Peptide Phage Display

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Abstract

Phage display is emerging as a promising strategy for the discovery of peptide ligands that are used for analytical tools, drug discovery, and target validations. Phage display technology can produce a huge number of peptides and generate novel peptide ligands. Phage display technology has successfully managed to create some peptide ligand. The structural analysis of ligands shows that the conformational design of peptides in library is important for structural relationship among peptides on phage, synthetic peptides and non-peptidic compounds as well as for selecting high-affinity ligands that bind to every target from a phage peptide library. Key issues concern constraints on the conformation of peptides on the phage and the development of chemically synthesized peptides derived from peptides on phage. This review discusses studies related to the conformation of peptides selected from phage display peptide libraries in addition to the conversion from peptides to non-peptides.

TECHNOLOGICAL OVERVIEW OF PHAGE DISPLAY

Fusion proteins can often retain the biological function of original proteins. The minor coat protein of filamentous bacteriophage can tolerate the fusion of peptides for display on the phage[1]. A vast number of peptides can be produced using one phage per peptide corresponding to foreign DNA inserts. The particles of fusion phages have been treated as peptide-immobilized beads in affinity purification and as organisms in amplification[2,3]. Therefore, phage display is a very cost-effective and time-saving technology. Filamentous phages have been extensively used to display random peptides as fusions of the minor coat protein pIII[2,3], the major coat protein pVIII[4], or the minor coat protein pVI[5]. There are two basic display formats that can be selected from phage-display vectors. Polyvalent display generates every copy of the capsid protein displaying the fusion using a viral vector[2,3]. Monovalent display generates a mosaic of the wild-type and recombinant capsid proteins using helper phage[6]. Phage-display vectors are critical in the construction of phage libraries because vectors determine the expression and incorporation of inserted peptides into virions. These display formats are mainly used for pVIII as well as pIII fusions. Other phages, such as bacteriophage λ[7] and bacteriophage T4[8], have been used in similar display systems. Peptides have also been displayed on the outer membrane of bacteria[9] and yeast[10-12] using several outer membrane proteins. Each display system has advantages in the binding capacity to targets as well as the production of peptides. It is unknown how the surface accessibility of organism particles globally influences the interaction to target proteins.

Since Scott and Smith reported a peptide display library on filamentous bacteriophage[2], phage display has been successfully innovated in vector design, screening methods, and applications. There is particular interest in investigating vector design because fusion proteins cause biological bias in the process of translocation, assembly, and folding. There is competition between the technologies imposed on the efficient expression of exogenous peptides on phage and the biological exclusion

of exogenous peptides by phage as well as host cells[13]. Phage display technology has been developed for application to protein-protein interactions in various fields. In the discovery of peptide ligands, there are many recent reports of applications such as the identification of epitopes or vaccines, enzyme substrates[14-18], DNA-binding peptides[19,20], enzyme inhibitors[21-29], protein binding peptides[30-42], receptors[43-51], dimerization peptide units[52], tissue-specific homing peptides[53-55], and cell- or organism-binding peptides[56-63]. There are also many general reviews on phage display[64] covering topics such as random peptide libraries[65, 66], constrained peptide libraries[67, 68], phage biology[13] and applications[69-71]. Moreover, phage display technology extends to the synthesis of artificial proteins with random sequences[72]. The selection of novel peptide ligands from random peptide libraries is usually performed by panning[2]. Panning processes have been developed to solve the problems caused by immobilized targets[73]. Panning generates peptides with consensus sequences. Peptides are chemically synthesized on the basis of the consensus sequences and are evaluated by bioassay and structural analysis.

Although peptide libraries are ideal for displaying equal frequencies of amino acids in random sequence, it is difficult to realize frequency control using the present technology. The factors underlying this issue have been discussed in detail[13]. The random peptide on a phage is expressed as a fusion protein in host cells and the biological and chemical properties of fusion proteins is a key issue in display technology.

MOTIFS IN PEPTIDE LIBRARIES

The first phage library was constructed with the design of linear random peptides by fusion to the N terminus of pIII of filamentous bacteriophage[2, 3]. Screening from the phage display peptide library was performed to determine the epitope of antibodies by repetitive affinity selection called panning. The peptides binding to an antibody were selected from 10 million of hexapeptides using several cycles of panning. The two monoclonal antibodies, Mab A2 and M33, which are specific for the hexapeptide DFLEKI of myohemerythrin, were used for the screening of the library. The peptides bound to these Mabs had the consensus sequence $DFLX_3$[2]. Independently, the random hexapeptides on the N terminus of pIII were screened for the 3-E7 monoclonal antibody, which is specific for the N terminus of β-endorphin YGGF[3]. The consensus sequence was identified as YGX_4. It is critical that the YGX_4 sequence contains Y-G on the N terminus. These results show that the phage libraries with linear random peptides can be applied to identify epitopes consisting of a continuous primary sequence. The subsequent identifications of epitopes have been successfully applied to epitope mapping[74-78] and to mimicking the carbohydrate structures of bacteria, cancer cells, or viruses[79-81]. Linear peptide libraries, which are constructed by the fusion of peptides to the N terminus of pVIII as well as pIII in filamentous phage, are useful to identify epitopes for monoclonal antibodies. The chain length of random sequences is the main consideration in the design of linear peptides. The selection of epitopes on the human tumor suppressor protein p53 revealed different sequences depending on the chain length of random peptides[82]. Monoclonal antibody PAb421 against p53 recognizes a ten-amino acid synthetic peptide, KKGQSTSRHK, equivalent to the C-terminal region of p53. The epitope of PAb421 is proposed to involve residues widely spaced in the primary sequence, constituting a discontinuous epitope. Three types of phage library, which consisted of 6-mer, 12-mer, or 20-mer random peptides fused to the N terminus of pIII, were used for the reaction of the monoclonal antibody PAb421. The KXXXSTSXHXK motif with p53-homologous sequences was isolated from the 12-mer and 20-mer libraries, and the selection from the 6-mer library led to the isolation of sequences with the R/KXXXK motif with no discernible homology to p53. The selection using monoclonal antibody PAb240 gave the RHS motif common to the three libraries. This work demonstrated the importance of the chain length of random peptides for the identification of discontinuous epitopes. Moreover, peptide libraries have to be designed based on the characteristics of the target and the choice of peptide such as epitope or mimotope.

Some linear peptides can form structural scaffolds based on both amino acid sequence and chain length of the peptide. A 15-mer library was constructed by the fusion of peptides to the N terminus of pIII on filamentous phage and was screened to identify the peptides that bind to streptavidin. The selected peptides had the consensus sequence HPQ and some of them were cyclic peptides[3]. The HPQ motif was subsequently also identified as a ligand of streptavidin[83, 84]. As the

proline residue in peptides is restricted by the N-C α torsion angle ϕ, motifs containing proline stabilize β-turn conformations. Disulfide bridges also constrain the conformation of peptides. Therefore, the consensus sequence HPQ on phage is very likely to generate peptides with β-turn conformations. In fact, crystal structures of streptavidin-bound linear and cyclic peptide ligands containing the HPQ sequence, FSHPQNT[85], cyclo-Ac-[CHPQGPPC]-NH_2 and cyclo-Ac-[CHPQFC]-NH_2[86] showed that the peptides bound in a β-turn conformation. The sequence containing HPQ is a common motif for binding to streptavidin. These studies illustrated the importance of turn conformation and led to the construction of peptide libraries containing a cystine bridge in phage display.

Many motifs are found in the sequences of natural proteins. As shown in Table 1, motifs have also been identified by selection from phage libraries. The consensus sequence is often developed into a peptide motif. To identify higher affinity ligands from peptide libraries, the motif of GP or DEY were applied to construct the library with a di- or tri-peptide in random sequence[41, 44].

TABLE 1 Targets and motifs of amino acid sequences selected from phage libaries

Target	Motif	Ref.
EPOR	GGTYSCHFGPLTWVCKPQGG	49
	DREGCRRGWVGQCKAWFN	44
IGFBP-1	CRAGPLQWLCEKYFG	41
Complement C3	ICVVQDWGHHRCT	29
Calmodulin	SCLRWGKWSNCGS	32
IgE receptor	XXCPXXCYX	45
Syntrophin PDZ domain	XXETCXAGXXC	42
Angiotensin-converting enzyme 2	CXPXRXXPWXXC	27
Streptavidin	HPG	3
Androgen receptor coregulators	FXXL(F/Y)	111
Alpha M beta 2 integrin	(D/E)(D/E)(G/L)W	112
Urokinase-type plasminogen activator	BXXSSXXB	113
V-type H+-ATPase	WLELRP	114
Antibodies from prostate cancer patients	NX(S/T)DK(S/T)	115
P-selectin	EWVDV	116
Peripheral-type benzodiazepine receptor	STXXXXP	117
FGF receptor	MXXP	118
Leukocyte-specific beta(2) integrins	LLG	119
X-inhibitor of apoptosis protein	C(D/E/P)(W/F/Y)-acid/basic-XC	120

B represents a basic amino acid and X represents any amino acid

It is dependent on peptide sequences whether motifs play a role in either the structural scaffold or surface contact with the target. Currently, this issue is being clarified by structural analysis using co-crystalization or NMR spectroscopy. A library with a general motif $X_4CX_2GPX_4CX_4$ led to the identification of a peptide with the sequence of CRAGPLQWLCEKYFG that bound to the insulin-like growth factor binding protein-1 (IGFBP-1). The synthetic peptide CRAGPLQWLCEKYFG was analyzed by NMR spectroscopy and adopted a turn-helix structure. The hydrophobic residues (two Leu, Trp, Tyr, Phe) provided direct contact to IGFPB-1 as well as stabilization of the peptide conformation. The structural scaffold attributed a turn structure to the Cys, Gly, and Pro residues, and a helix to the sequence of KYFG[87]. These results showed that the sequence motif might be essential for both the structural scaffold and direct contact with the target. As the peptides by displayed phage are small in size, some amino acid residues play a dual role in structural stabilization and direct contact with targets.

CYSTINE BRIDGE SCAFFOLD IN PEPTIDE LIBRARIES

It is a design goal that the conformation of peptide is such that a bioactive peptide is generated. Moreover, the characteristics of peptides are used for the development of non-peptide structures to create drug candidates. The conformation of peptides is basically determined by amino acid sequences. In linear peptide libraries, the sequences are random, but a longer peptide chain is required to generate a rigid conformation. A library containing 8-mer random peptides has a theoretical diversity of 2.6×10^{10} members. To construct this library, transformants with 1.1×10^{12} members is required in genetic diversity. This is generally an experimental limitation in the construction of a complete library. Even when a library of longer peptides is constructed, both the structural analysis and the development of non-peptide structures are more complex in correlation with the peptide chain length. Therefore, it is a practical approach that a peptide library has a structural scaffold. Ligands interacting with proteins biologically adopt a α-helix, β-strand, or loop structure. The α-helix and β-strand structures stabilize longer peptide chains. The α-helix structure is essential to the

biological functions of some peptide hormones[88, 89], which may be mimicked in the screening of peptide libraries with an alpha-helically homogenous conformation[90, 91]. Loop structures play an important role in the molecular recognition of protein-protein or protein-peptide interactions, and they may be a scaffold candidate for peptide libraries. Many libraries have been developed to introduce a constraint into peptides[67, 68] Random peptides sandwiched by a pair of cysteine residues are used to cause constraints. Employing a constraint caused by a cystine bridge resulted in the discovery of a ligand for the erythropoietin receptor (EPOR)[49]. A peptide bound to EPOR was identified by screening from a phage library of random octapeptides flanked by cysteines. By using synthetic peptides, it was shown that peptides with a consensus sequence $YXCX_2GPXTWXCXP$ had an agonistic activity against EPOR. One of the peptides, GGTYSCHFGPLTWVCKPQGG, was structurally analyzed with co-crystals[50]. The crystal structure of a complex of this peptide with an extracellular domain of EPOR generated important information for peptide design. The sequence of GPLTW is involved in both peptide-receptor and peptide-peptide interactions. The sequence of GPLT revealed a type I beta structure turn. This result agrees with the fact that both proline and glycine residues preferentially generate the turn conformation according to empirical prediction. These results indicated that the turn structure might be effective for the formation of a cystine bridge as well as bioactivity. In another report of EPOR agonist peptides, a consensus sequence of $CX_2GWVGXCX_2W$ was isolated from 38-mer random peptides of a phage library[44]. This sequence also contains glycine residues and a tryptophan residue in the cystine bridge. A library with the sequence motif $X_4CX_2GP_2X_4CX_4$ led to the identification of a peptide with the sequence of CRAGPLQWLCEKYFG that bound to IGFBP-1[41]. Although this motif is common to EPOR agonist peptides, the tertiary structures of these peptides in solution are very different[87]. The turn structure with a cystine bridge has various conformations in common motifs and is still flexible so as to be under the influence of constituent amino acid residues. The motifs identified using phage display are shown in Table 1. Interestingly, the sequences of ligands selected from phage libraries with cystine bridges have a high frequency of proline, glycine, or tryptophan residues as the preferential constituent amino acid residues in a random sequence. The abundance of proline residues may result from the predominant sequence bias due to the formation of a cystine bridge[92].

As shown in the structure of EPOR agonist peptides, most peptides from phage libraries do not have clearly distinguished binding sites from structural scaffold residues. The distinction can be made by designing non-peptides derived from peptides. Peptide hormones are unsuitable for drugs due to their poor oral bioavailability and less stability in circulation. On the other hand, many challenges have been encountered in the synthesis of non-peptides derived from peptides as a rational approach. Somatostatin is a well-known example of the development of non-peptide structures substituting for the peptide backbone. Somatostatin is a disulfide-linked cyclic tetradecapeptide, AGCKNFFWKTFTSC[93] and regulates the release of hormone such as growth hormone, glucagon, insulin, and gastrin through various somatostatin receptor subtypes[94]. The sequence of FWKT in somatostatin is essential for activity and the other amino acid residues constitute the peptide backbone. The peptide backbone was substituted with beta-D-glucose[95], mannitol[96], and biphenyl scaffolds[97]. Moreover, totally non-peptide somatostatin agonists were discovered[98, 99]. Therefore, the distinction of binding sites from the peptide backbone may be important for the design of peptide libraries to address pharmaceutical interests. The backbone of somatostatin is a design candidate for constructing peptide phage libraries because of the abundant knowledge regarding its medicinal chemistry as well as structural analysis.

In the design of peptide conformation, disulfide-linked cyclic peptides in phage libraries are predominantly considered. There are many naturally occurring peptides containing cystine bridges. Peptide hormones are often synthesized as large proprotein precursors. After processing, mature bioactive peptides are stored in secretory granules that are released by specific stimulations. Therefore, the backbone of mature peptide hormones seems to be formed in the proprotein. The partial sequences of peptide hormones are shown in Table 2. Surprisingly, there are no proline residues in the sequence sandwiched by cystine bridges in peptide hormones. The numbers of turns in peptide hormones may increase due to the global interaction of the peptide backbones. On the other hand, the over-abundance of

TABLE 2 Naturally occurring peptides and their sequences in cystine bridges

Peptide	Sequence in cystine bridges
Adrenomedulin	CRFGTC
Atrial natriuretic factor	CFGGRMDRIGAQSGLGC
Brain natriuretic peptide	CFGRKMDRISSSSLGC
Calcitonins	CGNLSTC
CGRP	CDTATC
Cortistatin-17	CRNFFWKTFSSC
Endothelin-1	CSCSSLMDKECVYFC
Endothelin-2	CSCSSWLDKECVYFC
Endothelin-3	CTCFTYKDKECVYYC
Orexin A	CCRQKTCSC
Oxytocin	CYIQNC
Somatostatin	CKNFFWKTFSC

CGRP, Calcitonin gene-related peptide

proline residues in a cyclic dodecapeptide library was reported in the analysis of amino acid residue occurrence patterns[92]. The proline residue has a high propensity to adopt β-turns because of the restriction by the N-Cα torsion angle. In fact, proline residues have been used in synthetic peptides to stabilize β-turn conformations[100]. Therefore, the sequences containing proline residues generate β-turns by themselves, which is dependent on local conformational effects, rather than by the peptide backbone constraints. All random peptides have been displayed on phage by fusion to the terminal end of phage proteins, pIII or pVIII, although there are many types of phage display systems[64]. The conformation of random peptides in phage display is little affected by parent proteins. This agrees with the fact that an abundance of proline residues was observed in a cyclic dodecapeptide library[92]. This also gives the advantage of simply designing synthetic peptides derived from peptides on phage. Phage peptide display should facilitate the synthesis of peptides. The bioactivity of cyclic peptides on phage is often identical to that of synthetic peptides that are designed based on the primary sequence. On the contrary, the search for bioactive peptide fragments in a protein is sometimes successful in the preparation of peptide fragments from the primary sequence, but difficult in relation to the conformation of bioactive regions. In this way, the constraint of disulfide-linked cyclic peptides in phage libraries is different from that of naturally occurring small peptides with a cystine bridge from the viewpoint of cyclic strain energy.

The design of cyclic random peptides for display on phage may be regarded as the optimization of the loop length. A strong inverse correlation was observed between loop length and stability in a four-helix-bundle protein[101]. Reducing the loop length led to the minimization of atrial natriuretic peptide (ANP) using phage display[102]. The natriuretic peptide receptor-A-binding determinants consist of 7 amino acid residues, Phe^{8}, Met^{12}, Asp^{13}, Arg^{14}, Ile^{15}, Leu^{21}, and Arg^{27} in the sequence of ANP, shown in Table 2 and reoptimized as a 15-mer cyclic peptide, MCHFGGRMDRISCYR. The sequences of both MCH and SCYR make the conformation of the continuous sequence FGGRMDRI identical to that of ANP. The conformations of the sequence FGGRMDRI may be almost identical between the 15-mer cyclic peptide and ANP. In this way, the sequence between the cysteine residues that form the cystine bridge may strongly affect the structural conformation in peptide libraries. The sequences that are 5' and 3' of the cystine residues forming the cyctine bridge may contribute to the conformation of inner random peptides, contact surface to target, or phage assembly. Libraries displaying peptides with various loops were constructed and used for the successful selection of targets of interest. In the present technolgy, the designs for cyclic random peptides are illustrated as the sequence $X_nCX_mCX_n$ in fusions of the terminal ends of phage proteins[103]. However, the disulfide-linked peptide library exhibits conformational as well as amino acid sequence diversities even in identical libraries.

CONFORMATIONALLY CONSTRAINED LIBRARIES USING THE LOOP STRUCTURE OF PROTEIN SCAFFOLD

The low flexibility of peptide molecules can reduce the entropic costs for binding to targets. The conformation of the loop structure is rigid in the tertiary structure of proteins. Phage libraries were constructed by substituting random peptides for the amino acid residues in the loop structure of proteins. Tendamistat is an inhibitor of α-amylase, and a 74-amino acid molecule comprised of two three-strand β sheets[104]. The loop structure of tendamistat has also been applied in the peptide chemistry of a combinatorial library[105]. Random

hexapeptides substituted for the loop extend between amino acids 60 to 65 of tendamistat were recognized by mAbA8, a monoclonal antibody against endothelin, to reveal the most frequent sequence of FNVTYA. On the other hand, the consensus sequence LEPW was selected by the mAbA8 antibody from random hexapeptides fused to pIII, but not from the tendamistat random peptide library[106]. The affinity of tendamistat phage containing FNVTYA to mAbA8 was a little higher than that of the phage displayed CWLEPWLC flanked by pIII. The peptides containing LEPW were selected from the libraries with or without a cystine bridge. There is no homology among the sequences of FNVTYA, WLEPWL, and endothelin. This means that the feature of peptide may be important for mimotopes rather than primary sequences. These results show that the constraint may not be imposed by a cystine bridge in N-terminal linked libraries, or may be largely affected by the constituent amino acid residues in N-terminal fusions. On the contrary, the constraint of peptides in the loop structures of proteins is dependent on the multiplicity of interaction in tertiary structures, and is relatively constant in a library. In another study, the tendamistat peptide library containing a fixed RGD sequence was used for the specific selection of ligands of the integrin family[107]. That study shows that tandemistat fusions may control the conformations of the RGD motif in minute detail similar natural ligands of integrin.

IN THE FUTURE

Since phage peptide libraries was first reported in 1990[2], phage display has been successfully used in various research fields. There have been many technical innovations in phage display systems particularly in the past decade[64]. Moreover, phage display technology has improved the binding properties of peptides to target proteins by designing the conformation of peptides. Structural stability plays a role in the determination of the local conformation of peptides. As a result, the consensus sequences are selected from linear or cyclic random peptide libraries. The random peptide $X_nCX_{n'}P$ $X_{n''}CX_n$ may be an excellent design for phage display as far as using N- or C-terminal fusions is concerned because of the successful selection of ligands to EPO R[50], IgE receptor[45], or IGFBP-1[41]. If the X_n residues are sufficiently long to interact with each other, the inclusion of proline residues will not be necessary. The reason for this is because the interaction of the Xn residues causes the peptide fragment within a cystine bridge to form a β-turn. The global conformation of tendamistat in a phage display peptide library imposes a rigid conformation on random peptides as in peptide hormones[106]. Therefore, the conformational rigidity of random peptides in a tendamistat library is uniformly generated independent of the random peptide sequence. Other protein scaffolds or novel fusions are expected to generate other rigid conformations in phage display systems.

As a promising approach for drug discovery, phage display technology will be implicated in the discovery of non-peptidic compounds through the structural requirements of peptides selected from phage libraries, as outlined in Fig. 1. We constructed a peptide library by insertion of peptides into pIII (Uchiyama *et al*., in preparation). The design of peptides on phage is a model based on the structure of somatostatin, and consists of the stem and loop structure in pIII. The stem region

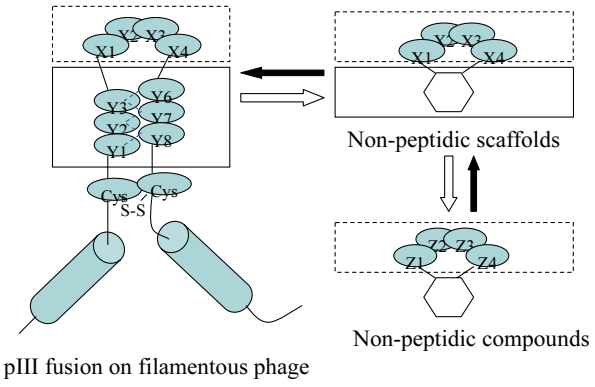

FIG. 1 Retro-design of the scaffold of random peptides. The rectangles with solid lines show the backbones which can convert from peptide regions inserted into pIII to non-peptide regions. The rectangles with dotted lines show the target binding sites which can convert from peptides to non-peptides. The circles with X or Y consist of amino acid residues and Z shows the functional group. The dotted lines between Y letters show the interaction between amino acid residues. The hexagons show non-peptidic and cyclic compounds. Conventional conversions are indicated by open arrows and the virtual conversions using the retro-design of the scaffold are indicated by closed arrows.

is a scaffold and is composed of six amino acid residues from Y1 to Y6. The binding region, from residues X1 to X4, makes direct contact with targets. The two regions in small peptides can be functionally distinguished. A somatostatin scaffold was used for the discovery of an antagonist to the tachykinin receptor 1[108], although there is no sequence homology between the tachykinin receptor 1 and somatostatin receptors. The role of a disulfide bond is not constrain the peptides, but to stabilize the interaction of the stem structure, because the introduction of a disulfide bridge was not as effective as the generation of a conformational constraint. The stem structure of peptides on phage can be converted to non-peptide scaffolds[95-98]. The binding region of peptides can be substituted with non-peptides to generate non-peptidic compounds[99]. These conversions are described in the foregoing example of somatostatin. It may be more efficient in drug discovery that as a retro-design, the scaffold of peptides on phage is designed in consideration of non-peptidic compounds. As a retro-design shown in Fig. 1, the conformational design of random peptides in phage libraries was performed on the basis of a non-peptidic scaffold.

The bioactivity of synthetic peptides based on the primary sequence is almost the same as that of peptides on phage in N- or C-terminal fusion libraries[41, 45, 50]. This is because N- or C-terminal fusion peptides are structurally independent of the parent protein. In the synthesis of peptides selected from the tendamistat library, the scaffold which is structurally equivalent to anti-parallel β strands of tendamistat should be coupled to both ends of the peptides. The tendamistat scaffold will be more useful in phage display for medicinal chemistry if a non-peptidic scaffold for the tendamistat library can be found.

In protease inhibitors, the protein-protein binding interfaces have been recognized to involve 10 to 15 residues of ligands by crystallographic studies, and the protein-protein complex is attributed to the rigid preformed binding loops[109]. A small subset of residues in a functional epitope was reported to dominate hot spots of the binding energy in protein-protein interfaces[110]. Designing the scaffolds of peptides for phage-display peptide libraries will become increasingly important for the analysis of protein interaction and enable to the discovery of mimics of the smaller functional epitopes. Phage display technologies are becoming more powerful in combination with other technologies. To address a rational approach for drug discovery, peptide designs are being performed under the integration of phage display technology, peptide chemistry, structural analysis, and medicinal chemistry.

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