Lasso Peptides

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Introducing Lasso Peptides as Molecular Scaffolds for Drug Design: Engineering of an Integrin Antagonist**

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Peptides combine a high specificity for their target receptor with a low toxicity and are therefore a promising source for drug leads.[1,2] However, their use has been limited because of undesirable physicochemical and pharmacokinetic properties.[2] To overcome these obstacles protein scaffolds, such as ultrastable ribosomally assembled peptides, can be used together with synthetic chemical strategies to present biologically active peptide epitopes, as shown recently by the conversion of the cyclotide kalata B1 into a vascular endothelial growth-factor-A antagonist.[3–7] In addition, bacterial lasso peptides have been under discussion as molecular scaffolds for drug design.[8–10] These ribosomally assembled peptides consist of 16–21 amino acids and share an N-terminal eight/nine-residue macrolactam ring through which the C-terminal linear tail is threaded and trapped by steric hindrance of bulky side chains.[11–15]

The currently known gene clusters of the lasso peptides microcin J25 (MccJ25) and capistruin consist of four genes, one coding for the precursor protein, two for the processing enzymes, and one for the export and immunity protein.[15,16] Mutational analysis of the precursor proteins of MccJ25 and capistruin revealed a high promiscuity of the biosynthetic machineries and the feasible heterologous production of various variants in *Escherichia coli*.[9,10] Therefore, lasso peptides combine unique characteristics relevant for their application as robust scaffolds for epitope grafting (Figure 1): 1) extraordinary stability against proteolytic degradation, temperature, and chemical denaturants; 2) gene-encoded lasso peptide precursor proteins; 3) a gene cluster of bacterial origin allowing heterologous production in *E. coli*; and 4) a promiscuous biosynthetic machinery tolerating various amino acid substitutions within the lasso peptide sequence.[9–11,15]

To prove their applicability as molecular scaffolds, we chose the integrin binding motif RGD as peptide epitope to be grafted onto a lasso peptide structure. Integrins are a large class of heterodimeric cell surface receptors and the RGD-binding integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have received increasing interest as therapeutic targets because of their role in tumor growth and angiogenesis.[17,18] As an insertion site the turn motif inside the threading tail was chosen to present: 1) the RGD epitope on the surface of the lasso structure to facilitate productive interaction with the binding site between the two integrin subunits; and 2) a conformationally restricted, kinked geometry responsible for the superior activity and selectivity profiles of cyclic RGD peptides compared to their linear representatives.[19–21] Thus, only MccJ25 was suitable as a molecular scaffold, since the biosynthetic machinery of capistruin does not tolerate substitutions within the noose.[9] Consequently, the tripeptide sequence Gly12-Ile13-Gly14 (numbering according to MccJ25) was substituted by Arg-Gly-Asp through site-directed mutagenesis of the precursor protein McjA, which is encoded alongside the processing enzymes McjB/McjC and the export and immunity protein McjD on the pTUC202 plasmid (Figure 1 in the Supporting Information).[16] HPLC–HRMS analysis of the culture supernatant of *E. coli* DH5α harboring the pTUC202 RGD plasmid revealed the successful maturation of the mutated precursor protein into the MccJ25 RGD triple mutant, which could be purified to homogeneity with a yield of 0.7 $mg\,L^{-1}$ (Figure 2 in the Supporting Information), consistent with the processing and production rate of the single mutants.[10] Tandem mass spectrometry (MS^2) fragmentation studies and carboxypeptidase Y digestion assays confirmed the lasso structure of MccJ25 RGD (see the Supporting Information).

The affinity of MccJ25 RGD towards $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$ integrins was analyzed by inhibition assays of integrin–extracellular matrix protein binding. The wild-type peptide MccJ25, the linear heptapeptide P1 (Ac-FVRGDTP-NH_2) corresponding to the sequence of the turn motif in MccJ25 RGD, the cyclic pentapeptide cilengitide (cyclo[RGDf-N(Me)V-]), and the non-peptide α_{IIb} integrin inhibitor tirofiban served as controls.[22–24] MccJ25 did not show biological activity towards $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$ integrins, thus proving the molecular framework to be inactive (Table 1). In contrast, the installation of the RGD epitope in the turn motif of the MccJ25 scaffold resulted in a remarkable increase of its binding affinity, with IC_{50} values of

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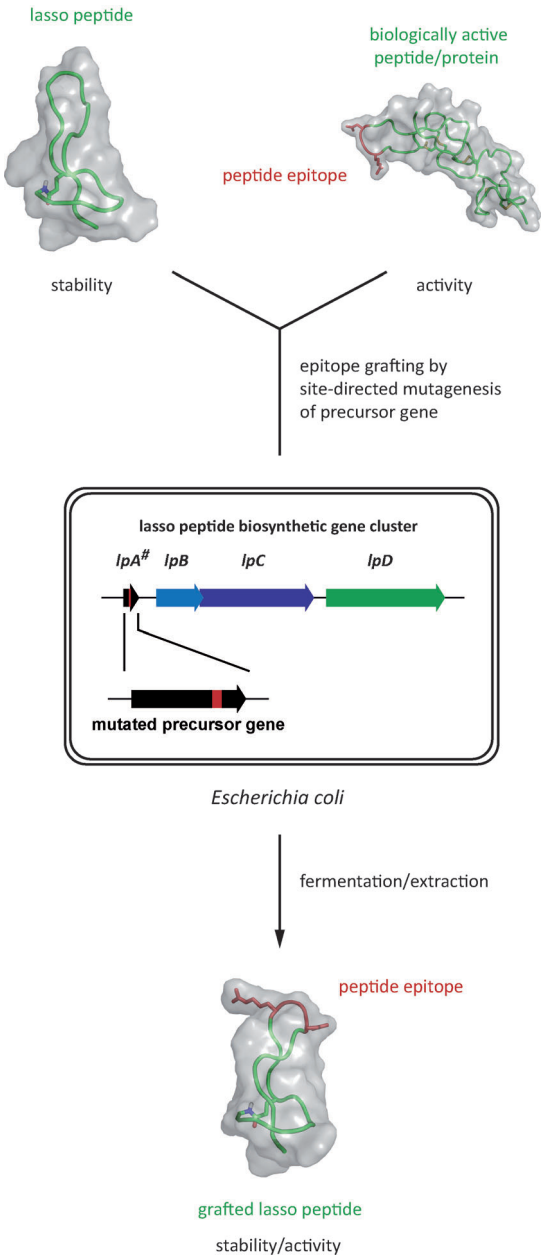

Figure 1. Introduction of novel biological activities into the stable lasso peptide scaffold by epitope grafting. A biologically active peptide epitope (red) consisting of proteinogenic amino acids is inserted into the lasso peptide scaffold by site-directed mutagenesis of the precursor gene (*lpA*$^{\#}$). The mutated lasso peptide precursor protein (LpA$^{\#}$) is converted into the grafted lasso peptide by the biosynthetic machinery (LpB/LpC) and subsequently secreted into the culture supernatant by the export and immunity protein (LpD) using *E. coli* as host system. The grafted lasso peptide can be extracted from the culture supernatant and combines the stability of the scaffold with the biological activity of the peptide epitope.

17 ($\alpha_v\beta_3$), 170 ($\alpha_v\beta_5$), 855 ($\alpha_5\beta_1$), and 29.7 nM ($\alpha_{IIb}\beta_3$). The linear peptide P1 displayed a high affinity towards the $\alpha_v\beta_3$ receptor ($IC_{50} = 43$ nM), moderate affinity towards $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ integrins ($IC_{50} = 654$ and 185 nM), but no inhibitory activity towards the $\alpha_v\beta_5$ integrin (Figures 7–10 in the Supporting Information). Cilengitide and tirofiban showed

Table 1: Affinity of MccJ25 RGD for $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$ integrins.[a]

Peptide	$\alpha_v\beta_3$	$\alpha_v\beta_5$	$\alpha_5\beta_1$	$\alpha_{IIb}\beta_3$
MccJ25	>10000	>10000	>10000	>10000
MccJ25 RGD	17±9	170±37	855±191	29.7±2.9
Ac-FVRGDTP-NH_2	43±14	>10000	654±122	185±146
cilengitide	0.92±0.19	25±8	8.2±0.6	206±92
tirofiban	–	–	–	0.6±2.3

[a] Shown are the IC_{50} values [nM] determined in isolated receptor binding assays. For comparison the affinities of MccJ25, cilengitide, tirofiban, and the linear heptapeptide P1 corresponding to the peptide sequence of the replacement site were determined.

IC_{50} values consistent with published data, thus proving the reliability of the assay.[22,24,25] Taken together, the grafting of the bioactive RGD epitope onto the inactive MccJ25 scaffold generated a nanomolar integrin inhibitor with an at least tenfold higher affinity for $\alpha_v\beta_3$ than $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins. However, the absence of selectivity for the platelet receptor $\alpha_{IIb}\beta_3$ indicates that the grafted lasso peptide requires further modifications (e.g. mutations of the flanking amino acids) to become a candidate for clinical applications.

As $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins play a crucial role in endothelial cell migration and blood vessel formation promoting tumor growth by removing waste products and providing nutrients, MccJ25 RGD was analyzed towards its influence on capillary formation of human umbilical vein endothelial cells (HUVECs) in comparison to the linear peptide P1 and cilengitide.[18] MccJ25 RGD did not show any inhibitory effect at 0.54 μM as the expected tube formation of HUVECs on Matrigel substrate was observed (Figure 2A). However, increasing concentrations of MccJ25 RGD suppressed the formation of capillaries in a dose-responsive manner with a minimal effective concentration of 2.3–4.6 μM (Figure 2). The linear peptide P1, which showed nanomolar affinity towards the $\alpha_v\beta_3$ integrin, had no effect on HUVEC tube formation up to 120 μM.

Stability studies in human serum revealed that the linear peptide P1 is completely degraded after 4 h, whereas more than 50% of MccJ25 RGD is present in the serum after 30 h (Figure 11 in the Supporting Information). Thus, as a result of its stability against proteolytic degradation, the affinity of MccJ25 RGD towards $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins observed in

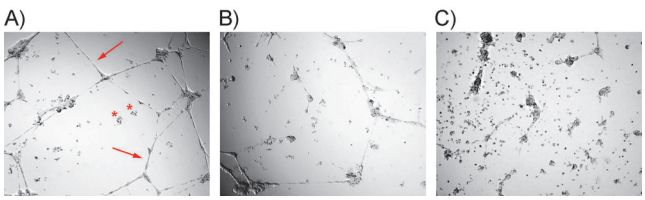

Figure 2. Inhibitory effect on capillary formation of MccJ25 RGD. Microscopic images of tube formation of HUVECs on Matrigel substrate. A) Capillary formation of HUVECs in the presence of 0.54 μM MccJ25 RGD. Red arrows point to capillaries formed. Red asterisks mark groups of single cells. B) Reduced tube formation in the presence of 5 μM MccJ25 RGD. C) Formation of capillaries is completely inhibited at 44 μM MccJ25 RGD. Only cell aggregation is observed.

ligand inhibition assays in vitro can be transformed into an inhibitory effect on capillary formation in cell culture assays, whereas the linear heptapeptide P1, which is susceptible to proteolytic degradation, only shows a productive interaction in the absence of proteases. Interestingly, cilengitide did not prevent the formation of capillaries up to 17 μM and consequently was not more potent than the grafted lasso peptide, although significantly higher affinities for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were observed (Table 1). Moreover, HUVEC proliferation was not compromised by MccJ25 RGD up to a concentration of 56 μM. A reduction in viability was only observed with higher concentrations resulting in an extrapolated IC_{50} of 225 μM. Thus, the suppression of tube formation by MccJ25 RGD is a specific integrin inhibitory effect without influencing the vitality of the cells. In addition, the grafted lasso peptide, in contrast to MccJ25, is not able to inhibit the growth of *E. coli* K12 MC4100 (Figure 12 in the Supporting Information), which is in agreement with the previously described inactive single mutant MccJ25 G14D.[10]

To investigate the influence of the RGD substitution on the lasso scaffold, the three-dimensional structure of MccJ25 RGD was determined by NMR spectroscopy (see the Supporting Information). The superposition of the 20 lowest-energy structures in Figure 3A shows that the grafted peptide adopts a well-defined lasso fold, confirmed by the low root-mean-square deviation (RMSD) for the backbone (0.2 Å) of the structure ensemble (Table 3 in the Supporting Information). As intended, the RGD epitope is presented on the surface of the lasso structure in a kinked conformation of the peptide backbone, thereby locking the most important functionalities (guanidine group of Arg12 and carboxyl group of Asp14) at an optimized distance (Figure 3B). This conformational restraint and the reduced flexibility of the RGD motif are most likely the explanation for the higher affinity of MccJ25 RGD compared to the linear peptide P1, which is devoid of any conformational restriction.[21] The structural alignment of the wild-type peptide and the grafted lasso peptide (Figure 3C) illustrates that the RGD substitution did not significantly alter the overall structure of the molecular framework, thus demonstrating the robustness of the lasso fold for epitope grafting of short peptide sequences.

In conclusion, the conversion of the lasso peptide MccJ25 into a nanomolar integrin inhibitor by RGD substitution proves that lasso-structured peptides are a promising molecular scaffold for the presentation of bioactive peptide epitopes. These privileged templates are devoid of cytotoxicity, display remarkable stability under physiological conditions, and are accessible by a direct fermentative route, which is time-saving, environmentally friendly, and economical.[26] Future investigations will involve the grafting of other peptide epitopes to prove a universal application of lasso peptides as molecular scaffolds, as well as further optimization of MccJ25 RGD by applying disulfide formation of introduced cysteine residues to rigidify the environment of the insertion site, as found in class I and class III lasso peptides.[27] The reduction of the conformational space may also improve the selectivity of the engineered lasso peptide towards distinct integrin subtypes. In addition, the introduction of nonproteinogenic amino acids into the lasso structure using orthogonal aminoacyl-tRNA synthetase/tRNA pairs remains to be explored and should expand the chemical space accessible through ribosomally assembled lasso peptides.[28]

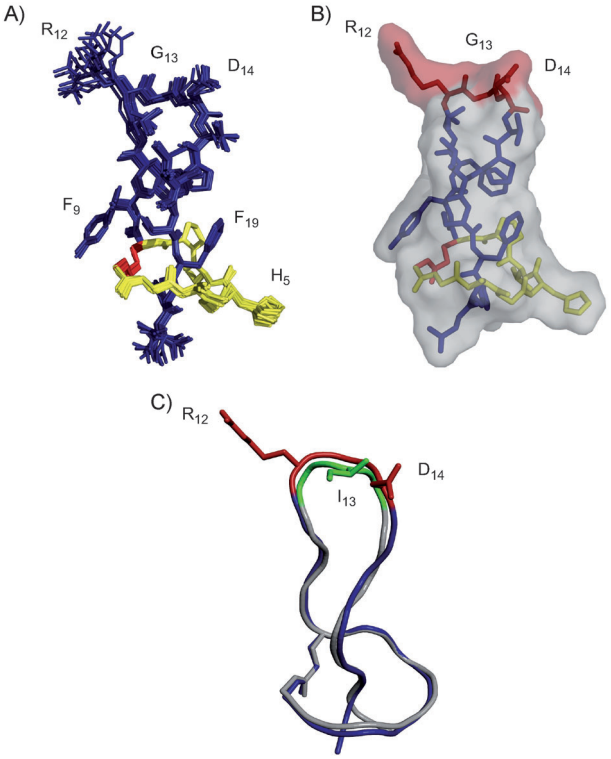

Figure 3. NMR structure of the grafted lasso peptide MccJ25 RGD. A) Superposition of the 20 lowest-energy structures of MccJ25 RGD shown as sticks. The isopeptide bond (red) between Gly1 and Glu8 generates an eight-residue macrolactam ring (yellow) through which the C-terminal tail (blue) is threaded. B) Average structure of MccJ25 RGD in solution. The solvent-accessible surface is shown in transparent gray and the grafted RGD epitope is highlighted in red. C) Structural alignment of MccJ25 (gray) and MccJ25 RGD (blue). The grafted RGD sequence is highlighted as red sticks and the substituted GIG sequence in MccJ25 is shown in green. Structural alignment was performed using RAPIDO and yielded an RMSD of 1.22 Å.[29]

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[1] A. K. Sato, M. Viswanathan, R. B. Kent, C. R. Wood, *Curr. Opin. Biotechnol.* **2006**, *17*, 638–642.
[2] Z. Antosova, M. Mackova, V. Kral, T. Macek, *Trends Biotechnol.* **2009**, *27*, 628–635.
[3] M. Werle, A. Bernkop-Schnurch, *Amino Acids* **2006**, *30*, 351–367.
[4] E. Biron, J. Chatterjee, O. Ovadia, D. Langenegger, J. Brueggen, D. Hoyer, H. A. Schmid, R. Jelinek, C. Gilon, A. Hoffman, H. Kessler, *Angew. Chem.* **2008**, *120*, 2633–2637; *Angew. Chem. Int. Ed.* **2008**, *47*, 2595–2599.
[5] M. Gebauer, A. Skerra, *Curr. Opin. Chem. Biol.* **2009**, *13*, 245–255.

[6] S. Gunasekera, F. M. Foley, R. J. Clark, L. Sando, L. J. Fabri, D. J. Craik, N. L. Daly, *J. Med. Chem.* **2008**, *51*, 7697 – 7704.
[7] A. E. Garcia, J. A. Camarero, *Curr. Mol. Pharmacol.* **2010**, *3*, 153 – 163.
[8] K. J. Rosengren, D. J. Craik, *Chem. Biol.* **2009**, *16*, 1211 – 1212.
[9] T. A. Knappe, U. Linne, L. Robbel, M. A. Marahiel, *Chem. Biol.* **2009**, *16*, 1290 – 1298.
[10] O. Pavlova, J. Mukhopadhyay, E. Sineva, R. H. Ebright, K. Severinov, *J. Biol. Chem.* **2008**, *283*, 25589 – 25595.
[11] S. Rebuffat, A. Blond, D. Destoumieux-Garzon, C. Goulard, J. Peduzzi, *Curr. Protein Pept. Sci.* **2004**, *5*, 383 – 391.
[12] K. J. Rosengren, R. J. Clark, N. L. Daly, U. Goransson, A. Jones, D. J. Craik, *J. Am. Chem. Soc.* **2003**, *125*, 12464 – 12474.
[13] M. J. Bayro, J. Mukhopadhyay, G. V. Swapna, J. Y. Huang, L. C. Ma, E. Sineva, P. E. Dawson, G. T. Montelione, R. H. Ebright, *J. Am. Chem. Soc.* **2003**, *125*, 12382 – 12383.
[14] K. A. Wilson, M. Kalkum, J. Ottesen, J. Yuzenkova, B. T. Chait, R. Landick, T. Muir, K. Severinov, S. A. Darst, *J. Am. Chem. Soc.* **2003**, *125*, 12475 – 12483.
[15] T. A. Knappe, U. Linne, S. Zirah, S. Rebuffat, X. Xie, M. A. Marahiel, *J. Am. Chem. Soc.* **2008**, *130*, 11446 – 11454.
[16] J. O. Solbiati, M. Ciaccio, R. N. Farias, J. E. Gonzalez-Pastor, F. Moreno, R. A. Salomon, *J. Bacteriol.* **1999**, *181*, 2659 – 2662.
[17] A. Meyer, J. Auernheimer, A. Modlinger, H. Kessler, *Curr. Pharm. Des.* **2006**, *12*, 2723 – 2747.
[18] C. J. Avraamides, B. Garmy-Susini, J. A. Varner, *Nat. Rev. Cancer* **2008**, *8*, 604 – 617.
[19] J. P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S. L. Goodman, M. A. Arnaout, *Science* **2002**, *296*, 151 – 155.
[20] M. Aumailley, M. Gurrath, G. Muller, J. Calvete, R. Timpl, H. Kessler, *FEBS Lett.* **1991**, *291*, 50 – 54.
[21] D. Heckmann, H. Kessler, *Methods Enzymol.* **2007**, *426*, 463 – 503.
[22] M. A. Dechantsreiter, E. Planker, B. Matha, E. Lohof, G. Holzemann, A. Jonczyk, S. L. Goodman, H. Kessler, *J. Med. Chem.* **1999**, *42*, 3033 – 3040.
[23] C. Mas-Moruno, F. Rechenmacher, H. Kessler, *Anticancer Agents Med. Chem.* **2010**, *10*, 753 – 768.
[24] G. D. Hartman, M. S. Egbertson, W. Halczenko, W. L. Laswell, M. E. Duggan, R. L. Smith, A. M. Naylor, P. D. Manno, R. J. Lynch, G. Zhang, C. T.-C. Chang, R. J. Gould, *J. Med. Chem.* **1992**, *35*, 4640 – 4642.
[25] S. L. Goodman, G. Holzemann, G. A. Sulyok, H. Kessler, *J. Med. Chem.* **2002**, *45*, 1045 – 1051.
[26] F. E. Lopez, P. A. Vincent, A. M. Zenoff, R. A. Salomon, R. N. Farias, *J. Antimicrob. Chemother.* **2007**, *59*, 676 – 680.
[27] T. A. Knappe, U. Linne, X. Xie, M. A. Marahiel, *FEBS Lett.* **2010**, *584*, 785 – 789.
[28] C. C. Liu, P. G. Schultz, *Annu. Rev. Biochem.* **2010**, *79*, 413 – 444.
[29] R. Mosca, T. R. Schneider, *Nucleic Acids Res.* **2008**, *36*, W42 – W46.

