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Genetically Encoded Libraries of Constrained Peptides

Tjibbe Bosma,^[a] Rick Rink,^[a] Markus A. Moosmeier,^[b] and Gert N. Moll^{*,[a, c]}

Many therapeutic peptides can still be improved with respect to target specificity, target affinity, resistance to peptidases/proteases, physical stability, and capacity to pass through membranes required for oral delivery. Several modifications can improve the peptides' properties, in particular those that impose (a) conformational constraint(s). Screening of constrained peptides and the identification of hits is greatly facilitated by the generation of genetically encoded libraries.

Recent breakthrough bacterial, phage, and yeast display screening systems of ribosomally synthesized post-translationally constrained peptides, particularly those of lanthipeptides, are earning special attention. Here we provide an overview of display systems for constrained, genetically encoded peptides and indicate prospects of constrained peptide-displaying phage and bacterial systems as such in vivo.

1. Introduction

In most diseases, peptides can have a therapeutic role.^[1] Peptides, however, can lack sufficient target specificity or have short half-lives in vivo, and their capacity to pass through gut epithelial membranes might be insufficient to allow for oral delivery. Incorporation of unnatural amino acids and the modification of peptides can enormously increase their structural diversity and chemical space. The introduction of conformational constraints can lead to different peptide structures. Constrained peptides have the potential to combine the best attributes of antibodies and small molecules, and several of them are in late-stage clinical trials.^[2] Peptides can be head-to-tail cyclized, internal amino acids can be linked, and crosslink(s) can be installed by use of externally added reagents. The length of the crosslink in combination with the distance between the crosslinked amino acids, has an impact on the structure. For instance, a lysinoalanine system and a β -lactam moiety form a longer bridge from backbone to backbone than a lanthionine unit.^[3] Conformational constraints can improve target specificity, target-binding affinity, peptidase resistance, and membrane permeability.

Target specificity can be crucial for the therapeutic potential of a peptide drug. Unmodified linear peptides can adopt multiple conformations that can allow interaction with targets other than the intended ones as, for example, related subtype tar-

gets. Introduction of a conformational constraint can lead to higher target specificity, as demonstrated, for instance, by the enhanced specificity of lanthionine-enkephalin for the δ -opioid receptor^[4] and the specificity of lanthionine-somatostatin for somatostatin receptor 5 (SSTR5).^[5]

Target affinity is of great importance for peptides that have to block their target or disrupt protein–protein interactions. Furthermore, the affinity of a peptide for a target can be enhanced by reducing its conformational freedom. Structural rigidity decreases the entropic cost of adopting the target-binding conformation, thereby improving target-binding affinity. In this way, high structural stability of peptides—achieved through hydrophobic or electrostatic intramolecular interactions and/or crosslinks—can lead to increased target binding affinity. In addition, the conformational constraint imposed on a peptide might induce a structure that is suitable or required for binding. A thioether-constrained streptavidin tag binds > 100 times more strongly to streptavidin than a corresponding linear tag.^[6] The two N-terminal cyclic structures of the lantibiotic nisin are essential for its binding of a pyrophosphate group of lipid II, nisin's target.^[7]

Introducing a conformational constraint can confer local resistance to peptidase-mediated breakdown.^[8] This can lead to a prolonged half-life in vivo and reduced frequency of administration. By reducing peptidase-mediated peptide breakdown in the gastrointestinal (GI) tract and by enhancing membrane passage, conformational constraints can also contribute to potential for oral delivery.^[9]

Especially in cases of chronic diseases, oral delivery of peptide drugs is highly preferable. Oral availability and gut epithelial membrane permeability of peptides appear to be dependent on parameters such as low molecular weight, partition coefficient, limited numbers of hydrogen receptors and donors, limited polar surface area, and restricted numbers of rotational bonds.^[10] Imposing a conformational constraint might favorably reduce the number of rotational bonds and allow for shielded internal hydrogen bonding, reducing the

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polarity as described in the case of cyclosporine A. Reduced polarity can thus favor epithelial gut membrane translocation needed for oral bioavailability.

Genetically encoded peptide libraries have advantages such as library size and ease of screening.^[11] The discovery of target-binding constrained peptides is indeed greatly facilitated by genetically encoded peptide libraries. Among such genetically encoded libraries of constrained peptides, here we consecutively consider 1) *in vitro* libraries that make use of mRNA display,^[12] 2) phage display systems of helix-turn-helix peptides^[13–15] and post-translationally constrained peptides, 3) bacterial and yeast display systems, and 4) prospects for important future developments.

2. In Vitro Libraries of Genetically Encoded Constrained Peptides

Genetically encoded constrained peptide libraries allow easy identification of target-binding hits. This might not be vastly applicable in the discovery of agonistic, receptor-stimulating peptides that not only bind to their receptors but also stimulate them. However, genetically encoded constrained peptide libraries are very utilizable in the discovery, for instance, of target-binding peptides such as receptor antagonists, enzyme inhibitors, protein–protein-interaction-disrupting peptides, diagnostic target-binding peptides, and peptide tags.

2.1. In vitro mRNA display systems

In mRNA display, translated peptides are associated with their mRNA progenitors through puromycin linkages. After screening, selected or enriched peptide sequences can be identified by reverse transcription and amplification of cDNA by PCR. mRNA display of constrained peptides—constrained through coupling of two cysteines with dibromoxylene, for instance—has been applied to discover, amongst other cases, constrained inhibitors of thrombin. Cyclic inhibitors with low nanomolar affinities for thrombin were identified, with the cyclic structures of these peptides being essential for the observed high-affinity binding. This is consistent with the model of increased affinity of cyclic peptides resulting from decreasing the entropic costs of binding.^[16]

Translation in *in vitro* mRNA display can be engineered to allow for the discovery of constrained peptides.^[17] This engineering involves replacing natural amino acids by chemically similar analogues that remain substrates for aminoacyl-tRNA synthetases. The limitations caused by the substrate specificities of the aminoacyl-tRNA synthetases can also be circumvented. For instance, to broaden the range of possibilities, selected aminoacyl-tRNA-synthetases in the *in vitro* system can be replaced by noncanonical aminoacyl-tRNAs that have already been charged with the desired unnatural amino acids. To generate libraries of constrained peptides, various ways to synthesize noncanonical aminoacyl-tRNAs can be followed.

An engineered ribozyme that is flexibly capable of esterifying activated amino acids on full-length *in vitro* transcribed tRNAs has been generated.^[18] As a result, peptides can be

composed of over 400 different constituents instead of the ordinary 20 amino acids. This broadly applicable permissive ribozyme, called flexizyme, thus allows for the generation of constrained peptides, amongst many other highly diverse structures.

2.2. In vitro mRNA display of lanthipeptide libraries

For the design of lanthipeptide libraries, *in vitro* display of nucleotide-linked libraries could be a powerful method. Lanthionines can be inserted into the translated peptide by use of chemical methods, for instance. In addition, *in vitro* translation allows substitution of natural amino acids by unnatural analogues.

The latter approach has been applied for the design of lanthipeptide libraries.^[19] Lysine was replaced by the unnatural analogue 4-selenalysine, which is converted under mild oxidative conditions into a dehydroalanine (Dha). Under reducing conditions, a nearby cysteine was deprotected and subsequently reacted with the Dha, thus forming a lanthionine moiety.

Chemical introduction of lanthionines usually lacks stereo- and regiospecificity. *In vitro* activity of enzymes capable of post-translationally installing lanthionine has been reconstituted in excellent studies by van der Donk.^[20] Therefore, future mRNA display of lanthipeptides with enzymatically installed lanthionines might significantly broaden the potential to generate lanthipeptide libraries with variable and complex ring patterns.

2.3. Phage display of constrained peptides

Disulfide-bridged peptides often have a nonimmunogenic character^[21] and potential for oral delivery.^[22, 23] Phage display of different scaffolds of disulfide-bridged peptides has been broadly applied as, for instance, in the discovery of a modestly potent inhibitor of interleukin-23.^[24] Phage display of complex peptides—so-called avimers, which are tricyclic disulfide-bridged peptides that exploit the avidity of multimer unit binding—has been described.^[25] Phage-displayed peptide libraries of both conformationally constrained α -helical structures^[13–15] and α -helical structures, stabilized by treating *i,i*+4 cysteine-containing (poly)peptides with α,α' -dibromo-*m*-xylene, have been demonstrated.^[26] Phages displaying bicyclic peptides have been developed.^[27, 28] These bicyclic structures were generated by treating peptides containing three cysteines with the chemical linker 1,3,5-tris(bromomethyl)benzene. Bicyclic peptides would be expected to be more rigid, and binding of both constrained structures might allow for higher target-binding affinity. Bicyclic inhibitors against the proteases plasma kallikrein and cathepsin have been obtained from phage libraries and showed high affinities towards both targets, having K_i values of 1.7 and 100 nM, respectively.^[27] Bicyclic peptide inhibitors against the proteases urokinase-type plasminogen activator (uPA)^[29] and coagulation factor XIIa^[30] were also successfully obtained. Furthermore, bicyclic ligands were discovered for receptors such as Her2^[31] and Notch1.^[11] Characterization of phage-selected bicyclic peptides indicated

Figure 2. Bacterial display of nisin on the *L. lactis* cell surface. Prenisin (leader peptide in purple, core peptide in yellow) is translationally fused to the LPXTG-containing cell wall anchor (blue). The N-terminal leader peptide is recognized by the modification enzymes, allowing modification of the propeptide. NisB-dehydrated amino acids are shown in red; NisC-formed thioether bridges in displayed prenisin are shown in blue. The modified precursor peptide anchor fusion is translocated across the cell membrane, while its C-terminal hydrophobic membrane anchor and charged tail (green) is retained within the cell membrane, after which sortase-catalyzed covalent attachment of the cell wall anchor through its LPXTG motif to the peptidoglycan takes place. B: NisB. C: NisC. T: NisT.^[38]

In contrast to the cell-surface display of modified peptides with a covalent linkage between the encoding DNA and the displayed peptide, it is also possible to generate intracellularly modified peptides that stay inside the cell and so remain together with their encoding DNA. An elegant recent study demonstrated the generation of a plasmid-encoded intracellular library of lanthipeptides within *Escherichia coli* by using the lanthionine-installing synthetase ProcM. This library was combined with a bacterial reversed two-hybrid system for the interaction between the HIV p6 protein and the UEV domain of the human TSG101 protein. A lanthipeptide inhibitor that successfully disrupted this protein–protein interaction, which is crucial for HIV budding from infected cells, was identified.^[39]

3.2. Yeast display of constrained peptides

Disulfide-rich peptides have been used in a yeast display system to afford an integrin-binding peptide containing the RGD motif.^[40] A truncated form of the Agouti-related protein, a 4 kDa cystine-knot peptide with four disulfide bonds, has been used as a scaffold for generating a yeast display library that was screened for binding to $\alpha v \beta 6$ integrins with high affinity and specificity.^[41] Yeast-displayed cystine-knot peptides have been optimized towards inhibitors of human matriptase-1.^[42] Van der Donk and co-workers developed C-terminal yeast display of the class II lanthipeptides lactacin 481 and halodura-

cin β . Randomization of the C ring of lactacin 482 was used to select tight binders to $\alpha v \beta 3$ integrin.^[34]

4. Summary and Outlook

The increasing development of new constrained peptides with new functions and strongly improved therapeutic potential clearly indicates that constrained peptides might one day play an important role in curing patients. Up until now, genetically encoded constrained peptide libraries have not been widely applied for the discovery of agonistic peptides. Agonistic peptides require not only binding of the peptide to the target receptor, but also the capacity to induce the transfer of a signal. These functional requirements often require free peptide termini. Furthermore, in screening procedures, peptide agonists need to be separated from peptides that only bind to the target. Nevertheless, phage-displayed peptide agonists have been reported.^[43] Because constrained peptides can have enhanced target affinity and enhanced target specificity, genetically encoded libraries of constrained peptide agonists might have additional value in deorphanizing orphan receptors.

L. lactis bacteria have been used for delivering therapeutic peptides in the GI tract.^[44] Constrained agonistic peptide-displaying bacteria (which can display a few thousand copies per cell) and phages (which display only a few constrained peptides per phage) might in themselves also constitute orally deliverable therapeutic systems for stimulating target receptors for diseases of the GI tract. Surprisingly, cyclic peptides can facilitate transcellular transport of entire phages across intestinal epithelium in vitro and in vivo,^[45] thus indicating application of constrained-peptide-displaying phage beyond the GI tract. Most interestingly, in vivo phage display has demonstrated that phages can be targeted in vivo by short linear displayed peptides.^[46] The resistance of constrained peptides to peptidase action in vivo, as well as the enhanced target specificity, strongly indicate that phage-displayed constrained peptides have great potential for such in vivo phage display to enable target and or tissue specificity. This might imply significant medical applications not only for development of in vivo target-specific peptides but also for the ligand-displaying phage themselves, such as in application of phages for local gene delivery or of autolytic phages against tumors.

Conflict of Interest

T.B., R.R., and G.N.M. are employees of LanthioPep (Lanthio Pharma); M.A.M. is an employee of MorphoSys AG. G.N.M. is manager of LanthioPep and Lanthio Pharma. T.B. and M.A.M. are inventors of patent applications related to some of the systems described in this manuscript.

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