From peptides to drugs via phage display

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In the past ten years, there has been a flurry of research generating and screening combinatorial peptide libraries. Molecular biologists have favored phage display as a means of generating millions to billions of different peptides for the purposes of mapping protein–protein interactions of antibodies, cell surface receptors and intracellular proteins. This article summarizes recent work on identifying peptide ligands via phage display and several methods by which they serve to promote drug discovery: design of peptidomimetics, biological validation of targets, and the establishment of high-throughput screens of chemical compound libraries. These methods provide powerful aids in the search for lead compounds of previously 'unscreenable' targets and for new targets discovered in genomics efforts.

In recent years, combinatorial peptide libraries have served as a rich source of ligands for a myriad of targets. Depending on the experience of the investigator, combinatorial peptide libraries can be generated on pins[1], beads[2], or in solution[3], expressed in bacteria attached to phage[4–6] or bacteria[7] or a DNA-binding protein[8], or synthesized *in vitro* off polysomes[9]. While each of these formats has its own advantages and disadvantages, they can all be screened for peptide ligands to the active sites of the target molecules of interest.

In phage display, peptides or proteins are fused to one of the capsid proteins and are accessible to molecular interactions[10]. Combinatorial peptides, encoded by degenerate oligonucleotides, can be expressed at the N-terminus of the major (i.e. 2500 copies) or minor (i.e. five copies) capsid proteins – pVIII or pIII, respectively – of bacteriophage M13. Figure 1 illustrates the types of display possible from pVIII and pIII, depending on the vectors utilized. Libraries of 10^8–10^{10} different recombinants can readily be constructed by electroporating large amounts of ligated DNA into *Escherichia coli* cells, which will then go on to secrete viral particles. In this manner, phage libraries have been constructed displaying combinatorial peptides 6–43 amino acids long. Although these libraries represent only a small fraction of the mathematical permutations (i.e. 10^{10} is much smaller than 20^{43}), they are still a rich source of peptide ligands, and several reviews of their utility have recently been published[11–14].

Screening phage libraries

Phage libraries are typically screened by affinity selection. Microtiter dish wells or plastic tubes are coated with protein targets, incubated with a library aliquot, and the phage particles that are retained in the wells are recovered by a simple pH denaturation step[15] (Fig. 2). Remarkably, bacteriophage M13 viral particles are resistant to exposure to pH 2 or 12 and are infectious once the pH has been neutralized. Another common approach is to add biotinylated target protein to tubes containing an aliquot of phage, and then to recover target–phage complexes with streptavidin-coated microtiter wells or magnetic beads[16]. Because of the incredible stability of the phage particles, it has also been possible to fractionate them over cell monolayers[17,18], column matrices[19] and even after injection into mice[20,21]. The recovered phage are then amplified by simple infection of bacteria, providing a ready source of DNA for

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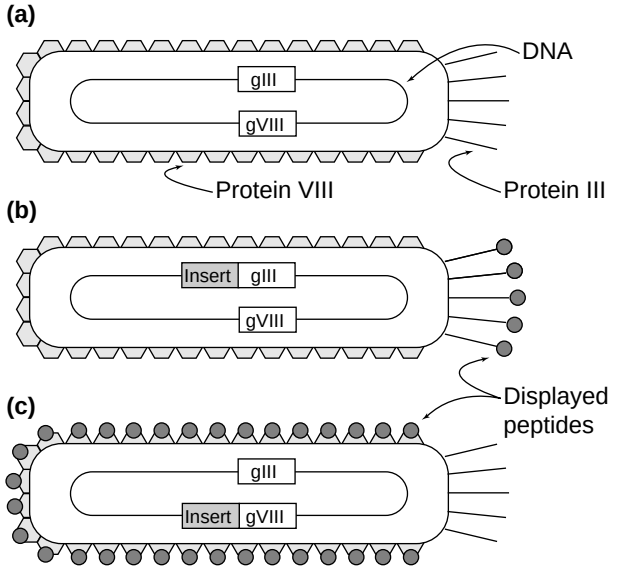

Figure 1. *Wild-type (a) and recombinant (b,c) bacteriophage M13 viral particles. Viral particles are composed of one copy of single-stranded DNA and five capsid proteins. DNA (gray boxes) can be inserted into genes III or VIII to generate chimeric proteins, with the resulting peptides 'displayed' at the N-terminus of either protein. There are approximately five and 2500 copies of proteins III and VIII, respectively, per phage particle. Particles that display on all copies of pIII or pVIII are termed type 3 (b) or type 8 (c), respectively[99]. Other display formats are described elsewhere[100].*

sequencing and prediction of the displayed binding peptides.

What makes peptides particularly effective in the drug discovery process is their apparent ability to 'home in' on the active or biologically relevant sites of protein targets. Even though phage-displayed peptide libraries are rather large, and would thus seem to have the potential to recognize many different aspects of a target molecule, they generally bind to only a few sites on a target molecule. Most importantly, these sites coincide with the active or binding sites of the target. This observation may be surprising at first glance; however, several reasons may account for the apparent specificity of the affinity-selected peptides for active sites. First, most proteins interact with other proteins, and short peptides from combinatorial libraries can act as 'surrogate' ligands. In addition, even though two interacting proteins can be rather large, the number of residues critical for binding can be rather small (i.e. three to ten)[22–24]. Thus, on some

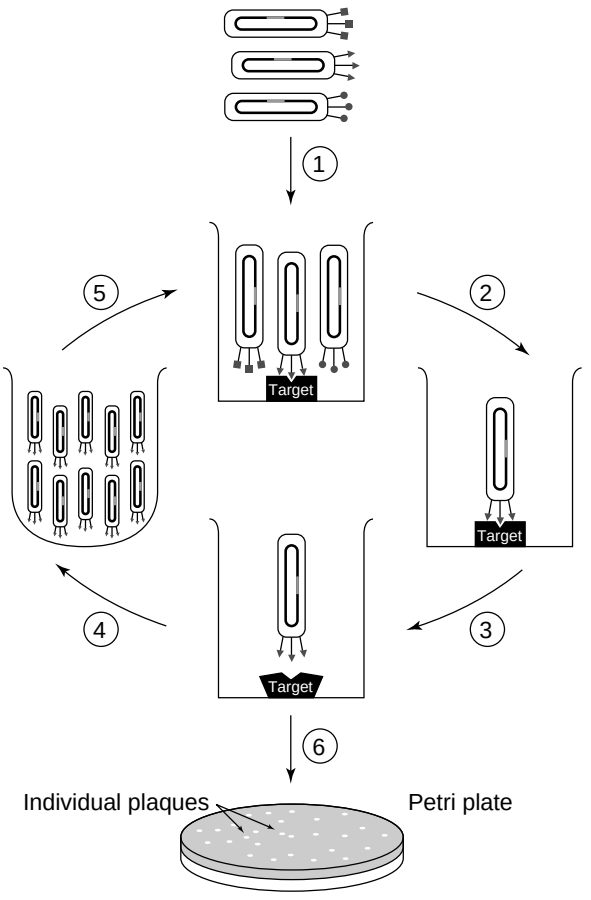

Figure 2. *A library of recombinant bacteriophage displaying different peptide sequences can be fractionated by affinity selection in six steps. Shown at the top of the figure is a simple mixture of three recombinant phage, with DNA inserts (gray line) into gene III, displaying different peptides (square, triangle, circle) at the N-terminus of the mature capsid protein, pIII. There are five copies of pIII per viral particle. In step ①, the phage particles are added to a microtiter well coated with target protein (target, shown with binding cavity). After incubation, non-binding phage are washed away (step ②). In step ③, the bound phage are released from the target by pH denaturation; the target is denatured but the phage particles remain intact. In step ④, the recovered phage are used to infect* Escherichia coli *and 6 h later each phage is amplified >10^7 fold. An aliquot of the amplified phage is then added (step ⑤) to a well containing fresh immobilized target. After repeating steps ②–⑤ twice more, the recovered phage are plated out in step ⑥, to yield individual plaques on bacterial lawns on Petri plates. Once the binding of the clones has been confirmed, their DNA is sequenced and the displayed peptide sequence predicted.*

occasions, peptides will have the capacity to provide, in proximity, the critical residues. Second, one of the driving forces for peptide binding to a target is the displacement of water from recesses or cavities in a target molecule; such sites predominate at the active or binding sites of a receptor molecule[25]. Third, affinity selection with phage necessitates that the phage bind strongly enough to their targets to resist the shear forces of washing. The typical K_d values of peptides recovered by phage display generally range from 20 μM to 10 nM when synthesized and tested in solution.

Proteins targeted with phage-displayed peptide libraries

Table 1 lists several examples published in the recent phage-display literature. The entries are arranged arbitrarily by category: earliest targets used, cell surface receptors, cytosolic proteins, viral proteins, and enzymes. Target molecules with recesses or pockets that constitute an active or binding site have yielded the successful isolation of phage-displayed peptides by affinity selection. Each of the entries will be discussed briefly below.

Table 1. Protein targets described in recent phage-display literature[a]

Target	Binding location	Activity
Streptavidin	Biotin binding site	None[5,26,74]
Antibodies	Antigen binding site	Antagonist[4,6]
Integrins	Extracellular matrix binding site	Antagonists[30,76,92,93]
Histocompatibility protein	Peptide binding site	None[94]
Thrombin receptor	Thrombin binding site	Antagonist[96]
Urokinase receptor	Ligand binding site	Antagonist[17]
E-selectin	Glycoprotein binding site	Blocks neutrophil adhesion[35]
IL-1 receptor	IL-1 binding site	Antagonist[36]
EPO receptor	EPO binding site	Agonist[37,39]
TPO receptor	TPO binding site	Agonist[40]
C3 complement	Not determined	Complement activation antagonist[41]
C4b binding protein	Protein S binding site	Inhibitor of protein S–C4b interaction[42]
Calmodulin	Protein–protein interaction site	Antagonist[43,54,46,96]
Endoplasmic reticulum chaperone BiP	Chaperone binding site	Stimulation of BiP ATPase activity[47]
Src	SH3 domain	Blocks protein–protein interactions[46,52,97]
Src	Kinase domain	Inhibitor[55]
Abl, Cortactin, Crk, Grb2, Lyn, p53BP2, PLCγ1, Yes	SH3 domains	Tested for Lyn SH3 ligand (blocked mast cell activation)[61]
Caveolin	Protein–protein interaction site	Blocks binding to G protein and autophosphorylation of EGF-R (Refs 58,59)
Vinculin	Talin binding domain	Blocks binding to talin[57]
DM2	p53 binding site	Antagonist[65.66]
Hepatitis B core antigen	Not determined	Blocks interaction between two capsid proteins[67]
Adenoviral capsid protein		Ref. 68
β-Glucuronidase	Active site	Inhibitor[69]
NADPH oxidase (p47)		Antagonist[98]
Phosphoenolpyruvate–sugar phosphotransferase	Active site	Inhibitor[70]

[a]Protein targets for phage display are listed together with properties of their sites of binding and the activity of synthetic peptides. This is just a subset of a growing literature: citations have been chosen based on historical precedence or recent appearance in the literature. In many cases, the site of peptide binding to the target is deduced either by resemblance to the ligand or its ability to antagonize/agonize the target protein's function. Thus, it is inferred that binding occurs at, or near, the active or biologically relevant site on the target molecule. BiP, endoplasmic reticulum chaperone molecule; DM2, double minute 2 protein; EGF-R, epidermal growth factor receptor; EPO, erythropoietin; IL-1, interleukin 1; SH3, Src homology 3 domain; TPO, thrombopoietin.

Early targets

One of the first targets used to select phage from a combinatorial library was streptavidin, the biotin binding protein from *Streptomyces avidinii*. The selected phage carried the motif HPQ (Ref. 5), which was later shown to bind to the top of the biotin binding pocket[26]. However, since streptavidin binds biotin with such an impressive affinity (K_d ~10^{14} M), the peptides did not inhibit the biotin-binding activity of streptavidin. Another early class of target, and one used by many investigators since, was antibodies; almost every successful screening experiment with phage libraries yielded isolates that bound to the antigen binding site. These peptides resembled epitopes of antigens; often the primary structures of the peptides could be found in the antigen sequence, and generally peptides synthesized according to the phage inserts could antagonize the interaction between antibodies and the authentic antigens[4,6]. The rapid elucidation of peptide ligands to antibodies offers significant opportunities in vaccine development, design of diagnostic reagents and autoimmune therapies[27–29].

Cell surface receptor targets

Another set of examples where phage-displayed peptide libraries have been used successfully has been the isolation of peptide ligands for cell surface receptors. One class of productive targets is the integrins, a family of heterodimeric proteins involved in binding various extracellular matrix molecules (e.g. fibronectin, laminin). Peptides that bind to the platelet integrin, gpIIb/IIIa and inhibit platelet aggregation have been isolated from a library of cyclized peptides with the motif CXXRGDC (Ref. 30). Peptide ligands for the $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins have also been isolated[31,32]. The major histocompatibility complex (MHC) proteins have been used to select peptides that bind to the promiscuous binding sites; such peptides have permitted identification of position-specific anchor residues, defining motifs for peptide binding to individual MHC molecules[33]. Peptides have been isolated that bind to the thrombin receptor of whole platelets and, when synthesized, found to inhibit the aggregation of platelets tenfold higher than previously reported peptide antagonists of the thrombin receptor[34].

Another class of molecules, selectins, which bind carbohydrates and glycoproteins on cell surfaces, has been a successful target. The neutrophil E-selectin was used to screen a phage library, and led to the isolation of peptides with nanomolar dissociation constants and which inhibited neutrophil cell adhesion *in vitro* and neutrophil cell migration to sites of inflammation *in vivo*[35]. Peptide ligands to three different cytokine receptors have been isolated and characterized at Affymax Research Institute (Palo Alto, CA, USA). Peptides were isolated from phage-display libraries that bound to the interleukin 1 (IL-1) receptor and antagonized the binding of IL-1 to the receptor[36]. Variants of these peptides had enhanced antagonist activity (IC_{50} = 2 nM) *in vitro* and could block IL-1-driven responses in human and monkey cells. Peptide ligands for the erythropoietin (EPO) receptor have been discovered from a library of cyclized combinatorial peptides[37]. While a particular 14-mer peptide lacked any obvious primary structural similarity to EPO, it bound within the receptor binding pocket, as shown by X-ray crystallography of the peptide–receptor complex[38], and was a potent agonist in cell assays and mice when prepared as a dimer[39]. A dimeric form of the peptide could compete against the binding of EPO to its receptor, with an IC_{50} of 2 nM. Peptides that bind to the thrombopoietin (TPO) receptor have recently been reported[40]. Peptides 14 amino acids long bound the receptor with a 2 nM dissociation constant, and when dimerized were as potent agonists as the 322 amino acid TPO molecule itself. Furthermore, the peptide dimer triggered proliferation and maturation of human megakaryocytes *in vitro* and promoted an increase in platelet count when injected into mice.

Two serum proteins have been used to select binding peptides through phage display. A 27-mer peptide that bound to C3b was selected from a library; C3b is the proteolytically activated form of complement component C3 (Ref. 41). The functional activity of the peptide was delineated to a cyclic 13-mer sequence that could inhibit complement activation by blocking C3 proteolytic cleavage *in vitro*. Complement protein C4b has also been used to select peptides from a phage library[42]. Comparison of the selected 15-mer peptides to the sequence of the vitamin K-dependent S protein, which forms tight 1:1 complexes with C4b in human serum, led to the discovery of four matching regions in the vitamin K-dependent S protein sequence. When a peptide was synthesized corresponding to one of those regions, it was observed to inhibit the interaction between vitamin K-dependent S protein and C4b. Thus, C4b target selected the same types of peptide sequences present in the S protein.

Cytosolic protein targets

A wide variety of cytosolic proteins have also served as targets for phage library screening experiments. Both linear[43–45]

and cyclized[46] peptides have been selected that bind in a Ca^{2+}-dependent manner and antagonize the action of calmodulin on effector molecules. The endoplasmic reticulum chaperone molecule, BiP, selected peptides that bind and elevate its ATPase activity[47]. Sequence analysis of the peptides was used to develop a scoring system that predicts potential BiP binding sequences in naturally occurring polypeptides. The cellular protooncogene product, Src, has been used in a variety of experiments to select peptide ligands from combinatorial peptide libraries. Peptides that bind to the Src-homology 3 (SH3) domain have the motifs RPLPPLP or PPVPPR, depending on whether the peptides bind in opposite orientations relative to the SH3 domain[48–52]. Furthermore, the peptides can antagonize the specific interaction of cellular proteins with the SH3 domain with IC_{50} values of 1–5 μM, and nearly identical sequences have been reported in Src SH3-domain-interacting proteins[53,54]. When full-length Src (60 kDa) was used to select phage, peptides that carried the motif GXXG, where X was frequently a hydrophobic residue, were isolated[55]. Nine-mer peptides carrying this motif were modest inhibitors (K_i = 24 μM) of the protein tyrosine kinase activity of Src (Ref. 55).

Peptides have been isolated that bind to the SH3 domains of many other proteins[54], and their sequences match regions resident in known SH3-domain-interacting proteins. Thus, the identification of the optimal ligand preferences of the SH3 domain allows one to predict potential interacting partners in the cell. The protein vinculin, which interacts with several proteins to link the actin cytoskeleton with integrins[56], was used to select binding peptides from a combinatorial library[57]. These peptides could compete against the binding of talin, but not of paxillin or α-actinin, to vinculin. Interestingly, in one of the peptides there were three sequence motifs that also occur in the region of talin previously mapped to bind to vinculin. Caveolin, which is a ~21 kDa integral membrane protein suggested to act as a scaffold in interacting with G proteins and tyrosine kinases, was used recently to screen a phage-displayed peptide library. The selected peptides shared several motifs: ΦXΦXXXΦ, ΦXXXXΦXXΦ, ΦXΦXXXΦXXΦ, where Φ is an aromatic residue (Trp, Phe, or Tyr). Peptides matching these motifs were found to be present in a variety of proteins that reside at the plasma membrane of cells[58]. Short peptides, corresponding to these motifs but derived from a G protein[58] and the epidermal growth factor receptor[59], were found to bind to caveolin, disrupt the interaction of the proteins with caveolin and block autophosphorylation of the epidermal growth factor receptor[59].

A particular protein–protein interaction that is important for normal cell growth regulation occurs between the tumor suppressor, p53, and the double minute 2 protein (DM2)[60,61]. In normal cells, p53 arrests the cell cycle in response to DNA damage to permit the opportunity for DNA repair. In the cell, DM2 binds to p53 and negatively regulates its functions in transcription, cell cycle arrest and apoptosis, by shortening its half-life through ubiquitin-directed proteolysis[62]. The importance of these molecules is underscored by the two observations that most human cancers contain mutations in both alleles of the *p53* gene[63] and that DM2 is overexpressed in many human sarcomas as a result of gene amplification[64].

The interaction of p53 and DM2 has been explored by a variety of means, such as protein truncation, mutagenesis, peptide synthesis and X-ray crystallographic experiments. It has also been possible to use phage-displayed combinatorial peptide libraries to define the interactions of DM2 with proteins. Screens of such libraries with the N-terminal 140 amino acids of DM2, the smallest segment of DM2 which binds p53, has led to the identification of interacting peptides. In published[65,66] and unpublished experiments from our laboratories, the identified peptides shared the motif XFXDXWXXLXX. Intriguingly, these peptides resemble the segment of p53 determined to interact with DM2 (Fig. 3). Thus, DM2 selected peptides that bound to one of its biologically relevant sites, as evidenced by its excellent correspondence to a sequence within the known interacting ligand, p53.

Viral protein targets

Another target source has been viral capsid proteins. These proteins interact with other capsid proteins for viral morphogenesis as well as cell surface receptors, and thus make excellent antiviral drug targets. The core antigen of hepatitis B virus has been used to affinity select peptides from a six-mer library[67]. One set of isolated peptides shared a motif, LLGRMK, which when chemically synthesized was capable of blocking the interaction of the core antigen protein with the hepatitis B viral envelope protein *in vitro*. Although there was not an obvious match between the primary structures of the peptides and the envelope protein, there were three regions of similarity in the region of the envelope protein shown to interact with the core antigen protein. The same phage library was also

screened with the adenovirus type 2 (Ad2) penton capsomer and fiber domains[68].

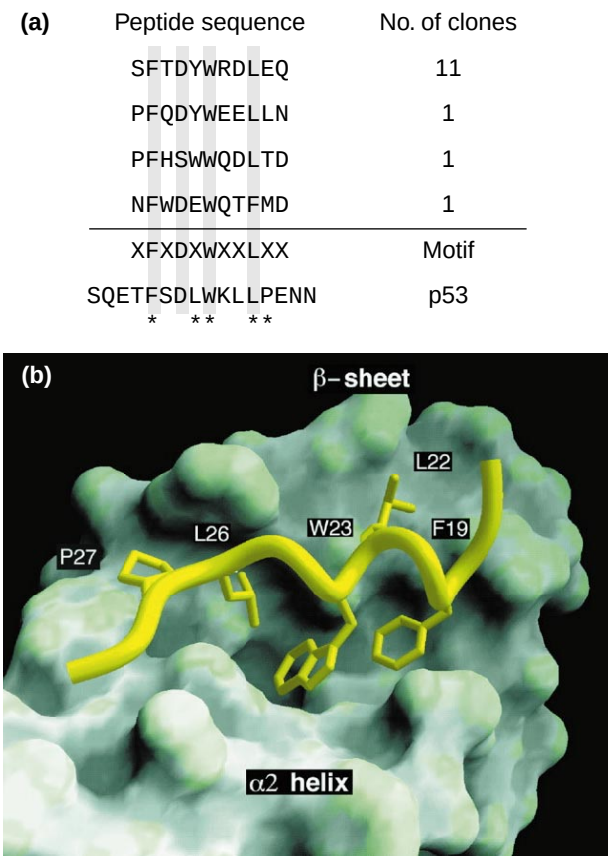

Figure 3. *(a) Alignment of peptides displayed by phage isolated by affinity selection with a segment of mouse double minute 2 protein (DM2; aa 1–140) fused to glutathione S-transferase (GST). There were 14 clones isolated from a library of 10^9 different phage, displaying X_5WX_5 peptides, where X is any amino acid encoded by NN(G/T) codons. One peptide was displayed by 11 clones, and three other peptide sequences were displayed by single isolates. Amino acids (aa) in the motif shared by the four peptides (XFXDXWXXLXX) are assigned by being frequent ≥ 3/4 times at a given position. The motif is aligned with a segment of human p53 (aa 15–30) with common residues boxed. Asterisks mark residues of p53 denoted in (b), which shows the structure of the complex between a segment of human p53 (aa 17–28) and mouse DM2 (aa 25–108) deduced by X-ray crystallography. DM2 is shown as a contour map with two β sheets and one α helix noted. The peptide backbone of the p53 segment has the side groups of selected amino acids shown. (Reproduced, with permission, from Ref. 101.)*

Enzyme targets

Enzymes have also served as successful targets for phage display, even though their substrates are not peptides. Peptides that bind specifically to bovine alkaline phosphatase or β-glucuronidase have been selected from a novel combinatorial peptide library consisting of exons[69]. While synthetic peptides targeting bovine alkaline phosphatase did not inhibit activity, the peptides that bound to β-glucuronidase inhibited its activity with a K_i of 17 nM. Peptides that bind bacterial phosphoenolpyruvate–sugar phosphotransferase have been selected from 6-, 10- and 15-mer libraries[70]. The peptides share a general amino acid character (basic with no acidic residues) and can inhibit the enzyme with IC_{50} values of 10 μM to 2 mM. We have been successful in isolating active site directed peptides for a wide range of enzymes, including tRNA synthetases, hexokinase, alcohol dehydrogenase, β-glucosidase, carboxypeptidase and phosphorylase *a* (to be presented elsewhere). Thus, peptides can be isolated that map the sites of protein–protein and protein–substrate interactions, making them extraordinarily valuable tools for biological research.

Peptide ligands in the drug discovery process

With peptide ligands in hand that bind active sites of target molecules, how does one convert them into drugs? In certain circumstances, such as cell surface receptors, it is probable that certain peptides can serve immediately as agonists or antagonists. However, because of the presence of proteases in serum, such peptides will be short-lived and useful only for the treatment of acute health problems. In addition, since such peptides will need to be injected into the blood stream, this form of peptide delivery is unpopular. These limitations have thus necessitated the development of nonpeptide therapeutics. It is also possible to design peptidomimetics, based on the identified combinatorial peptide ligands, that are protease-resistant, readily cross the plasma membrane and have desirable pharmacokinetic properties[71,72].

There are several prerequisites for peptidomimetic design. It is advantageous to have the smallest possible peptide ligand defined and for the peptide to be conformationally constrained. Once peptide ligands have been isolated from a phage-display library, the peptides can be truncated to define the minimum peptide size that retains activity. This can be accomplished by the construction of additional phage-display libraries that retain randomized

amino acids that flank a functional core motif[73], or by chemically synthesizing and testing shorter peptides[41]. Conformational constraint in phage-displayed peptides is typically achieved by positioning cysteine residues on each side of a combinatorial peptide sequence; such sequences form intramolecular disulfide bonds as the phage are secreted into the oxidizing environment of the bacterial periplasmic space. Typically, the binding strength of a peptide sequence to a target is enhanced when the peptides are cyclized[30,74,75]; such peptides have been developed, for example, as ligands for integrins[76] and the tumor necrosis factor receptor[77]. Finally, it has been proposed that drug design can be based on the structure of combinatorial peptides expressed in a protein scaffold. These peptides will have few conformations, which can be deduced by biophysical methods or computer modeling for the purpose of designing small organic compound mimics[78].

Validating protein targets with peptides

Peptides can also be utilized for target validation in the drug discovery process. Advances in genomics, primarily the sequencing of the human genome and the genomes of selected human pathogens, have provided a rich source of tantalizing targets. But how do we focus in on the best candidates for pharmacological intervention? Peptide ligands discovered using phage display can be a powerful tool to assess the suitability of a gene for drug discovery. Once peptide ligands have been identified for active or binding sites of intracellular targets, they can be introduced or expressed inside cells and their effect monitored. If the peptides inhibit a molecular interaction (such as between proteins or the active site of an enzyme), there may be a biological consequence. If the effect is specific and biologically relevant, then the target can be considered to be 'validated' and worthy of drug development. Target validation with peptides could be faster to achieve than with gene knockouts and may in fact offer a better model of drug action; a peptide will probably interfere with only one of several functions of a protein target, much like a drug, whereas a genetic knockout will result in the complete loss of a protein's function(s).

There are several means of validating a target with a peptide. For intracellular targets, one can express peptides inside cells, either alone or fused to an innocuous reporter protein (e.g. green fluorescent protein[79]), by utilizing recombinant DNA. However, only a few experiments using this approach have been published[80]. One successful route of introduction has been injection. When peptide ligands for the Src SH3 domain were injected into *Xenopus laevis* oocytes, there was an acceleration of progesterone-stimulated maturation[52]. Presumably, the peptides modulated the activity of an SH3 domain-containing protein in the oocyte cytoskeleton or a signal transduction pathway. It has also been possible to introduce peptides inside eukaryotic cells by electroporation. When peptide ligands specific for the Lyn SH3 domain were electroporated into mast cells, mast cell activation was blocked[81]; however, the inhibitory activity of the peptides was short-lived ($t_{1/2}$ = 25 min), presumably because of intracellular proteolysis. This problem can be overcome by the use of β-peptides[82] or D-chirality peptides, which are resistant to proteolysis. A D-chirality ligand for the Src SH3 domain has been isolated by 'mirror-image phage display', in which the 60 amino acid Src SH3 domain was synthesized from D-amino acids, followed by selection of a peptide from an L-amino acid phage-display library[83].

There is also a growing literature describing the linking peptides to other peptides or protein domains; these linking peptides have the capacity to cross the plasma membrane for the purpose of modulating target activity inside cells. For example, a 16 amino acid segment of the *Antennapedia* protein can be internalized by cells in culture in a receptor-independent manner[84–86]. When cultured cells were exposed to a fusion protein containing the C-terminal domain of p21WAF1 (an important regulator of the cell cycle) and the *Antennapedia* peptide, the cell cycle arrested due to inhibition of Cdk4 activity[87]. The Tat protein (an 86 amino acid protein involved in replication of human immunodeficiency virus type 1) can translocate across the plasma membrane and reach the nucleus[88]. It may be possible to fuse peptides to a 35 amino acid segment of Tat to concentrate peptide ligands in the nucleus to interact with nuclear target proteins[89]. Once the hybrid proteins have been introduced into the cytoplasm or nucleus, they have the opportunity to interact with their intended target and validate its importance to normal or abnormal cellular processes. Although there are several mysterious aspects to this route of delivery (i.e. mechanism, efficiency and half-life), it potentially offers a convenient means of introducing peptides into many different cell types.

Developing assays for chemical antagonists with peptides

The third and final way of utilizing peptides in the drug discovery process is in establishing high-throughput

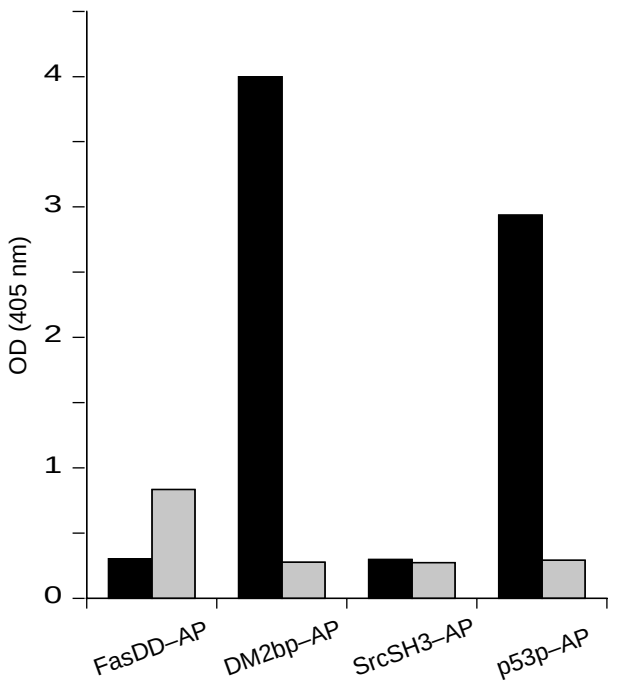

Figure 4. *Spectrophotometric analysis of protein–protein interactions. A double minute 2 protein (DM2) binding peptide (SFTDYWRDLEQ) isolated from a phage-display combinatorial peptide library, a segment of p53 (TFSDLWKLLPE), Src SH3 domain and death domain of Fas receptor were fused to the N-terminus of secreted bacterial alkaline phosphatase (AP). The binding of chimeric enzymes (DM2bp–AP, p53p–AP, SrcSH3–AP and FasDD–AP) to the N-terminal 140 amino acids of DM2 and the Fas receptor death domain (DD), purified as GST fusions and immobilized on glutathione-coated Sepharose beads, was followed spectrophotometrically with the chromogenic substrate* p*-nitrophenyl phosphate. Black bars, GST–DM2; Gray bars, GST–FasDD.*

screens (HTS) of small-molecule libraries. It is straightforward to format an *in vitro* assay where molecules are tested for their ability to displace the binding of the peptide ligands. Microtiter plate wells can be coated with target protein and the binding of the peptides to the immobilized target followed by several methods. We have monitored the interaction of peptides with their targets using spectrophotometry and chemiluminesence (using alkaline phosphatase or horseradish peroxidase), radiometry [^{3}H, ^{35}S or ^{125}I, in conjunction with scintillation counting – either scintillation proximity assay (Amersham) or Flashplate (New England Nuclear)] and time-resolved fluorescence. Alternatively, the peptide ligands can be genetically fused to alkaline phosphatase as a one-step reagent for monitoring peptide–protein interactions[90,91]. For example, the phage-displayed peptides that bind to mouse DM2 (described in Fig. 3) can be fused to alkaline phosphatase and tested for binding to immobilized DM2. As seen in Fig. 4, a fusion between the peptide ligand for DM2 and alkaline phosphatase binds strongly to DM2 but not to an unrelated protein. This interaction can serve as the basis of a HTS assay of a chemical library; wells with compounds that do not interfere with the interaction will turn yellow with *p*-nitrophenyl phosphate, whereas wells with compounds that do interefere (i.e. 'hits') will remain clear. With the help of automation, libraries with 10,000–100,000 different compounds can be conveniently screened for those compounds that can block the interaction between p53 and DM2 *in vitro*. Inhibition of the interaction of these two proteins in sarcoma cells, which commonly overexpress DM2, could permit p53 to act as a transcription factor and trigger cell death.

Concluding remarks

In conclusion, there is an ever-growing body of evidence supporting the premise that phage-displayed peptide libraries are of value not only in mapping protein–protein interactions but also in the drug discovery process. Peptide libraries provide useful avenues into the generation of peptide agonists and antagonists, target validation, the design of peptidomimetics and the formatting of HTS of chemical libraries. Experience has shown that this approach is successful with an extraordinarily wide range of protein targets, including structural proteins, signal transduction proteins, receptors, serum proteins, oncoproteins, viral gene products and enzymes. The ability of phage display to home in on functionally important sites of proteins will allow formatting of HTS of genomic targets with no known enzymatic function, as well as of proteins whose activities are difficult to measure in a HTS assay. Both the academic and the industrial pharmaceutical scientist can benefit greatly from this technology.

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