Phage display-derived peptides as therapeutic alternatives to antibodies

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Peptide-based drugs are now viable alternatives to biopharmaceuticals, such as antibodies. Most of the past limitations of peptides have been removed by new technologies, so that peptides now face similar hurdles to antibodies. Phage-display technology provides novel peptides that bind protein targets with high affinity and specificity. Most marketed peptide-based drugs are receptor agonists derived from natural peptides. To address the need for antagonists, novel strategies have been developed for inhibiting receptor–ligand interactions. We review results from phage display in finding peptide drug candidates and conclude with some business benefits of developing peptides.

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▼ Most biopharmaceutical drugs act by binding to a protein target, thereby changing the behavior of the protein or the cell that bears it. Currently, human antibodies are popular candidates for development into therapeutics [1] because they are assumed to have the advantages of high affinity and specificity, favorable pharmacokinetics, large-scale producibility, acceptable toxicity, low antigenicity and predictable, albeit high, cost.

Through the use of phage display, it is now possible to find peptides that bind protein targets with high affinity and specificity, in some cases comparable with that of antibodies. Phage display involves producing libraries of peptides displayed on phage. These can contain as many as 10^{10} different peptides (far surpassing combinatorial small-molecule libraries) and can contain fixed residues that impose a structure on the variable residues. Selection from such libraries can rapidly provide dozens to hundreds of binders to many different sites on a target protein.

Peptides have several advantages over antibodies as drug candidates including: (a) lower manufacturing costs (synthetic versus recombinant production), (b) higher activity per mass (15–60-fold, assuming 75 kDa for one combining site of an antibody and 10–50 amino acids), (c) lower royalty stack than antibodies because of a simpler intellectual property landscape during discovery and manufacturing, (d) greater stability (lengthy storage at room temperature acceptable), (e) less chance of unintended interaction with the immune system (assuming the peptide contains no known immune-system signaling sequence), and (f) better organ or tumor penetration [2].

Peptides offer some of the advantages of small molecules. Because the peptides considered here comprise 10–50 amino acids, their interaction with protein targets can be highly specific, more so than small molecules. Control over the strength and extent of the peptide–target interaction is highly desirable when peptide therapeutics are called upon to block a protein–protein interaction. To make a peptide into a therapeutic, however, the biological effect, the pharmacokinetic profile and a lack of unwanted antigenicity must be made adequate.

Most peptide therapeutics on the market are agonists and are thus needed only in small quantities to activate their targeted receptor. In addressing cancer and inflammation, however, antagonists that prevent the activation of receptors involved in disease progression are most commonly sought. Many such receptors (e.g. the IL-1 receptor, IL-1R) are activated by binding protein or peptide ligands. In these situations, high specificity and affinity do not guarantee an effective antagonist. To have a biological effect, a peptide therapeutic must either cover the active site of the target or distort the target enough to deprive it of its usual activity. To address this issue, we and others have developed strategies to streamline the discovery of potent peptide antagonists using the phage-display technique (Figure 1).

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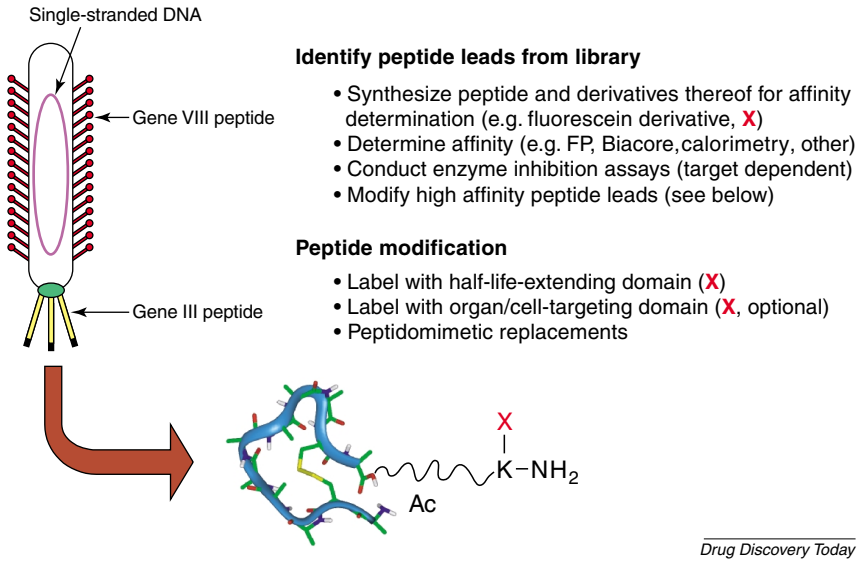

Figure 1. Phage or phagemid derived from M13 can display peptides on proteins III or VIII. DNA sequences of selected phage gives the amino-acid sequences of the binding peptides. Synthetic peptides can be blocked at the N-terminus with acetate and at the C-terminus by conversion to the amide. Linker groups, such as PEG, can extend the peptide at the C-terminus. An additional C-terminal lysine allows facile derivitization.

Selection of peptides

The phage-display technique can select peptides that bind protein targets with high affinity and specificity. For example, Huang *et al.* [3] reported selection of cyclic peptides that bind angiotensin-converting enzyme 2 (ACE2) from libraries displayed multiply on gIIIp of M13 phage. The selected peptides were grouped into families based on amino-acid sequence. The members of the family that contains the peptide DX-600 (GDYSH**CSPLRYYPWWKC**TYPDP, bold amino acids show the family motif; underscoring indicates disulfide ring closure) were complete inhibitors of ACE2 enzymatic activity, and the K_d of these peptides ranged from ~140–3 nM. Peptides having the **ALFCV(D/E)F**, **(F/Y)C(F/L/I)(D/E)F**, and **(D/E)C(E/D)W**XX(**F/W**) motifs showed weak or no inhibition. Presumably, each motif binds to a different site on ACE2.

Franklin *et al.* [4] used multimeric display on gVIIIp of M13 phagemids to find peptides that bind each of three members of the inhibitor of apoptosis (IAP) family. Melanoma IAP (ML-IAP) was the target; two IAP domains from XIAP were 'anti-targets'; hence, binding to the XIAP domains was to be avoided. The selected peptides have K_d values for ML-IAP in the range of 160–440 nM and some show modest selectivity among the three targets. Several peptide derivatives were synthesized; the best has K_d = 70 nM. From an X-ray structure of the peptide bound to the target, the authors designed a substitution with a non-encodable amino acid [(3S)-methyl-proline] that reduced K_d to 10 nM while improving specificity against XIAP. This process of replacing genetically encodable amino acids with other non-natural molecular fragments produces what is usually called a peptidomimetic. Such replacements often increase the plasma stability of peptides by preventing their cleavage by proteases [5]. Peptidomimetic replacements, therefore, are often a necessary step in making peptides into therapeutics.

In another study, Ashraf *et al.* [6] identified separate classes of peptides that bind to protein kinase Cα (PKCα), an intracellular target, only under activation conditions – that is, peptides that bind only to active and correctly folded PKCα and can thus be used as surrogate ligands in screening libraries of small molecules.

Hetian *et al.* [7] used the multiple display of peptides on gIIIp of M13 phage to find the peptide HTMYYHHYQHHL, which binds to the VEGF receptor kinase domain-containing receptor (KDR). This peptide slows the growth of breast carcinoma BICR-H1 tumors in mice. Oddly, the *in vitro* IC_{50} of this peptide is ~100 μM, but an *in vivo* dose of ~30 μM (60 μL of 500 μM peptide, assuming mouse blood volume of 1 mL) has an effect, but no pharmacokinetic data were given.

Karasseva *et al.* [8] found a peptide, p6.1, that binds to recombinant human ErbB-2 tyrosine kinase receptor, which is implicated in many human malignancies. Peptide p6.1 binds ErbB-2 with D_d = 30 μM and shows no detectable binding to three other proteins. Because the peptide can bind tumor cells that bear ErbB-2, they suggest this peptide could be developed into an imaging agent or therapeutic, although the binding constant of 30 μM would need to be improved. The affinity could be improved by making a phage-display library of peptides similar to p6.1 and selecting for high-affinity binding to ErbB-2.

Using libraries of between 10^9 and 10^{10} peptides, Dyax Corp. (http://www.dyax.com) and others have routinely found peptides with high specificity that bind target proteins with K_d values from 3nM–5 μM [3]. Most often, one or more families of related peptides are found. The common motif of one family can then be built into a secondary library and higher-affinity peptides selected. In most cases, these peptides have affinities in the range of 1–10 nM

[9,10]. Usually, several families of binders are obtained; of these, two or more families can often be found that do not compete for binding to the target, presumably because they bind different sites. When two molecules, such as peptides, that bind at non-overlapping sites are joined with a suitable linker, such as several units of PEG, the affinity of the heterodimer is usually much greater than the affinity of either component [11,12]. An added advantage of such a heterodimeric molecule is that it covers a larger area of the active site on the target and is more likely to interfere with protein–protein interactions. Interference with protein–protein interactions can also be fostered by using the natural ligand of the target or neutralizing antibodies during selection or screening. For example, if peptides that block interaction between IL-1R and IL-1 were wanted, one could select for peptides that bind IL-1R and use IL-1 to elute those that bind at the interface between the proteins. We have found that such heterodimeric peptides are much more efficient in blocking the binding of vascular endothelial growth factor (VEGF) to its receptor, KDR, than either of the component peptides [11,12]. Many tumors produce VEGF when they become anoxic and the interaction of VEGF and KDR on normal endothelial cells is an important event in angiogenesis [13].

Peptides can be selected in a manner that fosters finding binders to particular binding sites, for example sites that are involved in biological function.

Some researchers selected peptides that bind to biologically active sites by use of monoclonal antibodies that bind the active epitopes [14–16]. Others have used a natural ligand of the target to elute peptides meant to bind in the ligand-binding site of *Pseudomonas aeruginosa* MurC, an essential enzyme involved in the early steps of biosynthesis of peptidoglycan monomer [17–19]. Another method used competitive-binding studies with peptide 11 (CDPGYIGSR) or heparan sulfate that possessed the desired laminin-1-binding activity, to select for other peptides that bind at the same site [20]. The combination of selection and screening yielded several peptides that bind at the sites of interest. Ide *et al.* [21] sought peptides that bind H7 flagellin, a cell-surface protein of *Escherichia coli*; they captured phage that bind H7 flagellin-positive cells and released the flagellin-specific phage by competition with flagellin.

Engineering peptides

Many peptides have brief serum residences, usually owing to degradation or excretion. Rapid renal filtration can be reduced by PEGylation or by adding a group that adheres to serum albumin (SA) or other serum protein. Joining phosphate esters to the N-terminus of an anticoagulant peptide causes binding to SA and can increase the serum half-life by ~50-fold in rabbits; however, some masking of the potency of the modified peptide seems to occur [22]. Dennis *et al.* [23] showed that binding to SA causes peptides to have longer residency in blood. Koehler *et al.* [24] showed that uncharged albumin-binding non-peptide moieties attached to peptides could give these peptides longer half-lives. Other benefits can be derived; for example, de Serres *et al.* [25] report on the immunogenicity of thrombopoietin (TPO), AF15705, and GW395058. AF15705 is a TPO mimetic and GW395058 is the N-terminally PEGylated version of AF15705. Rabbits immunized with GW395058 did not raise antibodies that react with TPO, yet rabbits immunized with AF15705 did. Thus, PEGylation not only increased serum residence but also reduced immunogenicity. Outside the vaccine area, avoiding immunogenicity is vitally important for peptide drugs and biopharmaceuticals; a strong immune response at best eliminates the drugs effect and at worst causes serious toxicity.

Conversely, the cancer antigen MART-1(27–35), which was known to be an epitope for tumor-reactive cytotoxic T-lymphocytes (CTLs), has a very short half-life [26]. However, PEGylation of MART-1(27–35) at the N-terminus might allow the peptide to stay in the system long enough to act as a cancer vaccine. Brinckerhoff *et al.* proposed that use of PEGylation might increase the immunogenicity of the peptide and showed that *in vitro* binding of the peptide to tumor-reactive CTLs was not reduced by PEGylation but that half-life might be increased. If you are making a vaccine, cancer or otherwise, increased immunogenicity is a good thing.

Another method known to boost immunogenicity of peptides is coupling one peptide to a second arginine-glycine-aspartate (RGD)-containing peptide that binds cell-surface integrins, which greatly increases the immunogenicity of the first peptide [27]. This property might be put to good use if the first peptide were a part of a protein on cancer cells so that the immune system would attack the cancer.

Optimizing delivery

A few peptides have potential for organ-specific or cell-specific delivery. Penetratin, TAT, VP22, and other peptides have been used to deliver peptides, small molecules, and some small proteins intracellularly for inhibiting key cellular pathways, for example, those that play a role in cancer progression [28,29]. Targeting peptides have also been developed that deliver therapeutic peptides, proteins and small molecules to the brain [30]. Attaching PEG or adding an albumin-binding moiety can enhance serum residence

Table 1. Peptide drugs available on the market[a]

Product	Manufacturer	Year approved in USA	Indication(s)	Worldwide sales, 2002 (in million US$)
Humulin®	Lilly	1994	Diabetes	1004
Humalog® (human insulin)		1996		834
Lupron® (leuprolide)	TAP Pharmaceuticals	1985	Prostate tumor, endometriosis, fibrosis, precocious puberty	876
Zestril®	AstraZeneca	1988	Hypertension	877
Prinivil® (lisinopril)	Merck	1987	Congestive heart failure	480
Zoladex® (goserelin)	AstraZeneca	1989	Breast cancer, prostate cancer, endometriosis	794
Sandostatin® (octreotide)	Novartis	1988	Acromegaly, diarrhea	608
Miacalcin® (calcitonin)	Novartis	1991	Hypercalcemia, osteoporosis, Pagets disease	395
Integrilin® (eptifibatide)	Millenium	1998	Angina, myocardial infarction	304
Natrecor® (nesiritide)	Scios	2001	Congestive heart failure	107
Angiomax® (bivalirudin)	Medicines Company	2000	Angina	38

[a]This is a non-exhaustive list representing peptides that are currently marketed. Data obtained from: Investigational Drugs Database (IDdb [3]), available at http://www.iddb3.com, accessed 30 December, 2003; MedAdNews, May 2003 issue, available at http://www.medadnews.com, accessed December 30, 2003; FDA Orange Book Page. available at http://www.fda.gov/cder/ob, accessed 30 Dcember,2003; Scios Natrecor Page®, available at http://www.sciosinc.com, accessed 30 December, 2003; Medicines Company, Annual Report 2003, available at http://www.themedicinescompany.com, accessed 30 December, 2003.

and has been known to reduce immunogenicity in some, but not all, cases. Introducing non-encodable moieties can reduce the immune system's ability to respond. Although making peptide drugs is challenging, the reduction in cost realized via an increasing number of optimization techniques could be substantial.

Business and intellectual property advantages

Beyond the scientific advantages of peptides compared with antibodies, peptides' lower royalty stack confers business advantages. This results from the greater clarity in intellectual property surrounding peptide discovery. Peptides do not have the manufacturing-related royalty stack that exists with antibodies, (e.g. the Cabilly patents that claim methods for producing monoclonal antibodies from recombinant DNA [31]), nor do they require royalties to companies with proprietary vectors, strains and methods for GMP cell-based production. These royalties exist in addition to the antibody discovery-related royalty stack (e.g. from transgenic mouse, phage display or humanization-related intellectual property). However, peptides can have their own royalty stack if a royalty is owed on a technology for discovery or a technology to increase the peptide's half-life and stability. Although many peptides are relatively stable in plasma, they might need to be PEGylated or fused to a naturally occurring plasma protein, such as HSA or Fc region of IgG, to increase their half-life.

With the exception of royalties, the costs associated with peptide synthesis have decreased over time and many more manufacturers now offer large-scale GMP peptide synthesis. Although it takes 106 steps of chemical synthesis and 6–8 months to synthesize Fuzeon® (enfuvirtide) [32], the first peptide inhibitor on the market for HIV treatment, the development and eventual approval of this drug along with advances in peptide synthesis have helped reduce costs of large scale GMP peptide synthesis. Fuzeon® was discovered through a research agreement between Trimeris (http://www.trimeris.com) and Roche (http://www.roche.com) and is beneficial for HIV therapy because it blocks HIV-gp41-mediated fusion to cells [33]. Fuzeon® was launched in March 2003 and BioWorld recently reported Fuzeon® sales of US$18.3M in Q2 and Q3 of 2003, with an analyst estimate of US$250–US$500M at peak sales [34]. The higher activity per gram of peptide versus that of an antibody, combined with downstream advantages such as room-temperature storage, suggests that there is a lower cost of goods associated with peptides than that for antibodies. These features have led several biotechnology companies to pursue peptide therapeutics (see Table 1 for examples).

Although the number of peptide products on the market is still lagging behind the number of available antibodies, it is increasing and has recently exceeded ten. Supporting the view that peptides can be successful products, some of these have net sales in the US$100M to US$1B range (Table 1). The majority of marketed peptide products are peptide hormones or peptides that simulate their action (e.g. insulin). Nonetheless, peptides that are agonistic or antagonistic for receptors implicated in oncology and inflammation, peptides as antibiotics, or peptides that act as enzyme inhibitors in a variety of therapeutic indications are increasingly being tested for efficacy at the discovery and preclinical stages, suggesting that this class of drugs might soon occupy a larger niche in the marketplace.

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