargylglycine) in the second loop.9

In this paper we describe a novel approach to identify potent binding motifs with medium diversity (total of 672 peptides). These bicycles combine a “fixed” RGD loop (“Design”) with a second loop (XXX, “Random”) that further supports the RGD-integrin affinity and also brings selectivity into the peptide. Integrons represent a family of cell adhesion receptors11 that are potential targets for novel therapeutic agents resulting from their significant role in pathological processes. A major contribution to the investigation of integrin-binding peptides was made by Kessler and co-workers, who developed the potent α5β1 antagonist cilengitide,12 and other cyclopeptides such as α5β2, and α5β3.13a–g In addition, integrin affinity tuning via conformational confinement of the RGD peptide on surfaces has been reported.13d–f Recently, Cochran and co-workers described a family of high-affinity integrin-binding “cystine-knot” (knottin) RGD peptides, which are considered great candidates for drug development.14 However, these disulfide-rich peptides basically do not express any selectivity in binding to the integrins αβ1, αβ3, and αβ5. Here we describe a set of bicyclic RGD peptides that display both high affinities and outstanding selectivities for αβ3. Moreover, we also developed a similar set of binders with selectivities for the integrin αβ1, the results of which will be disclosed elsewhere. We consider this combined “random-design” approach also highly suited for identifying high-affinity and -selectivity binders to different ECM target proteins based on e.g. the laminin-binding YIGSR- or IKVAV-peptide motifs.

## Results and Discussion

### General Procedure for Library Screening.

Our approach involves the design of partially randomized libraries of small, RGD-containing bicyclic CLIPS (chemical linkage of peptides onto scaffolds)15 peptides to be used in an iterative affinity and selectivity optimization process for the integrin receptor αβ3. We used libraries of “label-free” peptides comprising acetylated N-termini and C-terminal amides, the individual αβ3-binding activities of which were evaluated by measuring the extent of inhibition of biotinylated knottin-RGD binding to integrin αβ3 using a recently published competition ELISA setup (see Table S-1 in the Supporting Information).16 A schematic representation of this setup is given in the Supporting Information of ref 16. At first, all 672 bicyclic RGD peptides were screened for inhibition at the highest concentration (1 mM). For the top 96 hits from the first screening (top 30 for second and third generations), which showed at least 90% inhibition at 1 mM, a second screening was performed at lower concentration (2.5–10 μM) to determine their affinities more accurately. Finally, the best binders were resynthesized and HPLC-purified, followed by determination of the IC50 values.

### Design and Synthesis of RGD Peptide Libraries.

We designed linear peptide libraries consisting of two separate binding motifs surrounded by three cysteines. The first motif contains the well-known RGD sequence that should provide the basal integrin affinity, while the second motif contains a randomized sequence “XXX”, which is intended to support binding of the RGD loop and also to provide integrin selectivity. The motifs are enclosed by cysteines, which ensure the double CLIPS cyclization (T3, Figure 1) and hence the formation of a bicyclic peptide comprising two different loops.

The primary challenge was to determine the proper size of both the RGD and the random X(X),X loop. The well-known integrin binder cilengitide (cycle-[V(N-Me)RGDf])12 consists of five amino acids. In a bicyclic peptide, a similar 3-mer loop is generated by enclosing the 3-mer RGD sequence with two cysteines; hence, the minimal integrin-binding motif CRGDC was selected. We kept the RGD loop size constant in the first-generation library and checked for further optimization at a later stage. For the second randomized loop, we considered a trimer XXX motif suitable to provide the required level of structural and conformational diversity. When all natural amino acids (except cysteine and methionine) are included, there are 324 (183) possible variants of the dimer motif XX, 5832 possible trimer sequences XXX, and 104976 variants for the tetramer motif XXXX. Therefore, 96 different XXX motifs would cover 1.8% of the total viable natural L-tripeptide space, which is reasonable when one considers the chance to overlook a high-affinity integrin binder after several rounds of optimization, in particular in comparison to the 0.09% coverage of structural space when a tetrameric XXXX motif is used.

The RGD motif was located either in the C-terminal/right loop and the XXX sequence in the N-terminal/left loop or vice versa. A total of 96 random XXX sequences were generated for each loop by using the software program R. In view of the apparent effect of D-amino acids on integrin binding affinity,12,17 additional sublibraries comprising different combinations of L- and D-cysteines were also designed. Hence, the entire first-generation library consisted of four sublibraries with...
96 random sequences in the left loop and three sublibraries with random sequences in the right loop, giving a total number of 672 different T3 bicycles (CT3XXXCCT3RGCDC3T3, C T3XXXXCT3RGDC3T3, C T3XXXXCT3RGDC3T3, C T3XXXXCT3RGDC3T3, CT3RGDC3T3XXXXCT3T3, C T3RGDC3T3XXXXCT3T3, C T3RGDC3T3XXXXCT3T3, C T3RGDC3T3XXXXCT3T3, C T3RGDC3T3XXXXCT3T3, Figure 1). After these libraries were screened for binding to integrin αvβ3 (method described below), various lead motifs for next-generation libraries were derived from the sequences of the best binders. The second loop in the second-generation libraries comprised (i) all first-generation lead sequences (e.g., QAD), extended by one amino acid (XQAD or QADX), and (ii) a full replacement set of the second amino acid (HXQ motif, in view of multiple positive hits: HEQ/2xHLQ). After the best second-generation αvβ3-binders were identified, a set of third-generation libraries was designed on the basis of (i) a full set of replacement variants (86 peptides) based on the HWQ motif and (ii) extension of the RGD loops on either or both ends with one amino acid (e.g., RGDX/XRGD) while the second loop HWQ was kept constant.

**Screening Bicyclic Peptide Libraries for αvβ3-Bindin.** About 4% of the 672 first-generation bicyclic RGD peptides showed OD405 values below 0.4, corresponding to more than 80% inhibition of knottin-RGD binding to αvβ3 (OD405 = 0.2, 100% inhibition; OD405 = 1.2, 0% inhibition). For the strongest αvβ3-binding bicycles, we observed an overrepresentation of peptides comprising the RGD motif in the C-terminal/right loop. Moreover, the best binders all had at least one D-Cys attached to the RGD sequence, either C-terminal or both N- and C-terminal, which is consistent with the results of Kessler et al., who reported enhanced integrin binding for (mono)cyclic peptides with a D-amino acid next to the RGD motif. Furthermore, we also found that peptides comprising the motif HXQ (X = any l-amino acid) in the left loop mostly showed significant inhibition. Surprisingly, approximately 50% of the bicyclic RGD peptides showed OD405 values of higher than 2.0, even though the OD405 of pure biotinylated knottin-RGD peptide was only ~1.2. Apparently, those bicycles...
somewhat activate either the $\alpha_{\beta_3}$ for knottin-RGD binding or vice versa. Another possibility might be that these bicyclic precursors bind to the integrin and streptavidin. In any event, at this point we did not further investigate those bicycles or considered them of interest as potent and selective $\alpha_{\beta_3}$ binders.

We then retested the best 96 hits from the first screening using only a 100-fold excess (10 $\mu$M) over the knottin-RGD peptide, and from these only 28% (27 peptides) showed $>$50% inhibition. The four strongest binders from both screenings ($C_{\alpha_1}QADC_{\beta_3}RGDC_{\alpha_1}$, $C_{\beta_3}QADC_{\alpha_1}RGDC_{\beta_3}$, $C_{\alpha_1}HEQC_{\beta_3}RGDC_{\alpha_1}$, $C_{\alpha_1}QWGC_{\beta_3}RGDC_{\alpha_1}$) were then resynthesized on a larger scale (20 $\mu$mol) and purified and their activities determined (195 nM $< IC_{50} < 835$ nM, Figure 2A). These values are still significantly higher than those determined for the knottin-RGD peptide itself (38 nM) and the cyclo-$KRGD$ peptide (35 nM). To further improve affinities, second-generation libraries were designed using the six best binders from first screenings as a lead, having (i) an extended (4-mer) second loops, e.g. XQAD or QADX (total of 216 peptides, see Figure 1), (ii) a full set of replacements for the glutamate (E) of the motif HEQ (25 peptides), (iii) a set of 12 non-natural variants of various potent lead sequences (see the Supporting Information), and (iv) the best 8 hits from first-generation screenings. From the total set of 261 RGD sequences, 31% (82 peptides) showed $>$50% inhibition at 5 $\mu$M, while only 7% (18) showed even $>$70% inhibition. To further narrow down the selection, we performed a rescreening of the best 30 hits at 2.5 $\mu$M, in which only 6% of the peptides (15) showed $>$50% inhibition and only 2% (6 peptides) showed $>$70% inhibition. None of the six best binders comprised an extended 4-mer sequence (such as XQAD or WGDX), indicating the choice for a trimer sequence in the left loop was correct. Instead, five peptides comprised the XHQ motif (with X = W(2x)/P/N/F) and one non-natural sequence $Q[Abu]$D (Abu = l-2-aminobutyric acid), with $IC_{50}$ values (measured with HPLC-purified peptides) ranging from 30 nM ($C_{\alpha_1}HPQ_{\beta_3}RGDC_{\alpha_1}$) to 225 nM ($C_{\alpha_1}HWQ_{\beta_3}RGDC_{\alpha_1}$, Figure 2A). Molecular structures of the best $\alpha_{\beta_3}$ inhibitors are shown in Figure 2B. Five of the six second-generation binders showed lower $IC_{50}$ values in comparison to the best first-generation binder ($IC_{50} = 195$ nM). The strongest binder, $C_{\alpha_1}HPQ_{\beta_3}RGDC_{\alpha_1}$, displayed an $IC_{50}$ value of 30 nM, which is comparable to that of knottin-RGD (38 nM) as reported by Kimura et al. $^{1,2a}$

We then designed third-generation libraries comprising (i) a complete set of single-replacement variants for the second-generation lead HWAQ (86 peptides) and (ii) 4-mer and 5-mer RGD loops extended by an additional amino acid (X) at either one or both ends, while the HWAQ loop was kept constant (57 tetramer and 56 pentamer sequences, in total 113 peptides; Figure 1). From the total of ~200 bicycles that were screened at 5 $\mu$M, 35% (79 peptides) showed $>$50% inhibition, 18% (35 peptides) showed $>$70% inhibition, and only 4% (8 peptides) showed $>$90% inhibition. In the rescreening of the best 20 hits (at 2.5 $\mu$M), 95% (19) of the peptides showed $>$80% inhibition, while only four peptides (20%) showed even $>$90% inhibition. The best six binders (i.e. $C_{\alpha_1}HPQ_{\beta_3}RGDC_{\alpha_1}$, $C_{\alpha_1}HFQC_{\beta_3}RGDC_{\alpha_1}$, $C_{\alpha_1}HRQC_{\beta_3}RGDC_{\alpha_1}$, $C_{\alpha_1}HFC_{\beta_3}RGDC_{\alpha_1}$, $C_{\alpha_1}HC_{\beta_3}RGDC_{\alpha_1}$, $C_{\alpha_1}HSCP_{\beta_3}RGDC_{\alpha_1}$) were resynthesized and purified for activity testing in a concentration-dependent manner. The most active binder ($C_{\alpha_1}HPQ_{\beta_3}RGDC_{\alpha_1}$, $IC_{50} = 31$ nM) differed only from the best second-generation binder, i.e. $C_{\alpha_1}HPQ_{\beta_3}RGDC_{\alpha_1}$ (Figure 2A), in the configuration of the second cysteine. The other third-generation binders showed $IC_{50}$ values ranging from 42 to 157 nM.

An alanine replacement study, in which each amino acid of the best $\alpha_{\beta_3}$ binder $C_{\alpha_1}HPQ_{\beta_3}RGDC_{\alpha_1}$ (85% inhibition at 1 $\mu$M) was sequentially replaced by l- or d-alanine, revealed significantly decreased affinities when R/G/D (<0%), H/P/Q (40–69%), or either one of the l-/d-cysteines residues formed monomeric peptides (4–25%, Figure 2C). Furthermore, a full replacement study on the HWQ loop of the second-generation binder $C_{\alpha_1}HWQ_{\beta_3}RGDC_{\alpha_1}$ (Figure 2D) clearly revealed the much higher importance of H (histidine) and Q (glutamine) in comparison to W (tryptophan). This obviously is the reason why so many W variants showed up as potentially improved binders.

**Essence of Cysteine Residues.** We also tested variants of the second-generation lead $C_{\alpha_1}HWQ_{\beta_3}RGDC_{\alpha_1}$ (27 peptides in total) in which one, two, or all three cysteines were replaced by the non-natural variants l- or d-homocystine (hc/hc), or l- or d-penicillamine (Pen/Pen) (Figure S2 in the Supporting Information). All mutations showed a clear decrease in $\alpha_{\beta_3}$ binding, but the decrease was much stronger for the Pen variants in comparison to the hc variants. For example, $C_{\alpha_1}HWQ_{\beta_3}(hc)_{\alpha_1}RGDC_{\alpha_1}$ and $C_{\alpha_1}HWQ_{\beta_3}(hc)_{\alpha_1}RGDC_{\alpha_1}$ showed 85/53% and 80/37% inhibition at 10 or 1 $\mu$M (88/55% for the lead), whereas the corresponding Pen variants $C_{\alpha_1}HWQ_{\beta_3}(Pen)_{\alpha_1}RGDC_{\alpha_1}$ and $C_{\alpha_1}HWQ_{\beta_3}(Pen)_{\alpha_1}RGDC_{\alpha_1}$ basically showed no measurable inhibition (i.e., 0%) at 10 $\mu$M. Occasionally the Pen variants did show activities comparable to those of the corresponding hc variants, as for ($Pen)_{\alpha_1}HWQ_{\beta_3}(Pen)_{\alpha_1}RGDC_{\alpha_1}$ (31%/8% at 10/1 $\mu$M) and (hc)_{\alpha_1}HWQ_{\beta_3}(hc)_{\alpha_1}RGDC_{\alpha_1}$ (20%/12% at 10/1 $\mu$M), but an improved affinity was never observed, suggesting that the additional methyl groups in penicillamine largely abolish the integrin–bicycle interaction.

**Single-Loop Controls.** To illustrate the essence of the bicyclic peptide format for binding activity, we synthesized and tested the single-loop variants of some of the first-generation binders in which one of the d-/l-cysteines was replaced by alanines. The single-loop variants ($C_{\alpha_1-A-1}$, $AQAD_{\alpha_1}RGDC_{\alpha_1}c/\alpha-2$, $CM_{\alpha_1}QAD_{\alpha_1}RGDC_{\alpha_1}c/\alpha-3$, $CM_{\alpha_1}\alpha_{3}QAD_{\alpha_1}RGDC_{\alpha_1}c/\alpha-3$, $CM_{\alpha_1}\alpha_{3}QAD_{\alpha_1}RGDC_{\alpha_1}c/\alpha-3$, $CM_{\alpha_1}\alpha_{3}QAD_{\alpha_1}RGDC_{\alpha_1}c/\alpha-3$) were synthesized using 1,3-bis-(bromomethyl) benzene (mT2), which is the bivalent half of the trivalent T3 scaffold (for more detailed information on peptides constrained via CLIPS scaffolds T3 and mT2, see refs 2, 3b, 4, 5, and 10). For $C_{\alpha_1}QAD_{\alpha_1}RGDC_{\alpha_1}$, $\alpha_{\beta_3}$ binding strongly decreased on opening the QAD loop (79% to 7% for C/A-1), the RGD loop (79% to 0% for c/3-3), or both (79% to 32% for c/2-2) (see Table S-2 in the Supporting Information). Likewise, a vast decrease in affinity was observed for single-loop variants of bicyclic peptides $C_{\alpha_1}HEQC_{\beta_3}RGDC_{\alpha_1}$ (from 92% to 22% (C/A-1), 28% (c/3), and 22% (c/2-2) and C_{\alpha_1}WGD_{\beta_3}RGDC_{\alpha_1} (from 75% to 52% (C/A-1), 8% (c/3), and 23% (C/A-2)), which exemplifies the essence of constraining both loops for optimal $\alpha_{\beta_3}$ inhibition. Moreover, the corresponding single-loop RGD peptides also showed much lower binding values at 10 $\mu$M in comparison to the best bicyclic binders ($CM_{\alpha_1}RGDC_{\alpha_1}mT2$, 23%; $CM_{\alpha_1}RGDC_{\alpha_1}mT2$, 57%; $CM_{\alpha_1}RGDC_{\alpha_1}mT2$, 41% (Table S-2 in the Supporting Information), which underscores the importance of the second loop for integrin $\alpha_{\beta_3}$ binding.
Binding of HPQ Bicycles to Streptavidin. The fact that we identified high-affinity αβ3 binders comprising the HPQ motif via a competition ELISA may complicate the assay interpretation, as this motif is known to bind to streptavidin under certain conditions.18 In order to exclude the possibility that bicycles containing HPQ or similar motifs directly interact with streptavidin, and so would falsely indicate a strong αβ3 interaction, we directly measured the ability of a series of bicyclic and monocyclic peptides comprising the HPQ motif to bind to streptavidin-HRP in a simple binding ELISA (Table S-3 in the Supporting Information). As expected, none of the bicyclic and monocyclic peptide mixtures showed detectable binding to Streptavidin-HRP in the 0.005–30 μM concentration range as used in the competition ELISA. Only the bicyclic peptides CmT2-HPQcT3-RGDcT3 (OD ≈ 0.2) and monocyclic control CmT2-HPQcT3 showed very weak binding to Streptavidin-HRP at 100 μM (OD ≈ 0.4), but this can by no means be held responsible for the fact that bicyclic CmT2-HPQfC3-RGDcT3 and CmT2-HPQcF3-RGDcT3 were identified as the most potent RGD bicycle binders to integrin αβ3.

Determination of Affinity Binding Constants (Kd). We also studied the bicycle-integrin interaction in a direct manner using surface plasmon-enhanced fluorescence spectroscopy (SPFS),19 an optical technique that combines surface plasmon resonance (SPR) with fluorescence spectroscopy. For that purpose, (i) the bicycle-peptide CmT2-HPQfC3-RGDcT3 (best third-generation binder), (ii) linear GRGDS, (iii) cyclo-[KRGDf] and (iv) knottin-RGD were resynthesized including an N-terminal linker (K-PPPSG[4-Abz]SG, Abz = 4-amino-cyclo-third-generation binder), (ii) linear GRGDS, (iii) cyclo-[KRGDf] and (iv) knottin-RGD were resynthesized including an N-terminal linker (K-PPPSG[4-Abz]SG, Abz = 4-amino-cyclo-third-generation binder) following earlier studies of Pallarola et al. showing this linker to be minimally invasive with integrin affinity.20 The N-terminal lysine was acetylated, while the lysine side-chain amine was deprotected. Since the use of conventional SPR lacked sensitivity (data not shown), probably due to the low molecular weight (~2 kDa) of the bicycles, the peptides were labeled with the fluorescent Cy5 tag via coupling of a Cy5-NHS active ester to the lysine side-chain amine, and the surface plasmon field intensity was applied at the wavelength coincident with the absorption band of Cy5 to locally excite a fluorescent signal in close proximity to the gold surface. This approach increases the fluorescence signal originating from the peptide binding to the surface with an immobilized integrin receptor, which allows kinetic measurements.19 Concentration-dependent fluorescence curves F(c) were normalized to ΔFmax (value measured at saturation concentration) and fitted via Langmuir isotherm (Figure 3A). The dissociation equilibrium constant (Kd) was determined for Cy5-labeled CmT2-HPQfC3-RGDcT3, which turned out to be comparable to that of Cy5-labeled knottin-RGD (0.6 nM, Figure 3B). These results perfectly align with the competition ELISA data (Figure 2A). For Cy5-labeled cyclo-[KRGDf], a slightly weaker affinity (Kd = 4.1 nM) was determined, indicating that Cy5 labeling affects αβ3 binding of cyclo-[KRGDf] more significantly than for knottin-RGD or the bicyclic peptide (see IC50 values, Figure 2A). The linear peptide Cy5-GRGDS did not show measurable binding to αβ3 in SPFS.

Selectivity of Bicycle Binding to αβ3. Finally, we compared the αβ3 binding abilities of CmT2-HPQfC3-RGDcT3, CmT2-HPQcF3-RGDcT3, and CmT2-HSQcF3-RGDcT3, the three highest-affinity bicycle peptides, and control peptides (knottin-RGD and cyclo-[KRGDf]) with the corresponding binding values for integrins αβ1 and αβ3 by measuring IC50 values in a competition ELISA. We found that none of the three bicycle peptides showed any measurable interaction with either αβ1 nor αβ3 (IC50 > 10000 nM), which highlights their outstanding selectivities for αβ3 (Table 1). In sharp contrast to this, cyclo-[KRGDf] did show strong binding to αβ3 (IC50 = 182 nM) while not to αβ1, whereas knottin-RGD bound to all integrins with equally strong affinity (αβ3, IC50 = 76 nM; αβ1, IC50 = 114 nM). These data reveal that bicyclic RGD peptides display αβ3 selectivities superior to benchmark RGD peptides, such as knottin-RGD, cyclo-[KRGDf] and cilengitide.12 Recently, Neubauer et al. described peptidomimetics with αβ3/αβ1 selectivity ratios of ~0.006 and ~0.007 (ratio of corresponding IC50 values),13 which is still at least 2-fold less selective in comparison to the highest-affinity bicyclic αβ3 binder CmT2-HPQfC3-RGDcT3, for which this ratio is ≤0.003.

Table 1. IC50 Values Determined Using Competition ELISA for Three Best-Binding RGD Bicycle Peptides, cyclo-[KRGDf], Cilengitide, and Knottin-RGD for Different Integrins

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC50 (nM)</th>
<th>αβ1</th>
<th>αβ3</th>
<th>αβ5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CmT2-HPQfC3-RGDcT3</td>
<td>30 ± 4</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td></td>
</tr>
<tr>
<td>CmT2-HPQcF3-RGDcT3</td>
<td>31 ± 2</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td></td>
</tr>
<tr>
<td>CmT2-HSQcF3-RGDcT3</td>
<td>42 ± 6</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td></td>
</tr>
<tr>
<td>cyclo-[KRGDf]</td>
<td>35 ± 6</td>
<td>182 ± 29</td>
<td>&gt;10000</td>
<td></td>
</tr>
<tr>
<td>cilengitide</td>
<td>121 ± 18</td>
<td>26 ± 5</td>
<td>&gt;10000</td>
<td></td>
</tr>
<tr>
<td>knottin-RGD</td>
<td>38 ± 5</td>
<td>76 ± 7</td>
<td>114 ± 8</td>
<td></td>
</tr>
</tbody>
</table>

*Each concentration was tested in triplicate. IC50 values were calculated via nonlinear regression analysis using GraphPad.*
Structural Analysis via 2D-NMR Spectroscopy. Bicycle peptide C_{fl}HPQCC_{fl}RGDC_{fl} was studied in detail by NMR using different techniques (COSY/ROESY/TOCSY/HSQC). Individual residues were identified through their $^1$H and $^{13}$C chemical shifts (from edited HSQC) as well as chemical shifts of the side-chain resonances (2D TOCSY). From these data it can be concluded that the peptide largely adopts a random coil structure rather than an ordered secondary structure (Figure 4). This is fully in line with data from circular dichroism spectroscopy (data not shown) that were also representative of mainly random-coil structures in solution. Another possibility is that the peptide adopts a different folded structure—apart from $\alpha$-helix or $\beta$-sheet—that cannot be detected with 2D NMR techniques. Interestingly, the histidine residue shows a double set of aromatic proton resonances at 8.66/8.60 and 7.33/7.29 ppm, which according to 1D/2D-ROESY experiments are belonging to different conformers in chemical exchange. Given the slight differences in $^1$H and $^{13}$C chemical shifts of the exchanging histidines, it is unlikely the effect arises from $\pi/\tau$ tautomerism. Rather, a more likely explanation is that two different proline conformations coexist (≈3:1 ratio), which influences the chemical shifts of neighboring residues, such as histidine and the $N$-acetyl methyl resonance (1.91/2.03 ppm). This is supported by the significant differences in the proline diastereotropic H$_3$ resonances (3.92 vs 3.66 ppm and 3.75 vs 3.50 ppm), and the effect diminishes as one moves farther away from the proline. Considering the sharpness of the resonances, relaxation measurements were not pursued, as the structure is most likely monomeric.

Membrane Binding on Integrin-Expressing Cells (HT29). In order to prove that RGD bicycles also bind to integrins in their natural environment of the cell membrane, we labeled human colon cancer cells (HT29, express integrin $\alpha_v$ subunit) with the Cy5-functionalized bicycle peptide C_{fl}HPQCC_{fl}RGDC_{fl}, knottin-RGD, and cyclo-[KRGDf] and detected fluorescence emission via confocal microscopy. Surprisingly, the bicycle shows very high staining levels, much higher than that of the Cy5-labeled benchmark knottin-RGD (Figure 5), while the Cy5-labeled cyclo-[KRGDf] peptide is virtually silent under these conditions. Thereby, the peptide is clearly located on the cell membrane and is hardly internalized. Control studies with scrambled RGD bicycles showed did not show any traces of cell staining (data not shown), which proves that binding is clearly sequence specific. These data illustrate the potential of RGD bicycles as powerful markers of integrin expression on live cells.

CONCLUSION

In this study, we presented bicyclic RGD “CLIPS” peptides as a novel platform for high-affinity and -selectivity integrin $\alpha _3\beta _3$ binders. These peptides offer a straightforward, cost-effective, and versatile alternative for established binders, such as knottin-RGD and cyclo-[KRGDf]. The observed high selectivity for integrin $\alpha _3\beta _3$ suggests applications in integrin-mediated in vivo tumor staining, cancer diagnostics, and/or cancer therapeutics.
MATERIALS AND METHODS

Reagents and Chemicals. Incubation and washing buffers were prepared using standard protocols. Recombiant human integrins were purchased from R&D Systems (Minneapolis, USA). Strept-HRP (Streptavidin-Horseradish Peroxidase conjugate, Southern-Biotec, Birmingham, USA), was diluted 1:1000 for ELISA experiments. Amino acids were purchased from Iris Biotech (Marktredwitz, Germany) and Matrix Innovation (Quebec, Canada). Resins were purchased from Rapp Polymerc (Tübingen, Germany) and Merck (Darmstadt, Germany). MnCl₂·4H₂O, 1,3,5-tris(bromomethyl)benzene (T₃), 1,3-bis(bromomethyl)-benzene (mT₂), 2,2-dithiobis(5-nitropryidine) (DTNP), ethyl(dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine, Tween20, ethylene glycol, and acetic acid and sodium acetate for the preparation of acetate buffer were purchased from Sigma-Aldrich (Steinheim, Germany). CaCl₂·2H₂O, MgCl₂·6H₂O, and phosphate-buffered saline (PBS) were purchased from Merck (Darmstadt, Germany). Tween80 was purchased from Faryon (Capelle, The Netherlands), and I-Block was purchased from Tropix (Bedford, USA). Disulfo-Cys-S-NHS ester was purchased from Cyandye (Sunny Isles Beach, USA). Dithiolaromatic PEG₆-carboxyly (thiol-COOH; SPT001A46) and dithiolaromatic PEG₃ (thiolPEG); SPT-0013) were purchased from SensoPath Technologies (Bozeman, USA). Sodium p-tetrafluorophenyl-sulfonate (TFPS) and S-3-(benzoylphenoxy)propyl ethanethioate (thiol-benzophenone) were synthesized at the Max Planck Institute for Polymer Research (Mainz, Germany) according to the literature. Poly(N-isopropylacrylamide)-based terpolymer with a 94.5:1 molar ratio of N-isopropylacrylamide, methacrylic acid, and 4-methacryloyloxy benzophenone (pNIPAAm) were synthesized as previously described.

Peptide Synthesis. Synthesis of crude bicyclic peptide libraries, purified bicyclic and monocyclic peptides, and Cys-labeled peptides was performed using standard Fmoc-based peptide synthesis. All 672 peptides were synthesized and collected separately (synthesis on 2 μmol scale on solid phase, collection in 96-well polypropylene storage plates (each 2 mL). For more detailed information, see the Supporting Information.

ELISA. For buffer compositions and concentrations, see Table S-1 in the Supporting Information.

Integrin Coating and Blocking. Plates were coated with 100 μL of a 0.25–0.5 μg/mL integrin solution in coating buffer onto 96-well NUNC Polysorp plates (overnight, 4 °C) followed by blocking with 150 μL of 1% I-Block in blocking buffer (60 min, room temperature) and 3× washing with 400 μL of washing buffer.

Library Screening. In each screening experiment, 96 or 192 different peptides were tested. Bicyclic peptide libraries (2 μmol) were dissolved in DMSO (10 mM stock solutions) and further diluted to working concentrations (1 mM to 2.5 μM) with incubation buffer. After incubation with a fixed concentration of biotinylated knottin-RGD peptide in incubation buffer (minimum 15 min, room temperature), the individual bicycle solutions were added to the integrin-coated plates (90 min, room temperature), followed by washing with washing buffer. Then the plates were incubated with 100 μL of 1:1000 Strept-HRP in Strept-HRP buffer (60 min, room temperature). After incubation they were washed with 150 μL of substrate buffer containing 0.91 mM ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 0.006% H₂O₂ in substrate buffer (0.2 M Na₃HPO₄ adjusted to pH 4 using 0.2 M citric acid). Absorbance (405 nm) was measured for 4 min using a Molecular Devices Spectramax M2 plate reader.

IC₅₀ Determination. Peptides were mixed in eight different concentrations (each 3-fold dilutions) with a fixed concentration of biotinylated knottin-RGD (both in incubation buffer, 15 min, room temperature), followed by incubation of the plates with peptide/biotinylated knottin-RGD solutions for 90 min at room temperature. Strept-HRP and ABTS incubation steps were performed as described for library screening. All concentrations were tested in triplicate. IC₅₀ values were calculated via nonlinear regression analysis using GraphPad Prism software and represent the peptide concentration at which 50% inhibition of biotinylated knottin binding is observed.

Surface Plasmon-Enhanced Fluorescence Spectroscopy (SPFS). For the description of the optical system, sensor chip preparation, and the immobilization of the ligand, see the Supporting Information.

Measurement of Equilibrium Dissociation Constant Kₑ. For measurement of the binding affinity of Cys-labeled peptides to immobilized integrin ligands, PBS with 1 mM CaCl₂, 0.5 mM MnCl₂, 1 mg/mL BSA, and 0.05% Tween20 was used as the running buffer. Different concentrations of the peptide (0.1, 1, 5, 10, 50, 100, and 1000 nM) were sequentially flushed over the sensor surface. The sample at each concentration was allowed to react with the integrin for 30 min followed by rinsing the surface with running buffer solution for 10 min. The binding of the target analyte was monitored in real time by measuring the fluorescence intensity F(t) originating from the close proximity to the sensor surface that was probed by resonantly excited surface plasmons (Figure S-4B in the Supporting Information). The fluorescence signal F gradually increased upon binding of target analyte, and for each concentration, the equilibrium fluorescence signal ΔF was determined as a difference to the fluorescence baseline after 10 min of rinsing with running buffer. The titration curve was established on the basis of these values, and it was fitted with a Langmuir isotherm model (function ΔF = ΔFₘₐₓc/(Kₑ + c)) in order to determine the equilibrium dissociation constant Kₑ.

NMR. A detailed description of the acquisition of the NMR spectra, devices, methods, and spectrum editing, as well as the ¹H NMR spectrum of the bicycle C₇₃HPQ₇₃RGDc₇₃ (Figure S-5) is depicted in the Supporting Information. T₁ measurements were performed by properly calibrating the 90° pulse length and then performing estimates using the 1D inversion recovery sequence with excitation sculpting water suppression. After the longest T₁ was determined to be approximately 2 s, a pseudo-2D inversion recovery experiment was performed with 10 separate delays of 8 scans each with a total longitudinal relaxation time of 10.3 s. T₂ measurements were acquired by first performing estimates using the 1D PROJECT-CMPG sequence with presaturation water suppression. After the longest T₂ was determined to be approximately 1 s, a pseudo-2D PROJECT-CMPG sequence experiment with presaturation was performed with 12 separate delays of 8 scans each and a cycle time of 4 ms with a total longitudinal relaxation time of 10.3 s.

Cell Integrin Staining and Confocal Microscopy. Human colorectal adenocarcinoma cells (HT29) were obtained from The Netherlands Cancer Institute (NKI) and grown using standard procedures and conditions. For the
mM MgCl₂, and 0.5 mM MnCl₂) to remove nonadhered cells, followed by adding cold HCG buffer and cooling of the glass coverslips to 4 °C. Afterward, the Cy5-labeled peptides were added and allowed to incubate at 1 µM for 30 min at 4 °C, followed by at least five washing steps with HCG buffer, fixation with 4% paraformaldehyde solution in PBS pH 7.4 (20 min), and another four washing steps with HCG buffer. Subsequently, the cells were analyzed via confocal microscopy using a Leica TCS SP8 confocal microscope equipped with a supercontinuum white light laser (NKT Photonics) and water immersion objectives (63x W PL APO CS2, NA 1.2/40x W PL APO CS2, NA 1.1). The excitation wavelength was set to 633 nm while fluorescence was detected from 646 to 778 nm. All images were acquired under identical imaging conditions and processed via ImageJ (LUT: Fire).

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscombsci.8b00144.

Structures of (bionylated) knottin-RGD and cyclo-[KRGDf], detailed information about peptide synthesis, analysis, and purification and amino acids used in peptide libraries, additional peptides screened, and structure of bionylated knottin-RGD peptide, parameters varied in the competition ELISA setup, inhibition values for selected bicyclic peptides and monomeric analogues, position-replacement analysis for cysteines, ELISA protocol for testing HPQ-streptavidin binding and results, description and schematic of optical instrument used for SPFS and ligand immobilization, SPR sensorgrams and SPFS measurement for Cy5-functionalized bicycle C₃T₃HPQ₄₃RGD₄₅ additional NMR information, and ¹H NMR spectrum of bicycle C₃T₃HPQ₄₃RGD₄₅ (PDF)

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*Author Contributions*

The manuscript was written through contributions of all authors. D.B. and P.T. conceived the concept and analyzed the data. D.B. performed peptide syntheses, competition and binding ELISA experiments, and in vitro integrin staining experiments. V.J., N.G.Q., and J.D. designed, performed, and analyzed the SPR experiments. P.B.W. performed and analyzed the NMR experiments. D.B., K.J. and P.T. evaluated the confocal images. D.B., P.B.W., and P.T. wrote the manuscript. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare the following competing financial interest(s): Pepscan is the inventor of the CLIPS technology and holds a patent on the synthesis of bicyclic peptides using 2-CLIPS technology.

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**REFERENCES**


