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Bioactive Peptides from Libraries

Review

Chiara Falciani, Luisa Lozzi, Alessandro Pini, and Luisa Bracci*
Department of Molecular Biology
University of Siena
Via Fiorentina, 1
I-53100 Siena
Italy

Summary

New ligands for a variety of biological targets can be selected from biological or synthetic combinatorial peptide libraries. The use of different libraries to select novel peptides with potential therapeutic applications is reviewed. The possible combination of molecular diversity provided by combinatorial libraries and a rational approach derived from computational modeling is also considered. Advantages and disadvantages of different approaches are compared. Possible strategies to bypass loss of peptide bioactivity in the transition from ligand selection to in vivo use are discussed.

Introduction

Combination of a relatively small number of amino acids is the strategy used by nature to construct proteins, which are the most variable and adaptable molecules of life. No other molecule can be organized in such a large number of different structures and carry out so many different functions as proteins. Proteins are the principal mediators of molecular "crosstalking." Their extreme variability makes them suitable mediators of specific molecular interactions. Interactions between proteins, or between proteins and other molecules, mediate all the physiological and pathological phenomena of life and as such can be the targets of specific drugs to modify physiological or pathological events.

Evolution of chemical and "biological" technologies, for the production of peptides and proteins in the laboratory, has made it possible to use the same natural molecular approach to construct "new" peptide sequences, which recognize specific targets. The intrinsic variability of peptide sequences and their ability to specifically recognize other molecules can therefore be used to design peptide drugs.

The principle for selecting of new ligands for a variety of biological targets by means of combinatorial peptide libraries exploits the natural diversity of protein-protein and protein-ligand interactions. Different combinatorial approaches have been applied since the mid-'80s to generate molecular diversity through synthetic or phage-display libraries [1–3] of many different peptide sequences that can be used to select lead bioactive compounds for drug discovery and basic research.

Multiple peptide synthesis techniques provide the basis for the synthetic combinatorial library approach. The optimization of chemistry and the automation and miniaturization of solid-phase peptide synthesis enabled peptide libraries to be built using different solid supports, such as resin beads [1], pins [4], glass chips [5], tea bags [6], and cellulose membranes [7]. Peptide libraries can be used to "fish" active ligands from a large variety of structural combinations, making it possible to identify peptide mimotopes [8], which mimic the structure and function of a native protein. Peptide mimotopes, with a sequence generally different from that of native epitopes, can mimic antigen-antibody and protein-ligand binding sites in general.

Synthetic combinatorial peptide libraries have been used successfully to identify bioactive peptides, including antimicrobial peptides [9], opioid receptor antagonists [10], ligands for cell surface receptors [11], protein kinase inhibitors and substrates [12, 13], T cell epitopes [14, 15], peptides binding to MHC molecules [16], and peptide mimotopes of receptor binding sites [17].

Libraries of peptides displayed on biological surfaces, such as bacteriophage particles, have become a widely studied laboratory procedure for the identification of specific ligand molecules in research and drug discovery. The technology is based on an in vitro selection procedure in which a peptide gene is genetically fused to a bacteriophage coat protein, resulting in display of the peptide on the surface of the viral particle. From the mid-'80s, when the first phage peptide library was constructed, random peptide libraries displayed on phages have been used for a variety of biological and biotechnological applications, including ligands to target receptors [18, 19], specific ligands for DNA sequences [20], enzyme inhibitors [21, 22], peptides that mimic carbohydrate structures [23], protein-protein interfaces [24–28], and receptor binding sites [29, 30]. Phage-display libraries have led to selection of peptides against cancer cells [31–33] and against cancer-associated proteins [34]. In vivo screening of phage-display libraries has enabled identification of peptides that target a specific organ or tissue [35, 36] and that specifically bind to the endothelium of tumor blood vessels [37–39] and lymphatic vessels [40].

The combinatorial peptide library approach is mainly based on three methods. In one, peptide libraries are synthesized and cleaved from a solid support to be screened as free compounds [2]. In a second, synthetic combinatorial libraries of peptides are assayed on their solid support [1, 8]. The third method is based on phage display, which enables selection of clones of interest rather than screening, because large phage libraries can be panned against a target molecule by standard protocols (for reviews see [41] and [42]), allowing enrichment of only a few hundred specific phages that can easily be screened for positive ligands in a single test. Specific phages are then treated for DNA sequencing and peptide genes are revealed for subsequent chemical synthesis.

The development of technologies enabling construction of combinatorial libraries of a huge number of dif-

*Correspondence: braccil@unisi.it

ferent molecules posed the problem of quick and reliable methods for high throughput screening of active ligands.

The above three technologies for selection of peptide ligands fall into two groups: libraries that allow the selection of ligands in their final unlabeled and soluble form and those by which peptides are selected while still linked to their "support," either synthetic or biological, or to a labeling molecule.

Here we review the use of different libraries to select new peptides with potentially therapeutic applications. The drawbacks and advantages of different approaches are compared and we focus particularly on strategies to bypass loss of peptide activity during the different steps from ligand selection to in vivo use. We also look at the possibility of combining molecular diversity provided by combinatorial libraries and a rational approach derived from computational modeling, during different stages of novel peptide drug selection and optimization.

Generation of Molecular Diversity

Upgraded technologies for generating peptides by chemical synthesis or molecular biology techniques are generating an increasing number of different sequences. The simultaneous development of bioinformatics and computer modeling, plus the increasing availability of three-dimensional molecular structures, have increased the power and reliability of rational molecular design. New bioactive peptides can therefore be obtained by generating libraries containing the highest possible number of different sequences and then fishing for the desired function, or by rational design of an appropriate structure, hopefully with the desired function. These two opposite approaches can now be combined at different steps of the selection procedure and the relative contributions of rational and irrational selection depend on available knowledge about protein and peptide structure-function and may change at different steps of the process.

Synthetic Combinatorial Libraries

The first validation of the combinatorial approach was obtained using both synthetic [1, 2] and phage-display peptide libraries [43, 44] to identify B cell epitopes using well-defined monoclonal antibodies. The combinatorial approach identified peptides having no sequence homology with the native antigen, when antibodies, receptors, or other proteins that bind to conformational epitopes or active sites were used to screen the libraries [45, 46].

The first use of synthetic peptide libraries was reported by Geysen and colleagues in 1984 [4], using the pin method, and since then many papers describing different methods of synthesizing and screening peptide libraries have been published.

Synthetic combinatorial libraries, especially synthetic peptide libraries, are generally prepared by two different methods, the "divide, couple and recombine" (DCR) method [1, 2, 47], also known as "portion mixing"

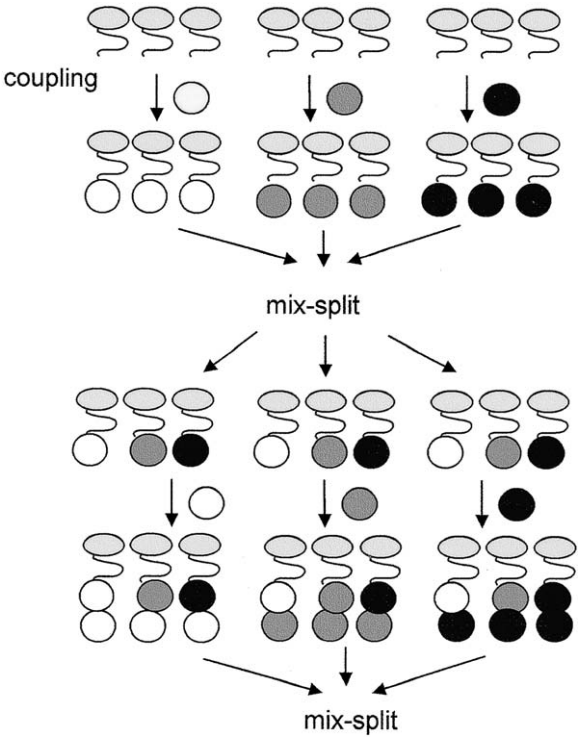

Figure 1. Portion Mixing Method for Synthetic Combinatorial Libraries

The resin is divided and recombined at every coupling step.

(Figure 1), and the "amino acid mixture" method [48]. The DCR method involves dividing resin into pools, coupling amino acids to individual aliquots of resin, mixing them and dividing them again for next amino acid coupling. This process generates "one-bead one-compound (OBOC)" libraries containing millions of random peptides [1], each bead expressing only one peptide and each peptide having equal distribution in the library.

Up to 10^8 different peptides, which can be simultaneously selected for a given target, can be synthesized by this technique, the main drawback being the identification of the peptide sequence of the selected bioactive bead. Microsequencing and mass spectrometry, performed directly on the beads, are possible approaches for peptide identification, together with chemical encoding, a method based on tagged peptides in which the tag enables the peptide sequence to be retraced.

In order to bypass the need for peptide sequencing, combinatorial peptide libraries with both randomized and defined positions can be produced using the amino acid mixture method. In mixture libraries, peptides are sequences of randomized (*X*) and defined (*O*) positions. *X* positions are obtained by coupling mixtures of amino acids in a predetermined molar ratio in order to have equal representation of each amino acid. Single defined amino acids are inserted in *O* positions. Identification of bioactive peptide sequences in mixture libraries is obtained by deconvolution.

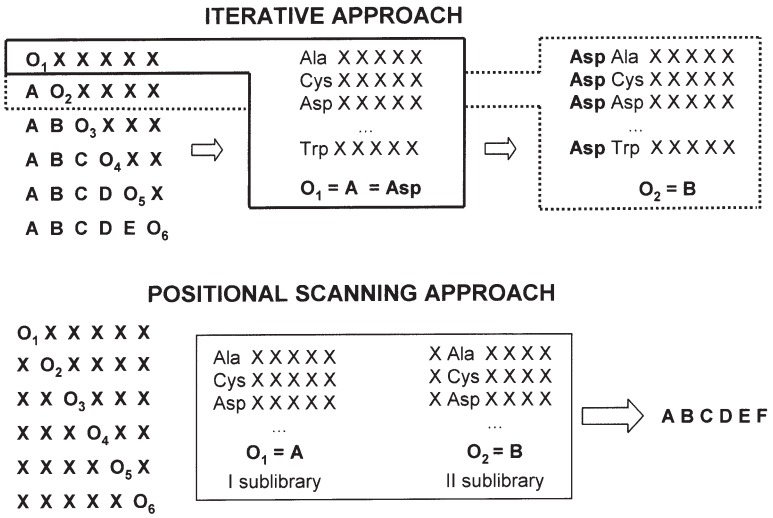

Figure 2. Descriptive Model of Iterative Process and Positional Scanning Deconvolution Approaches

See text for details.

There are two main deconvolution approaches: an iterative process [1, 2] and a positional scanning [49] (Figure 2). In the first approach a progressive selection is performed, choosing one amino acid at a time for each position. Sublibraries are generated on the basis of results of the previous one. In positional scanning all sublibraries can be assayed at the same time, because each variable position is tested independently of the others. In the case of positional scanning, the sequence is reconstructed at the end of the process, whereas in the iterative process the sequence is obtained step by step.

Deconvolution methods evaluate the contribution of each residue at each sequence position to biological activity. They can easily be used with arrays of peptide mixtures obtained using different supports. In particular, spatially addressable parallel peptide libraries, such as peptide libraries synthesized on solid support by SPOT synthesis, pin technology and peptide microarray, enable identification of the sequences of individual bioactive peptides by the deconvolution process, since each peptide mixture is well localized on the solid support. The large number of peptides that can be produced and screened simultaneously by this approach enables synthesis of complex libraries [50].

Dynamic combinatorial libraries (Figure 3) are a recent development of combinatorial chemistry [51]. Traditional combinatorial libraries consist of stable compounds, whereas dynamic combinatorial libraries are based on reversible reactions that produce "dynamic mixtures" in which reactants and products are present in thermodynamic equilibrium. The biological target of these mixtures selects the best binding structures and shifts the equilibrium of the reaction by subtracting the product.

Dynamic combinatorial libraries therefore do not have a deconvolution step and ligands are selected directly in the reaction mixture. Although innovative and promising, dynamic combinatorial libraries are so far limited to a small number of reversible reactions and libraries of moderate size.

In the "libraries from libraries" approach (Figure 4) [52], combinatorial libraries of peptides are built on a solid phase and are subsequently modified to maximize diversity: oxidations, reductions, alkylations, and acylations are performed, multiplying the number of new small organic compounds exponentially.

These second generation combinatorial libraries may give rise to more stable active compounds, but usually have different physical, chemical, and functional characteristics [53] from the first generation.

Biological Libraries

Molecular biology techniques make it possible to build polypeptide libraries expressed by different biological

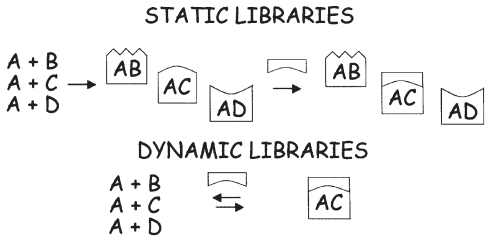

Figure 3. Dynamic Libraries

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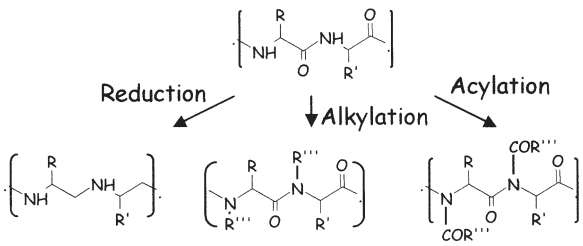

Figure 4. Example of Libraries from Libraries

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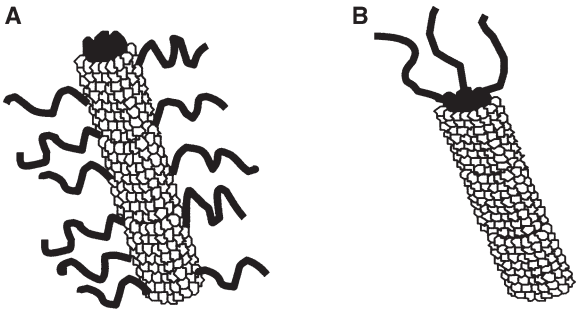

Figure 5. Peptides Displayed on M13 Phage Surface as Fusion to the Amino Terminus of pVIII (A) or pIII (B) Coat Proteins
Proportions are not observed in this picture.

entities including bacteria [54], phages, and ribosomes [55]. Biological peptide libraries are mainly based on phage exposition with peptides displayed by fusion with a viral coat protein. Many phages are used for displaying peptides [56, 57] but the most widely used is the filamentous bacteriophage M13. Peptides displayed on M13 virus can be exposed on the phage surface fused to the amino terminus of pIII or pVIII surface proteins (Figure 5).

Peptides fused to pIII usually give higher affinity ligands than peptides fused to pVIII, mainly due to the high avidity produced by the great number of pVIII copies (about 2700 copies, versus only 3–5 copies of pIII), which results in strong binding, even for peptides with low affinity.

In some cases, ligand affinity can also be predetermined (within a range) by modifying the stringency of selection protocols. Selections are usually performed using purified target molecules, although direct panning on cells [58, 59] and in vivo panning [60] have been described.

Target molecules (proteins, sugars, or fatty acids) are coated onto tubes (immunotubes) under conditions similar to those employed in ELISA [28, 61–64]. Phage libraries are then allowed to bind to the tube and specific ligands are eluted by pH change.

Low-density target coating and extensive washing of the tube enrich for high-affinity binders; high-density immunotube coating and less stringent washing procedures may otherwise be used to select low-affinity ligands. In some cases, the avidity of target molecule binding, associated with the multivalent display of binders on phages, makes it difficult to distinguish between medium- and high-affinity clones in the selection procedure. Selection schemes making use of streptavidin-coated magnetic beads allow the reaction of biotinylated molecules with phages in solution, followed by addition of streptavidin-coated beads. This decreases avidity effects to a large extent and makes it possible to dictate conditions for the selection of binders with suitable kinetic constants. In this case, however, a subset of the isolated binders is directed against streptavidin, rather than against the biotinylated antigen. The problem is typically negligible for large target molecules, but may be serious for small biotinylated molecules such as haptens. Recovery of binders to streptavidin can be eliminated by panning in the presence of a soluble excess of the streptavidin, or alternatively, by using target molecules biotinylated with biotin disulfide N-hydroxysuccinimide ester. This enables elution of positive phage ligands by incubation with dithiothreitol, which reduces the disulfide bridge between antigen and biotin, leaving the targeted protein bound to the phage particle without impairing the subsequent infection.

Another effective system made possible by phage display is selection by competition. Specific phage binders may be detached from the “antigen” by means of a competitor molecule, which binds the antigen naturally. This method was tested by us for the production of a nicotinic acetylcholine receptor scFv mimotope [65], but can be used for the general recovery of ligands interfering with binding of molecules, interaction of which triggers a pathological mechanism. An exhaustive example of this system is described by Mourez et al. [28] in which specific anti-anthrax toxin peptides are selected by incubating a phage library with the target molecule and eluting specific binders with competitive molecules.

Identification of a specific peptide sequence from a selected phage is possible because phage display libraries enable pairing of phenotype with genotype. This is possible because the phage carries an expression vector, termed phagemid, on which the peptide gene is encoded as a fusion gene with the viral coat protein. Sequencing of the peptide gene insert enables the amino acid sequence to be recognized.

The great advantage of phage libraries is the very large number of different peptide sequences (over 10^9) that can be displayed on viral surfaces. Moreover, phage libraries display their ligands in multiple copies due to the multiplicity of coat proteins to which they are fused. This, however, affects the binding capacity of peptides mounted on a phage and, in some cases, the corresponding synthesized monomeric peptide behaves differently when tested as a soluble molecule. Of course, only L-amino acid can be encoded and the construction of phage peptide libraries containing non-natural amino acids is not possible.

Selection of Active Compounds

Given the large number of different sequences that can be generated in peptide libraries, high-throughput screening is necessary to select novel peptides with the desired biological activity. Binding or functional assays can be used to screen synthetic or phage display peptide libraries, using a biological target, such as a protein, an enzyme, or a whole cell.

In phage display, ligand selection is divided into the two distinct steps of panning and screening. This makes it possible to combine the extremely high molecular diversity of these libraries with accessible biological tests for binding or for biological activity of selected clones.

SPOT synthesis of peptides on microarrays reveals direct links between peptide activity and sequence. Although phage peptide libraries have a higher molecular diversity, even compared to the most recent miniaturization of SPOT synthesis, they still require gene se-

quencing of selected clones, which is rather expensive and time consuming. The one-bead-one-peptide approach allows direct identification of active beads; however, an additional identification step of the active ligand sequence displayed on the bead is required after screening.

In the binding assays, target-ligand interactions can be detected by incubating immobilized peptide libraries with the target, which can be visualized taking advantage of suitable groups, such as enzymes, fluorescent probes, dyes, radio nuclides, or biotin. The peptide-bound target can be detected by colorimetric assays [66], fluorescent microscopy, or flow-cytometry [67].

Solid-phase-linked peptide libraries can also be screened using whole cells or microorganisms, such as a virus or bacterium and the cell-ligand interactions can be visualized directly by microscope.

High-throughput functional assays of enzyme activity have been used for the selection of substrates or inhibitors of kinases by radionuclide-based screening [12, 13] or by phage-display peptide phosphorylation [68] and for the selection of substrates of proteolytic enzymes by a fluorescence-quenching approach [69], using peptides immobilized on different solid supports [70].

The major drawback shared by all peptide libraries, in which selection of bioactive ligand is performed on support-linked peptides, is that the activity of the selected sequences, once these are synthesized as soluble compounds, may be different from that of the immobilized peptides. Moreover, the screening of support-linked synthetic libraries and phage display libraries can be affected by multivalent binding and thus give little information on the real affinity of the final soluble peptides. On the other hand, when performing high-throughput screening of free peptides in solution, automation, miniaturization, and very sensitive detection methodologies are required. Different assays such as ELISA, cell-based cytotoxic assay, antimicrobial assay, affinity chromatography, radiometric and fluorescence-based assays can be used.

ELISA screening of compounds is now using 384-well microplates, or larger, handled automatically. Increased sensitivity of the fluorescence and radioactivity detection technologies is therefore required as assay volume decreases [71, 72].

New fluorescence-based detection methods have been developed with improved sensitivity and selectivity. Examples of the most widely used are fluorescence resonance energy transfer (FRET), fluorescence polarization (FP), and fluorescence correlation spectroscopy (FCS) [71]. FRET is based on energy transfer between appropriate energy donor and acceptor molecules. It is used in monitoring enzyme activity: typically, a short peptide corresponding to the sequence for a natural cleavage site of the enzyme is synthesized and labeled at opposite ends with appropriate donor and quencher molecules. Before cleavage, the donor and quencher are very close and the effective fluorescence detected is low; once the two parts drift apart the fluorescent signal increases.

FP experiments allow measurement of changes in the emitted light intensity of small labeled probes on binding to larger molecules: the sample is excited with polarized light and when a binding equilibrium is established, the observed polarization of the emitted light increases.

FCS is an emerging detection technique for high-throughput screening: measurements are carried out using confocal optics to provide the highly focused excitation light and background rejection required for miniaturized samples. This technique is used to monitor binding interactions as well as other molecular events, since binding of a fluorescent probe to a molecule results in a change in its effective light emission.

Mass spectrometry [73] and BIACORE [74] are powerful techniques for screening soluble and unlabeled peptides. Particularly, BIACORE allows real-time detection of soluble peptide binding not only to proteins but to different biomolecules, like nucleic acids or sugars and also to cell membranes or whole cells. Moreover, the BIACORE allows the one-step measurement of kinetic rates and affinity of binding, thus providing an affinity ranking of functional peptides. This approach was used by our group to select peptide mimotopes of the nicotinic receptor ligand site [17, 75].

Combining Molecular Diversity with Rational Design

Structure-based molecular design and construction of molecular diversity are not mutually exclusive, but can be combined for the development of new specifically targeted drugs. The availability of structure-function information on the molecular components of the targeted biological reaction can be used for rational design of bioactive peptides. A rational approach can be the first step for generation of new bioactive peptides and can be combined, from the beginning of the process, with the molecular diversity offered by peptide libraries. Structure-function information can be used to limit the number of variables in a positional scanning library, thus enabling synthesis of libraries composed of longer peptides [17] or to design structurally constrained chemical [76, 77] or biological [78] libraries. On the other hand, multiple peptide synthesis can increase the number of computational analysis-derived sequences and structures to be tested as possible active compounds. As a general rule, the rational approach can help limit “randomness,” whereas generation of molecular diversity can help extend the range of rational approaches.

The step in which combination of rational design and generation of molecular diversity is generally most effective is the optimization of lead compounds. Optimization of the activity of a peptide lead compound involves careful analysis of the correlation between peptide sequence, and when possible active structure, and activity, pinpointing critical positions and residues. The most commonly used experimental techniques are systematic residue replacement (such as alanine scanning) and progressive deletion of peptide sequence. Definition of peptide active three-dimensional structure is obviously additional important information, which may enable accompanying experimental information by computer modeling. Once information has been gathered on peptide functional constraints, subsequent peptide libraries can be generated with the aim of obtaining second generation products with increased activity. Even at this stage, a combination of rational ap-

proach and generation of diversity can be used. The ratio between the two apparently opposite procedures depends first of all on availability of structure-function information on the peptide and its target. Amino acids not critical for activity can be replaced with others, generally the 20 natural amino acids, but nonnatural amino acids can also be included. Alternatively, molecular modeling can help reduce variability, giving indications about more suitable replacements in noncritical or even critical positions. Two alternative pathways for obtaining high-affinity bioactive peptides using a combination of computer modeling and molecular diversity have been proposed [75, 79].

Rational optimization of lead peptides can include introduction of structural constraints and generation of peptides with higher affinity that are also more stable to biological degradation [79, 80].

A combination of random selection, usually starting from phage display libraries, and further optimization of leads by rational design and/or chemical synthesis of molecular variants has been used for selection of bioactive products for a wide range of applications including peptide inhibitors of β-lactamase [81], human P-selectin antagonists [82], α4β7 integrin antagonists [83], peptide antagonists of Grb2-SH2 domain [84], and many others.

Phage-derived lead peptides can be optimized by mutating the sequence of the original peptide by introducing DNA substitutions, insertions, and deletions to construct new phage display sublibraries that are then panned against the target molecule. We cite two exhaustive examples in which (1) alanine scanning mutagenesis was performed by constructing new minilibraries of mutated peptides displayed on a phage [85] and (2) improved mimetic properties of peptides was obtained by generating new phage libraries, using 39-random mutagenic oligonucleotides, by a procedure that resembles natural immune system affinity maturation [86].

An essentially similar combination of molecular diversity and rational design can be applied when the lead peptide is a natural endogenous peptide, the functional properties of which can be mimicked or inhibited by a novel bioactive peptide. Important examples of strategies that can be applied in such a case come from mimicry of enkephalins [87]. The recent discovery that membrane receptors for several endogenous regulatory peptides are reexpressed or overexpressed in certain tumors has dramatically amplified research on novel bioactive peptides derived from natural sequences for tumor targeting and diagnosis [88].

Maintenance of Peptide Bioactivity from Selection to In Vivo Use

A limitation of peptide combinatorial libraries is that selected biologically active ligands that are L-peptides are not suitable as potential therapeutic drugs, mainly due to their short half-life in vivo because of rapid proteolytic cleavage. Several strategies have been used to preserve peptide bioactivity in the transition from the peptide selection by synthetic or phage libraries to use in vivo.

They include introduction of D- [89] or unnatural amino acids, introduction of structural constraints, cyclization, or conversion of lead peptides into small molecules [90] or into peptidomimetics [91] including in this term either amide bond mimetics or nonpeptide structures that mimic the binding interactions of the related peptides.

Nonnatural amino acids libraries can be obtained either by coupling nonnatural amino acids in standard peptide chemistry or by modifying all L-amino acid peptides by N-alkylation or acylation to produce stable linear or cyclized analogs. Structural constraints are usually introduced to stabilize active conformers and then to enhance bioactivity of compounds. In addiction, constraints have the advantage of stabilizing peptides against proteolytic action. In constrained libraries, peptide secondary structure is blocked in an "active conformation." For this purpose, libraries may be built on constrained scaffolds in order to display, for example, a β-turn (Figure 6). Intramolecular cyclization is another way of preserving peptides in a particular conformation, together with disulfide bond formation (Figure 6). All these techniques have been successfully used for identifying active ligands [92, 93]. The drawback of these approaches is that the biological activity of peptides can be profoundly modified by such structural modifications.

In previous studies we showed that the Multiple Antigen Peptide (MAP) [94, 95] dendrimeric forms of peptide mimotopes of the nicotinic receptor binding site were strong antidotes for the neurotoxin α-bungarotoxin, whereas related monomeric peptide mimotopes were not effective in vivo, despite in vitro activity identical to the corresponding dendrimers [75, 96]. The remarkably higher in vivo efficiency of the dendrimeric form with respect to the monomeric peptide was due to acquired resistance to protease and peptidase activity [97].

Our results, obtained comparing peptides of different origin and length, confirmed that the synthesis of bioactive peptides in MAP dendrimeric form can be a general method of imparting resistance to protease and peptidase activity, resulting in a dramatically increased half-life. All the dendrimeric peptides we tested retained the full biological activity of the native peptide [97].

Once produced as soluble compounds, peptides selected by phage display or solid phase-linked libraries can again present the problem of the maintenance of biological activity. This problem can be circumvented by synthesizing selected peptides in MAP form. Peptides in this form retain the full biological activity of the selected phage or support-linked peptide. This may be due to similarity between the MAP dendrimeric forms and phage or solid phase-linked peptides. Phage and solid phase-linked peptide libraries display peptides with the same orientation, from C-terminal to N-terminal, as in MAPs, where the peptide sequences are linked to the lysine core by their C-terminal (Figure 7). Moreover, the MAP molecule contains many peptide copies, allowing multivalent binding and increased binding efficiency, as for phage and solid phase-linked peptides.

This suggests that synthesis of peptides in MAP form can be a general strategy for maintaining biological activity in the transition from solid-phase or phage selection of peptide sequences to production of peptides

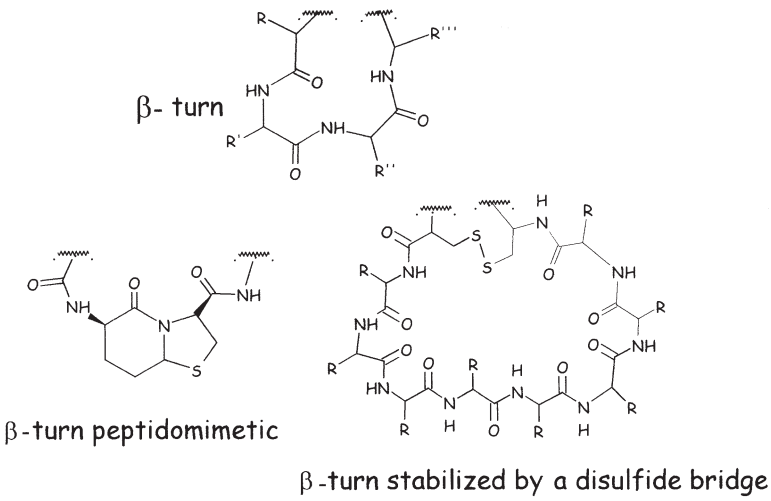

Figure 6. Examples of Constrained Structures

See text for details.

as soluble compounds. Moreover, MAPs are stable to peptidases and proteases, and this can be of primary importance in the development of new peptide drugs.

The use of peptides as drugs has not only been limited by their natural susceptibility to proteolytic degradation, but also by the general difficulty of delivery inside cells. Peptides generally have a limited ability to cross the plasma membrane, with the notable exception of antimicrobial peptides and cell-penetrating peptides (CPP) [98], like Tat, penetratin, and VP22. Most CPP identified so far are cationic sequences, derived from natural viral or animal sequences, or identified by chemical synthesis. They share the ability to cross cell membranes and work as carriers, transporting peptides, proteins, and even larger molecules or particles inside cells. CPP have largely been used to deliver peptides to intracellular targets [98]. The drawback of irreversible conjugation of peptides to CPP is the possible intracellular mislocalization of peptides, driven by the CPP sequence itself. This was recently solved in an elegant manner by synthesis of intracellular acting synthetic peptides, reversibly conjugated to Tat [99].

Future Perspectives

Peptides are versatile molecules with enormous potential for therapeutic applications. Their small size, compared to that of macromolecules like antibodies, enhances their tissue permeability. They are readily prepared by chemical synthesis and are easily modified and linked to different functional moieties.

Molecular diversity can be generated by biological or synthetic combinatorial peptide libraries combined with rational molecular design, to select lead bioactive peptides for a variety of biological targets. The combination of these two approaches can take place at different steps in the selection process and the preponderance of rational or irrational depends on the availability of structural-functional information on the peptide and its target.

The main limit to the use of peptides as drugs seems to be their short half-life due to proteolysis.

Several strategies can be used to maintain the peptide bioactivity in the transition from peptide selection by combinatorial libraries to use in vivo; however, the biological activity of second generation compounds may be profoundly altered. The synthesis of selected bioactive peptides in MAP dendrimeric form can be an alternative method of conserving biological activity by inducing resistance to degradation by proteases and peptidases.

MAPs composed only of natural amino acids but with a longer half-life than natural peptides have major applications as therapeutic drugs. An important possible application of protease-resistant MAPs may be in tumor therapy and diagnosis. Endogenous regulatory peptides, the receptors of which are overexpressed in different types of cancer, can be prepared and used in MAP form as tumor receptor-targeting agents.

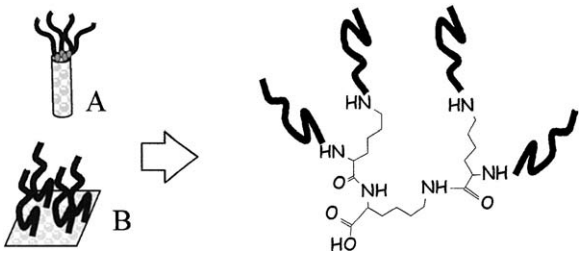

Figure 7. Schematic Representation of the Similarity of MAP to Phage Displayed (A) and Membrane Bound (B) Peptides

See text for details.

References

1. Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.J., Kazmierski, W.M., and Knapp, R.J. (1991). A new type of synthetic peptide library for identifying ligand-binding activity. Nature *354*, 82–84.
2. Houghten, R.A., Pinilla, C., Blondelle, S.E., Appel, J.R., Dooley, C.T., and Cuervo, J.H. (1991). Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. Nature *354*, 84–86.
3. Smith, G.P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science *228*, 1315–1317.

4. Geysen, H.M., Meloen, R.H., and Barteling, S.J. (1984). Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA *81*, 3998–4002.
5. Fodor, S.P., Read, J.L., Pirrung, M.C., Stryer, L., Lu, A.T., and Solas, D. (1991). Light-directed, spatially addressable parallel chemical synthesis. Science *251*, 767–773.
6. Houghten, R.A. (1985). General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proc. Natl. Acad. Sci. USA *82*, 5131–5135.
7. Frank, R. (1992). Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron *48*, 9217–9232.
8. Geysen, H.M., Rodda, S.J., and Mason, T.J. (1986). A priori delineation of a peptide which mimics a discontinuous antigenic determinant. Mol. Immunol. *23*, 709–715.
9. Blondelle, S.E., Perez-Paya, E., and Houghten, R.A. (1996). Synthetic combinatorial libraries: novel discovery strategy for identification of antimicrobial agents. Antimicrob. Agents Chemother. *40*, 1067–1071.
10. Dooley, C.T., Ny, P., Bidlack, J.M., and Houghten, R.A. (1998). Selective ligands for the mu, delta, and kappa opioid receptors identified from a single mixture based tetrapeptide positional scanning combinatorial library. J. Biol. Chem. *273*, 18848–18856.
11. Bae, Y.S., Park, E.Y., Kim, Y., He, R., Ye, R.D., Kwak, J.Y., Suh, P.G., and Ryu, S.H. (2003). Novel chemoattractant peptides for human leukocytes. Biochem. Pharmacol. *66*, 1841–1851.
12. Songyang, Z., Carraway, K.L., 3rd, Eck, M.J., Harrison, S.C., Feldman, R.A., Mohammadi, M., Schlessinger, J., Hubbard, S.R., Smith, D.P., and Eng, C. (1995). Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. Nature *373*, 536–539.
13. Wu, J., Ma, Q.N., and Lam, K.S. (1994). Identifying substrate motifs of protein kinases by a random library approach. Biochemistry *33*, 14825–14833.
14. Hernandez, J., Schoeder, K., Blondelle, S.E., Pons, F.G., Lone, Y.C., Simora, A., Langlade-Demoyen, P., Wilson, D.B., and Zanetti, M. (2004). Antigenicity and immunogenicity of peptide analogues of a low affinity peptide of the human telomerase reverse transcriptase tumor antigen. Eur. J. Immunol. *34*, 2331–2341.
15. La Rosa, C., Krishnan, R., Markel, S., Schneck, J.P., Houghten, R., Pinilla, C., and Diamond, D.J. (2001). Enhanced immune activity of cytotoxic T-lymphocyte epitope analogs derived from positional scanning synthetic combinatorial libraries. Blood *97*, 1776–1786.
16. Nino-Vasquez, J.J., Allicotti, G., Borras, E., Wilson, D.B., Valmori, D., Simon, R., Martin, R., and Pinilla, C. (2004). A powerful combination: the use of positional scanning libraries and biometrical analysis to identify cross-reactive T cell epitopes. Mol. Immunol. *40*, 1063–1074.
17. Bracci, L., Lozzi, L., Lelli, B., Pini, A., and Neri, P. (2001). Mimotopes of the nicotinic receptor binding site selected by a combinatorial peptide library. Biochemistry *40*, 6611–6619.
18. Yanofsky, S.D., Baldwin, D.N., Butler, J.H., Holden, F.R., Jacobs, J.W., Balasubramanian, P., Chinn, J.P., Cwirla, S.E., Peters-Bhatt, E., Whitehorn, E.A., et al. (1996). High affinity type I interleukin 1 receptor antagonists discovered by screening recombinant peptide libraries. Proc. Natl. Acad. Sci. USA *93*, 7381–7386.
19. Cwirla, S.E., Balasubramanian, P., Duffin, D.J., Wagstrom, C.R., Gates, C.M., Singer, S.C., Davis, A.M., Tansik, R.L., Mattheakis, L.C., Boytos, C.M., et al. (1997). Peptide agonist of the thrombopoietin receptor as potent as the natural cytokine. Science *276*, 1696–1699.
20. Isalan, M., Patel, S.D., Balasubramanian, S., and Choo, Y. (2001). Selection of zinc fingers that bind single-stranded telomeric DNA in the G-quadruplex conformation. Biochemistry *40*, 830–836.
21. Hyde-DeRuyscher, R., Paige, L.A., Christensen, D.J., Hyde-DeRuyscher, N., Lim, A., Fredericks, Z.L., Kranz, J., Gallant, P., Zhang, J., Rocklage, S.M., et al. (2000). Detection of small-molecule enzyme inhibitors with peptides isolated from phage-displayed combinatorial peptide libraries. Chem. Biol. *7*, 17–25.
22. Dennis, M.S., Eigenbrot, C., Skelton, N.J., Ultsch, M.H., Santell, L., Dwyer, M.A., O'Connell, M.P., and Lazarus, R.A. (2000). Peptide exosite inhibitors of factor VIIa as anticoagulants. Nature *404*, 465–470.
23. Lesinski, G.B., Smithson, S.L., Srivastava, N., Chen, D., Widera, G., and Westerink, M.A. (2001). A DNA vaccine encoding a peptide mimic of Streptococcus pneumoniae serotype 4 capsular polysaccharide induces specific anti-carbohydrate antibodies in Balb/c mice. Vaccine *19*, 1717–1726.
24. Ferrer, M., and Harrison, S.C. (1999). Peptide ligands to human immunodeficiency virus type 1 gp120 identified from phage display libraries. J. Virol. *73*, 5795–5802.
25. Li, F., Dluzewski, A., Coley, A.M., Thomas, A., Tilley, L., Anders, R.F., and Foley, M. (2002). Phage-displayed peptides bind to the malarial protein apical membrane antigen-1 and inhibit the merozoite invasion of host erythrocytes. J. Biol. Chem. *277*, 50303–50310.
26. Fairlie, W.D., Uboldi, A.D., Hemmings, G.J., Smith, B.J., Martin, H.M., Morgan, P.O., and Baca, M. (2003). A family of leukemia inhibitory factor-binding peptides that can act as antagonists when conjugated to poly(ethylene glycol). Biochemistry *42*, 13193–13201.
27. Norris, J.D., Paige, L.A., Christensen, D.J., Chang, C.Y., Huacani, M.R., Fan, D., Hamilton, P.T., Fowlkes, D.M., and McDonnell, D.P. (1999). Peptide antagonists of the human estrogen receptor. Science *285*, 744–746.
28. Mourez, M., Kane, R.S., Mogridge, J., Metallo, S., Deschatelets, P., Sellman, B.R., Whitesides, G.M., and Collier, R.J. (2001). Designing a polyvalent inhibitor of anthrax toxin. Nat. Biotechnol. *19*, 958–961.
29. Balass, M., Katchalski-Katzir, E., and Fuchs, S. (1997). The alpha-bungarotoxin binding site on the nicotinic acetylcholine receptor: analysis using a phage-epitope library. Proc. Natl. Acad. Sci. USA *94*, 6054–6058.
30. Chirinos-Rojas, C.L., Steward, M.W., and Partidos, C.D. (1998). A peptidomimetic antagonist of TNF-alpha-mediated cytotoxicity identified from a phage-displayed random peptide library. J. Immunol. *161*, 5621–5626.
31. Shadidi, M., and Sioud, M. (2003). Identification of novel carrier peptides for the specific delivery of therapeutics into cancer cells. FASEB J. *17*, 256–258.
32. Takahashi, S., Mok, H., Parrott, B., Marini, F.C., III, Andreeff, M., Brenner, M.K., and Barry, M.A. (2003). Selection of chronic lymphocytic leukemia binding peptides. Cancer Res. *63*, 5213–5217.
33. Oyama, T., Sykes, K.F., Samli, K.N., Minna, J.D., Johnston, S.A., and Brown, K.C. (2003). Isolation of lung tumor specific peptides from a random peptide library: generation of diagnostic and cell-targeting reagents. Cancer Lett. *202*, 219–230.
34. Pan, W., Arnone, M., Kendall, M., Grafstrom, R.H., Seitz, S.P., Wasserman, Z.R., and Albright, C.F. (2003). Identification of peptide substrates for human MMP-11 (stromelysin-3) using phage display. J. Biol. Chem. *278*, 27820–27827.
35. Pasqualini, R., and Ruoslahti, E. (1996). Organ targeting in vivo using phage display peptide libraries. Nature *380*, 364–366.
36. George, A.J., Lee, L., and Pitzalis, C. (2003). Isolating ligands specific for human vasculature using in vivo phage selection. Trends Biotechnol. *21*, 199–203.
37. Arap, W., Pasqualini, R., and Ruoslahti, E. (1998). Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science *279*, 377–380.
38. Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Stryhn, A., Ashmun, R.A., Shapiro, L.H., Arap, W., and Ruoslahti, E. (2000). Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res. *60*, 722–727.
39. Porkka, K., Laakkonen, P., Hoffman, J.A., Bernasconi, M., and Ruoslahti, E. (2002). A fragment of the HMGN2 protein homes to the nuclei of tumor cells and tumor endothelial cells in vivo. Proc. Natl. Acad. Sci. USA *99*, 7444–7449.
40. Laakkonen, P., Porkka, K., Hoffman, J.A., and Ruoslahti, E. (2002). A tumor-homing peptide with a targeting specificity related to lymphatic vessels. Nat. Med. *8*, 751–755.

41. Smith, G.P., and Scott, J.K. (1993). Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol. *217*, 228–257.
42. Pini, A., Giuliani, A., Ricci, C., Runci, Y., and Bracci, L. (2004). Strategies for the construction and use of peptide and antibody libraries displayed on phages. Curr. Protein Pept. Sci. *5*, 487–496.
43. Cwirla, S.E., Peters, E.A., Barrett, R.W., and Dower, W.J. (1990). Peptides on phage: a vast library of peptides for identifying ligands. Proc. Natl. Acad. Sci. USA *87*, 6378–6382.
44. Scott, J.K., and Smith, G.P. (1990). Searching for peptide ligands with an epitope library. Science *249*, 386–390.
45. D'Mello, F., Kairo, S.K., Howard, C.R., and Partidos, C.D. (2000). Mapping the specificity of an anti-feline immunodeficiency virus monoclonal antibody using a filamentous phage displayed peptide library. Vaccine *18*, 371–375.
46. Wrighton, N.C., Farrell, F.X., Chang, R., Kashyap, A.K., Barbone, F.P., Mulcahy, L.S., Johnson, D.L., Barrett, R.W., Jolliffe, L.K., and Dower, W.J. (1996). Small peptides as potent mimetics of the protein hormone erythropoietin. Science *273*, 458–464.
47. Furka, A., Sebestyen, F., Asgedom, M., and Dibo, G. (1991). General method for rapid synthesis of multicomponent peptide mixtures. Int. J. Pept. Protein Res. *37*, 487–493.
48. Ostresh, J.M., Winkle, J.H., Hamashin, V.T., and Houghten, R.A. (1994). Peptide libraries: determination of relative reaction rates of protected amino acids in competitive couplings. Biopolymers *34*, 1681–1689.
49. Pinilla, C., Appel, J.R., Blanc, P., and Houghten, R.A. (1992). Rapid identification of high affinity peptide ligands using positional scanning synthetic peptide combinatorial libraries. Biotechniques *13*, 901–905.
50. Kramer, A., Keitel, T., Winkler, K., Stocklein, W., Hohne, W., and Schneider-Mergener, J. (1997). Molecular basis for the binding promiscuity of an anti-p24 (HIV-1) monoclonal antibody. Cell *91*, 799–809.
51. Otto, S., Furlan, R.L., and Sanders, J.K. (2002). Recent developments in dynamic combinatorial chemistry. Curr. Opin. Chem. Biol. *6*, 321–327.
52. Nefzi, A., Ostresh, J.M., Yu, J., and Houghten, R.A. (2004). Combinatorial chemistry: libraries from libraries, the art of the diversity-oriented transformation of resin-bound peptides and chiral polyamides to low molecular weight acyclic and heterocyclic compounds. J. Org. Chem. *69*, 3603–3609.
53. Hoesl, C.E., Nefzi, A., Ostresh, J.M., Yu, Y., and Houghten, R.A. (2003). Mixture-based combinatorial libraries: from peptides and peptidomimetics to small molecule acyclic and heterocyclic compounds. Methods Enzymol. *369*, 496–517.
54. Benhar, I. (2001). Biotechnological applications of phage and cell display. Biotechnol. Adv. *19*, 1–33.
55. Amstutz, P., Forrer, P., Zahnd, C., and Pluckthun, A. (2001). In vitro display technologies: novel developments and applications. Curr. Opin. Biotechnol. *12*, 400–405.
56. Greenwood, J., Willis, A.E., and Perham, R.N. (1991). Multiple display of foreign peptides on a filamentous bacteriophage. Peptides from Plasmodium falciparum circumsporozoite protein as antigens. J. Mol. Biol. *220*, 821–827.
57. Sternberg, N., and Hoess, R.H. (1995). Display of peptides and proteins on the surface of bacteriophage lambda. Proc. Natl. Acad. Sci. USA *92*, 1609–1613.
58. de Kruif, J., Terstappen, L., Boel, E., and Logtenberg, T. (1995). Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc. Natl. Acad. Sci. USA *92*, 3938–3942.
59. Marks, J.D., Ouwehand, W.H., Bye, J.M., Finnern, R., Gorick, B.D., Voak, D., Thorpe, S.J., Hughes-Jones, N.C., and Winter, G. (1993). Human antibody fragments specific for human blood group antigens from a phage display library. Biotechnology (N. Y.) *11*, 1145–1149.
60. Coomber, D.W. (2002). Panning of antibody phage-display libraries. Standard protocols. Methods Mol. Biol. *178*, 133–145.
61. Thomas, C.J., Sharma, S., Kumar, G., Visweswariah, S.S., and Surolia, A. (2003). Biopanning of endotoxin-specific phage displayed peptides. Biochem. Biophys. Res. Commun. *307*, 133–138.
62. Mummert, M.E., Mohamadzadeh, M., Mummert, D.I., Mizumoto, N., and Takashima, A. (2000). Development of a peptide inhibitor of hyaluronan-mediated leukocyte trafficking. J. Exp. Med. *192*, 769–779.
63. Phalipon, A., Folgori, A., Arondel, J., Sgaramella, G., Fortugno, P., Cortese, R., Sansonetti, P.J., and Felici, F. (1997). Induction of anti-carbohydrate antibodies by phage library-selected peptide mimics. Eur. J. Immunol. *27*, 2620–2625.
64. Hafner, C., Samwald, U., Wagner, S., Felici, F., Heere-Ress, E., Jensen-Jarolim, E., Wolff, K., Scheiner, O., Pehamberger, H., and Breiteneder, H. (2002). Selection of mimotopes of the cell surface adhesion molecule Mel-CAM from a random pVIII-28aa phage peptide library. J. Invest. Dermatol. *119*, 865–869.
65. Bracci, L., Pini, A., Lozzi, L., Lelli, B., Battestin, P., Spreafico, A., Bernini, A., Niccolai, N., and Neri, P. (2001). Mimicking the nicotinic receptor binding site by a single chain Fv selected by competitive panning from a synthetic phage library. J. Neurochem. *78*, 24–31.
66. Lam, K.S., Lehman, A.L., Song, A., Doan, N., Enstrom, A.M., Maxwell, J., and Liu, R. (2003). Synthesis and screening of "one-bead one-compound" combinatorial peptide libraries. Methods Enzymol. *369*, 298–322.
67. Kessels, H.W., Ward, A.C., and Schumacher, T.N. (2002). Specificity and affinity motifs for Grb2 SH2-ligand interactions. Proc. Natl. Acad. Sci. USA *99*, 8524–8529.
68. Deng, S.J., Liu, W., Simmons, C.A., Moore, J.T., and Tian, G. (2001). Identifying substrates for endothelium-specific Tie-2 receptor tyrosine kinase from phage-displayed peptide libraries for high throughput screening. Comb. Chem. High Throughput Screen. *4*, 525–533.
69. Meldal, M., Svendsen, I., Breddam, K., and Auzanneau, F.I. (1994). Portion-mixing peptide libraries of quenched fluorogenic substrates for complete subsite mapping of endoprotease specificity. Proc. Natl. Acad. Sci. USA *91*, 3314–3318.
70. Rychlewski, L., Kschischo, M., Dong, L., Schutkowski, M., and Reimer, U. (2004). Target specificity analysis of the Abl kinase using peptide microarray data. J. Mol. Biol. *336*, 307–311.
71. Sundberg, S.A. (2000). High-throughput and ultra-high-throughput screening: solution- and cell-based approaches. Curr. Opin. Biotechnol. *11*, 47–53.
72. Gauglitz, G. (2000). Optical detection methods for combinatorial libraries. Curr. Opin. Chem. Biol. *4*, 351–355.
73. Wang, P., Fu, H., Snavley, D.F., Freitas, M.A., and Pei, D. (2002). Screening combinatorial libraries by mass spectrometry. 2. Identification of optimal substrates of protein tyrosine phosphatase SHP-1. Biochemistry *41*, 6202–6210.
74. Brinker, A., Scheufler, C., Von Der Mulbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and Hartl, F.U. (2002). Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70 x Hop x Hsp90 complexes. J. Biol. Chem. *277*, 19265–19275.
75. Lozzi, L., Lelli, B., Runci, Y., Scali, S., Bernini, A., Falciani, C., Pini, A., Niccolai, N., Neri, P., and Bracci, L. (2003). Rational design and molecular diversity for the construction of anti-alpha-bungarotoxin antidotes with high affinity and in vivo efficiency. Chem. Biol. *10*, 411–417.
76. Spatola, A.F., and Crozet, Y. (1996). Rediscovering an endothelin antagonist (BQ-123): a self-deconvoluting cyclic pentapeptide library. J. Med. Chem. *39*, 3842–3846.
77. Ogbu, C.O., Qabar, M.N., Boatman, P.D., Urban, J., Meara, J.P., Ferguson, M.D., Tulinsky, J., Lum, C., Babu, S., Blaskovich, M.A., et al. (1998). Highly efficient and versatile synthesis of libraries of constrained beta-strand mimetics. Bioorg. Med. Chem. Lett. *8*, 2321–2326.
78. Bonnycastle, L.L., Mehroke, J.S., Rashed, M., Gong, X., and Scott, J.K. (1996). Probing the basis of antibody reactivity with a panel of constrained peptide libraries displayed by filamentous phage. J. Mol. Biol. *258*, 747–762.
79. Kasher, R., Balass, M., Scherf, T., Fridkin, M., Fuchs, S., and Katchalski-Katzir, E. (2001). Design and synthesis of peptides that bind alpha-bungarotoxin with high affinity. Chem. Biol. *8*, 147–155.
80. Balass, M., Kalef, E., Fuchs, S., and Katchalski-Katzir, E. (2001). A cyclic peptide with high affinity to alpha-bungarotoxin pro-

tects mice from the lethal effect of the toxin. Toxicon *39*, 1045–1051.
81. Huang, W., Beharry, Z., Zhang, Z., and Palzkill, T. (2003). A broad-spectrum peptide inhibitor of beta-lactamase identified using phage display and peptide arrays. Protein Eng. *16*, 853–860.
82. Appeldoorn, C.C., Molenaar, T.J., Bonnefoy, A., van Leeuwen, S.H., Vandervoort, P.A., Hoylaerts, M.F., van Berkel, T.J., and Biessen, E.A. (2003). Rational optimization of a short human P-selectin-binding peptide leads to nanomolar affinity antagonists. J. Biol. Chem. *278*, 10201–10207.
83. Dubree, N.J., Artis, D.R., Castanedo, G., Marsters, J., Sutherlin, D., Caris, L., Clark, K., Keating, S.M., Beresini, M.H., Chiu, H., et al. (2002). Selective alpha4beta7 integrin antagonists and their potential as antiinflammatory agents. J. Med. Chem. *45*, 3451–3457.
84. Long, Y.Q., Lung, F.D., and Roller, P.P. (2003). Global optimization of conformational constraint on non-phosphorylated cyclic peptide antagonists of the Grb2–SH2 domain. Bioorg. Med. Chem. *11*, 3929–3936.
85. Nakamura, G.R., Reynolds, M.E., Chen, Y.M., Starovasnik, M.A., and Lowman, H.B. (2002). Stable "zeta" peptides that act as potent antagonists of the high-affinity IgE receptor. Proc. Natl. Acad. Sci. USA *99*, 1303–1308.
86. Urbanelli, L., Fortugno, P., Bartoli, F., Nuzzo, M., De Tomassi, A., Felici, F., and Monaci, P. (2000). "Affinity maturation" of ligands for HCV-specific serum antibodies. J. Immunol. Methods *236*, 167–176.
87. Shreder, K., Zhang, L., Dang, T., Yaksh, T.L., Umeno, H., DeHaven, R., Daubert, J., and Goodman, M. (1998). Synthesis and biological activity of a novel methylamine-bridged enkephalin analogue (MABE): a new route to cyclic peptides and peptidomimetics. J. Med. Chem. *41*, 2631–2635.
88. Schmitt, A., Bernhardt, P., Nilsson, O., Ahlman, H., Kolby, L., Maecke, H.R., and Forssell-Aronsson, E. (2004). Radiation therapy of small cell lung cancer with 177Lu-DOTA-Tyr3-Octreotate in an animal model. J. Nucleic Med. *45*, 1542–1548.
89. Dooley, C.T., Chung, N.N., Wilkes, B.C., Schiller, P.W., Bidlack, J.M., Pasternak, G.W., and Houghten, R.A. (1994). An all D-amino acid opioid peptide with central analgesic activity from a combinatorial library. Science *266*, 2019–2022.
90. Toogood, P.L. (2002). Inhibition of protein-protein association by small molecules: approaches and progress. J. Med. Chem. *45*, 1543–1558.
91. Eguchi, M., Shen, R.Y., Shea, J.P., Lee, M.S., and Kahn, M. (2002). Design, synthesis, and evaluation of opioid analogues with non-peptidic beta-turn scaffold: enkephalin and endomorphin mimetics. J. Med. Chem. *45*, 1395–1398.
92. Cochran, A.G., Tong, R.T., Starovasnik, M.A., Park, E.J., McDowell, R.S., Theaker, J.E., and Skelton, N.J. (2001). A minimal peptide scaffold for beta-turn display: optimizing a strand position in disulfide-cyclized beta-hairpins. J. Am. Chem. Soc. *123*, 625–632.
93. Becker, J.A., Wallace, A., Garzon, A., Ingallinella, P., Bianchi, E., Cortese, R., Simonin, F., Kieffer, B.L., and Pessi, A. (1999). Ligands for kappa-opioid and ORL1 receptors identified from a conformationally constrained peptide combinatorial library. J. Biol. Chem. *274*, 27513–27522.
94. Tam, J.P. (1988). Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA *85*, 5409–5413.
95. Tam, J.P. (2002). Peptide dendrimers: applications and synthesis. J. Biotechnol. *85*, 195–229.
96. Bracci, L., Lozzi, L., Pini, A., Lelli, B., Falciani, C., Niccolai, N., Bernini, A., Spreafico, A., Soldani, P., and Neri, P. (2002). A branched peptide mimotope of the nicotinic receptor binding site is a potent synthetic antidote against the snake neurotoxin alpha-bungarotoxin. Biochemistry *41*, 10194–10199.
97. Bracci, L., Falciani, C., Lelli, B., Lozzi, L., Runci, Y., Pini, A., De Montis, M.G., Tagliamonte, A., and Neri, P. (2003). Synthetic peptides in the form of dendrimers become resistant to protease activity. J. Biol. Chem. *278*, 46590–46595.
98. Trehin, R., and Merkle, H.P. (2004). Chances and pitfalls of cell penetrating peptides for cellular drug delivery. Eur. J. Pharm. Biopharm. *58*, 209–223.
99. Begley, R., Liron, T., Baryza, J., and Mochly-Rosen, D. (2004). Biodistribution of intracellularly acting peptides conjugated reversibly to Tat. Biochem. Biophys. Res. Commun. *318*, 949–954.