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Bicyclic Peptide Antagonists Derived from Genetically Encoded Combinatorial Libraries

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Abstract: Ligands based on bicyclic peptides can combine favourable properties of antibodies (good binding affinity and target specificity) and small molecule ligands (stability, access to chemical synthesis, diffusion properties) and might be suitable molecular structures for the development of therapeutics. By using a combinatorial methodology based on phage display and a chemical cyclisation reaction, we are generating bicyclic peptide antagonists of protein targets with therapeutic applications in mind.

Keywords: Combinatorial chemistry · Cyclic peptides · Encoded libraries · Peptide therapeutics · Phage display · Protease inhibitors · Serine proteases

From Antibodies to Bicyclic Peptides

In my laboratory, we are generating bicyclic peptide ligands to disease-related targets with the aim of developing therapeutics that combine the exquisite binding properties of antibodies and qualities of small molecule ligands. My interest in high-affinity binders originates from a PhD in the research team of Prof. Dr. Dario Neri at the ETH Zurich where I was exposed to the fields of therapeutic antibodies and combinatorial chemical libraries. As a post-doc in the research group of Sir Greg Winter at the Laboratory of Molecular Biology (LMB) in Cambridge, UK, I had proposed a strategy to generate large phage-encoded combinatorial libraries of multicyclic peptides. Sir Greg Winter and I had speculated that the diversity of binding sites in antibodies which is restricted to a relatively small region (the complementarity determining regions) can be mimicked by bicyclic peptide structures (Fig. 1). Together, we had developed an approach based on phage display that allows the generation and genetic encoding of billions of bicyclic peptides and the subsequent identification of ligands to targets of choice as described in the following section.[1]

Encoded Combinatorial Libraries of Bicyclic Peptides

Large genetically encoded bicyclic peptide libraries are generated by cyclising linear peptides displayed on phage in a chemical reaction. The peptides are designed to have cysteine residues on each end, and another one in the middle that separates two random peptide regions. Reaction of the sulfhydryl groups of the cysteine residues with a small molecule having three thiol-reactive groups (*e.g.* tris-(bromomethyl)benzene, TBMB[2]) yields a bicyclic peptide structure (Fig. 2). The reaction taking place in aqueous solution is quantitative and selective yielding a single product per phage. Importantly, the reaction conditions applied are mild, spearing the phage particles that enclose the genetic information coding for the peptide. With this strategy, combinatorial repertoires with more than 10 billion different bicyclic peptides can be generated. To identify bicyclic peptides binding to targets of interest, the libraries are subjected to typically 2–3 iterative rounds of phage production, chemical cyclisation, affinity selection and amplification. In a first proof of concept experiment, the phage selection strategy was successfully applied by Sir Greg Winter and myself to generate

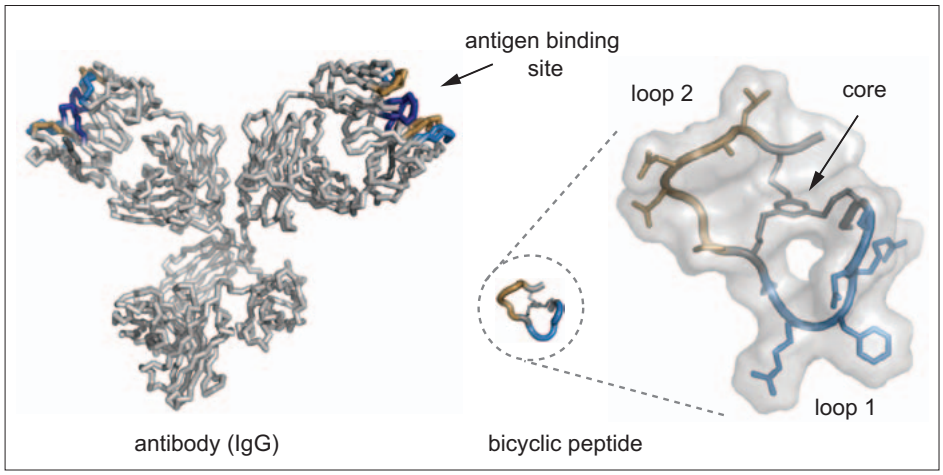

Fig. 1. Comparison of a bicyclic peptide with an antibody. In antibodies (150 kDa) the antigen binding sites are restricted to a small space (highlighted in colour). In contrast, in the nearly 100-fold smaller bicyclic peptides (1–3 kDa) the two binding loops represent the major component of structure.

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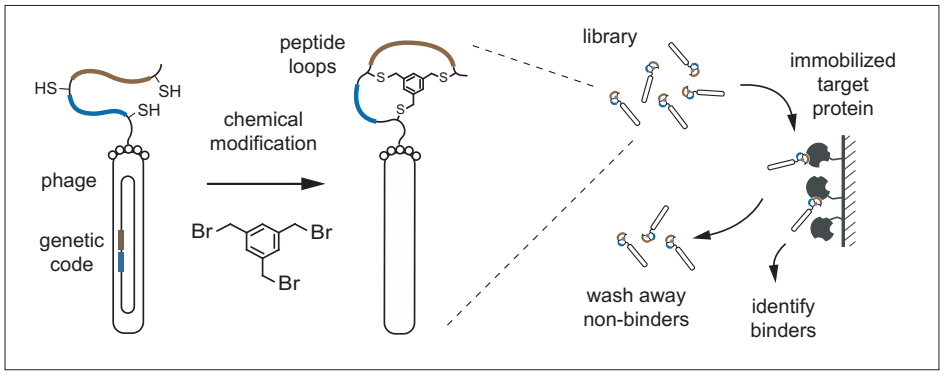

Fig. 2. Schematic drawing of the bicyclic peptide phage selection strategy. Linear peptides displayed on phage are cyclised by reacting three cysteine side chains with tris(bromomethyl) benzene (left). A combinatorial repertoire of more than a billion phage-encoded bicyclic peptides is subsequently subjected to affinity selections (right). After 2–3 iterative rounds, the bicyclic peptides are identified by sequencing the DNA enclosed in the phage particles.

inhibitors of the human serine proteases plasma kallikrein and cathepsin G.[1] More recently, my team had isolated binders to other protease classes as well as a receptor. The first bicyclic peptide ligands isolated by phage panning are all composed of the same small molecule core (mesitylene) and two peptide loops of six amino acids flanked by the cysteine residues. We are currently developing chemically diverse thiol-reactive small molecules to increase the structural diversity of bicyclic peptide libraries and in parallel, we are generating phage-encoded combinatorial bicyclic peptide libraries with variable loop lengths to generate more diverse topologies and to screen a larger sequence space. Also, we are interested in combining multiple, orthogonal cyclisation strategies to generate highly constrained multicyclic peptide structures. Recently, we had established a selective, enzyme-catalysed peptide cyclisation strategy based on the ligation of glutamine and lysine side chains by a bacterial transglutaminase.[3] By combining the transglutaminase-based ligation reaction with a chemical cyclisation reaction we could generate tricyclic peptides.

Binding Properties of Bicyclic Peptides

Most of the bicyclic peptides that have been isolated to date by phage panning are binding with nanomolar affinities to their targets. In affinity maturation approaches that were typically applied to improve one of the two peptide loops, the potency of some of these molecules could be further improved. The best binder generated so far, PK15, binds with a K_i of around 2 nM to the human serine protease plasma kallikrein (Fig. 3).[1] Compared to monocyclic peptide ligands, the bicyclic structures can potentially interact through two peptide loops with protein targets and hence develop stronger binding interactions. In fact, most of the disulfide-cyclised monocyclic peptides isolated by phage display or other selection strategies have binding affinities in the micromolar range and only a few highly affine monocycles with nanomolar affinities could be generated.[4] For the development of peptide therapeutics, high binding affinities in the low nanomolar or subnanomolar range are ideally needed, and this is more likely achieved with cyclic peptides having multiple binding loops.

In addition to their good binding affinity, the isolated bicyclic peptides were found to be highly specific. For example the above-mentioned inhibitor of human plasma kallikrein did not inhibit a panel of other, structurally highly related serine proteases including the mouse orthologue that shares 79% sequence identity with human plasma kallikrein.[1] While the high target specificity is desired to prevent undesired side effects, it complicates the testing of the bicyclic peptides in animal disease models. Bicyclic peptides that are species cross-reactive may be generated by alternating the orthologous targets in different phage selection rounds.

To understand the binding mode of bicyclic peptides, we are performing structural studies using X-ray crystallography and NMR. A question that interests us is whether both of the peptide loops interact directly with the target proteins. Other questions that we address in our studies are whether bicyclic peptides form well-

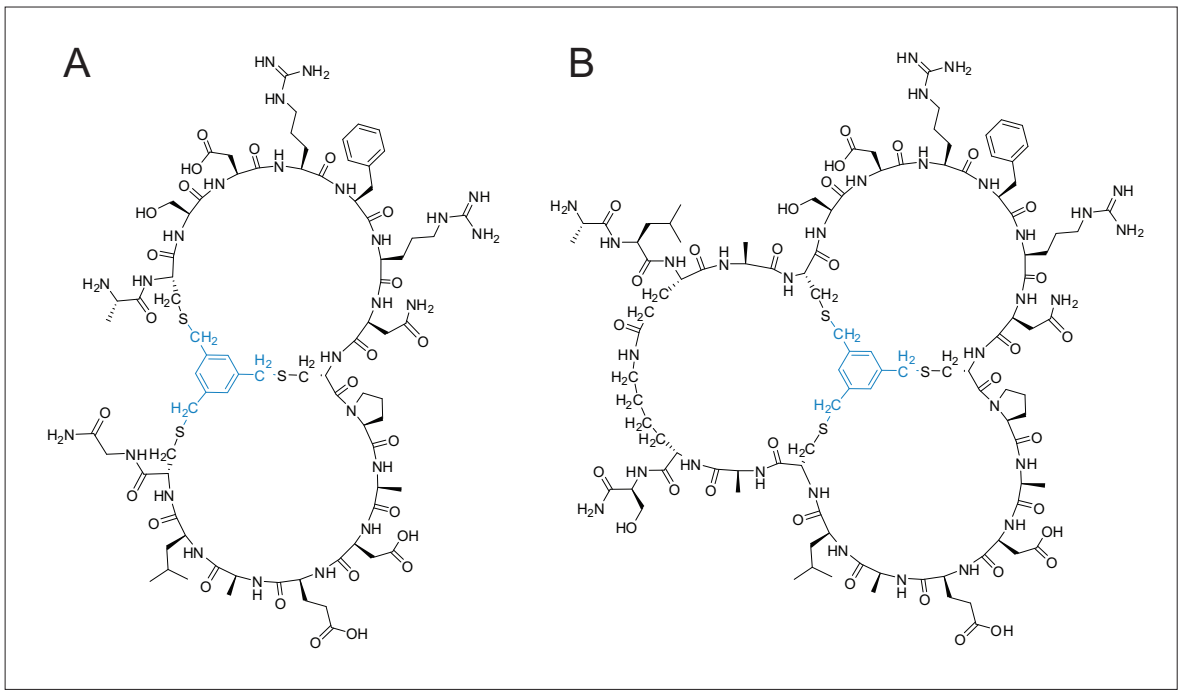

Fig. 3. Chemical structures of (A) a bicyclic peptide isolated against human plasma kallikrein (peptide PK15)[1] and (B) a tricyclic peptide generated by enzymatically linking a glutamine and lysine residue at the termini of a bicyclic peptide with a transglutaminase.[3]

defined structures in solution and if so, whether they retain the same overall conformation upon binding to their target. Also, we hope to find out whether the synthetic small molecule core in the bicyclic peptides forms noncovalent interactions with amino acids of the peptide loops and hence serves as a structural scaffold.

Towards the Generation of Therapeutics

The main research objective of my group is the testing of the therapeutic potential of bicyclic peptides. Towards this end, we are generating antagonists for indications where we believe that the molecule format of bicyclic peptides has an advantage over the two major drug classes, the small molecules and the monoclonal antibodies. Compared to small molecules, the bicyclic peptides have larger surfaces to form stronger and more specific interactions. The larger molecular mass of 1–3 kDa may enable bicyclic peptides to disrupt protein–protein interactions, which has been difficult to achieve with small molecules. An attractive property is also the generally low toxicity of peptide-based drugs. Compared to large monoclonal antibodies (150 kDa), the bicyclic peptides have nearly a 100-fold smaller molecular mass which may bring several advantages. Firstly, the peptidic structures can be chemically synthesized which facilitates their production as well as allows the incorporation of non-natural building blocks such as labels. Secondly, although not tested yet, the relatively small size should allow the bicyclic peptides to penetrate into tissue to reach targets efficiently as for example those expressed on tumour cells. Thirdly, the small and robust cyclic structures might allow application through more convenient routes than injection.

Specifically, we are currently developing bicyclic inhibitors of cancer-related proteases including the serine protease urokinase-type plasminogen activator (uPA). The uPA is overexpressed in various tumour cell lines and its catalytic activity is implicated in tumour growth and invasion. While potent small molecule inhibitors of uPA were generated by academic research groups and pharmaceutical companies, they lack high selectivity due to the structural similarity of paralogous proteases. Bicyclic peptide inhibitors should be able to inhibit the proteases in a specific manner. At the same time, we hope that small size of the peptidic inhibitors will allow them to extravasate and distribute homogenously in the extracellular matrix of tumour tissue to block uPA activity.

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