Drug discovery and vaccine development using mixture-based synthetic combinatorial libraries

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The approaches and concepts that encompass combinatorial chemistry represent a paradigm shift in drug discovery and basic research. Viewed initially as a curiosity by the pharmaceutical industry, combinatorial chemistry approaches are now recognized as essential drug discovery tools that decrease the time taken for discovery and increase the throughput of chemical screening by as much as 1000-fold. Although the use of mixture-based synthetic combinatorial libraries was one of the first approaches presented, its inherent strengths are only recently being recognized. Numerous mixture-based libraries of peptides, peptidomimetics and heterocycles have been synthesized and deconvoluted using the positional scanning approach. Mixture-based library approaches for drug discovery and vaccine development will be reviewed herein.

Combinatorial chemistry has fundamentally changed the conduct and scope of drug discovery. Synthetic combinatorial chemistry methods were first presented by Frank (simultaneous synthesis of large numbers of oligonucleotides)[1], Geysen (individual compounds chemically synthesized on plastic 'pins')[2] and Houghten (simultaneous multiple compound synthesis on polystyrene resin in polypropylene mesh packets – 'tea bags')[3]. All of these methods are based on Merrifield's pioneering work in solid-phase peptide synthesis[4]. The limiting factor in drug discovery screening remained the number of available individual compounds in virtually all studies, even with the advent of synthetic automation, regardless of whether the compounds required were peptides, peptidomimetics, or classical heterocycles or other small molecule organic compounds. It is noteworthy that Leznoff[5,6] and Rapoport[7] first carried out the synthesis of heterocycles on the solid phase in 1973 and 1976, respectively, but this powerful approach remained unappreciated until Ellman's work[8] in 1992.

The value of the parallel synthesis of large numbers of peptides using the tea bag approach was shown in a 1988 study by the authors' laboratory, in which over 500 analogs of the 23-residue antibacterial peptide magainin were prepared[9]. This increased synthetic capability (at the time, approximately tenfold greater for peptides) led to the successful clinical trial of the magainin analog Cytolex (MSI78) and other therapeutic peptides[10]. These approaches have also made possible a variety of basic research studies that were previously impractical, owing to economic or timeframe factors[11–13].

All combinatorial chemistry approaches, including the pin and tea bag methods for solid-phase synthesis, were first used to prepare peptides[2,3,14–17]. These and other

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solid-phase synthetic approaches are now widely used to prepare large individual compound libraries of virtually all types of compounds, including peptidomimetics[18–22], oligonucleotides[23,24], oligosaccharides[25–27] and heterocycles[8,28,29]. The widespread success of solid-phase synthesis is because reactions can be driven to completion (often >99.8%) on polymer supports, excess reagents or starting materials can be readily removed and it is suitable for automation. Despite the ten- to 100-fold increase in synthesis capability made possible by the pin and tea bag techniques, it soon became clear that the barrier in most studies remained the number of compounds that could be synthesized. This led to the synthesis and use of mixtures of compounds to increase the inherent throughput of synthetic and screening approaches.

Combinatorial methods

Over the past 15 years, a wide range of combinatorial methods have been developed and are currently being used for the identification of ligands that are important in drug discovery and basic research. Table 1 summarizes these synthesis and screening approaches, as well as the deconvolution methods used to identify individual compounds from the libraries. Informative references for these approaches are shown. The successful preparation of mixture-based combinatorial libraries, in combination with earlier parallel-array synthesis approaches, has now pushed aside many of the synthesis limitations that are inherent in the drug-discovery process. Although the utility of mixture-based combinatorial libraries is still questioned by some in the scientific community, it is clear from recent publications that scientific groups in industry and academia that had used parallel-array synthesis exclusively are now using mixture-based approaches.

The Molecular Design and Diversity group at Merck Research Laboratories (Rahway, NJ, USA) has identified selective agonists for the somatostatin receptor using a mixture-based small-molecule combinatorial library with iterative deconvolution[38,39]. In addition, they have reported the use of positional scanning libraries to define the substrate specificities for caspases and granzyme B (Refs 40,41). More recently, Ellman's group has reported the elucidation of substrate specificities for plasmin and thrombin using a fluorescent labeled tetrapeptide positional scanning library[42]. These recent publications, as well as several publications from more than 100 separate studies carried out by approximately 50 separate research groups (recently compiled[34]), reflect the increasing acceptance and the broad utility of mixture-based libraries. This review will concentrate primarily on our experiences using mixture-based combinatorial libraries and their deconvolution by positional scanning approaches in drug discovery and vaccine development.

Mixtures are not a new concept in drug discovery

The screening of natural product extracts is an accepted and productive source of therapeutic compounds. Such extracts are typically made up of hundreds to thousands of different compounds in varying concentrations. Highly active individual compounds, sometimes present in extracts at only one part per 100,000 or less, have been found in natural product extracts. Despite the fact that natural products and, indeed, the very nature of biological interactions are inherently mixture-based (and the fact that these interactions do not occur in environments made up of single compounds and single acceptors), an intense debate continues on the relative merits of the preparation and screening of mixture-based synthetic combinatorial libraries versus individual compound arrays of the same compounds. The central difference between the screening of individual compounds and synthetic mixtures is the balance between the time and cost necessary to acquire complete information about the activity of interest of every compound that makes up an individual compound array, and the ability to acquire a somewhat less complete information set very rapidly and cost effectively from the same compounds in a mixture-based library. Over the past 15 years, efforts from our laboratory, as well as dozens of other research groups, have demonstrated that the generation and use of mixture-based synthetic combinatorial libraries is an extremely effective, versatile and cost efficient means by which to generate highly active, therapeutically relevant compounds[34].

Table 1. Combinatorial library synthesis and screening

Method	Synthesis	Screening	Deconvolution	Refs
Parallel arrays	Solid- and solution-phase	Individual compounds	None required	1–3,30
One bead–one compound	Solid-phase	[a]On bead	Sequencing/LC-MS/tagging	31–33
Mixtures	Solid-phase	In solution	Iterative, positional scanning, LC-MS	34–37

[a] Compounds can also be cleaved from the beads and screened in solution.

Mixture synthesis

Divide–couple–recombine (split-mix) synthesis

An important factor in preparing mixture-based synthetic combinatorial libraries is the need to obtain as close to equimolar representation as possible of all individual compounds within the library. For peptides, therefore, the various amino acids must be incorporated into each of the library positions in a ratio as close to equimolar as possible. When using resin beads as the solid support for library synthesis, this can be achieved using a process labeled 'divide–couple–recombine'[35], 'split-and-mix synthesis'[31] or 'portioning mixing'[43]. This process, now commonly known as 'split-mix', involves the coupling of each protected amino acid to separate portions of resin, followed by combining and mixing all resin portions, before dividing the resin again for the next coupling step. By repeating this process, for example, for a total of five couplings, and using 20 amino acids as building blocks, a library of 3,200,000 (20^5) pentapeptides can be readily prepared. Owing to the physical separation of the resins prior to incorporating the individual amino acids, the split-mix process yields libraries that contain an individual, unique compound on each individual resin bead and, if cleaved from a mixture of resin beads, equimolar mixtures of the same compounds. If the peptides are not cleaved from the resin (the 'one bead, one compound' approach pioneered by Lam's group[31,32]), then a method of tagging is used to identify the active beads. The beads that have an active compound attached to them are typically visualized with a colorometric reagent and individually isolated from the other beads by their color. The active compound on the bead is then sequenced or identified by various tagging methods[33].

The split-mix method has also been used for the solid-phase synthesis of mixture-based libraries that have defined and mixture positions. The resulting resin-bound mixtures are then cleaved from their respective resins to generate equimolar mixtures of compounds that can be dissolved and used directly in solution with existing assays. The deconvolution of mixture-based libraries of this type was carried out using an iterative process[35]. A practical limitation when initially synthesizing mixture-based libraries is that the defined diversity positions can only be easily incorporated in the last steps of the synthesis.

Reagent-mixture method

The coupling of mixtures of incoming reagents, such as protected amino acids[44], is an alternative means for the introduction of mixture positions. Owing to the differences in coupling rates of the various amino acids or other reagents, coupling of an equimolar amino acid mixture to resin-bound amino groups produces non-equimolar incorporation of amino acids into the mixture positions. This will ultimately result in a highly non-equimolar distribution of individual compounds within the library. To overcome this problem, the ratio of protected amino acids within the coupling mixture is adjusted according to their different coupling rates, that is, the higher the coupling rate of a particular amino acid, the lower the concentration of that amino acid in the coupling mixture[44,45]. The desired equimolar ratios are established by adjusting the relative concentration of each amino acid according to its incorporation ratio after coupling of an equimolar amino acid mixture, as determined by amino acid analysis or reverse phase (RP)-HPLC. This concept can be applied equally well to mixtures of other incoming reagents such as aldehydes and carboxylic acids. The reagent-mixture method has also been applied to the synthesis of positional scanning combinatorial libraries of heterocyclic compounds, including cyclic ureas, cyclic thioureas[46] and bicyclic guanidine libraries[29,34].

Finding the needle in the haystack – deconvolution

Iterative approach

Geysen's early use of large peptide mixtures immobilized on pins, while clearly of conceptual importance, has not found widespread use. To deconvolute pin-immobilized peptide mixtures, Geysen used an iterative approach[45], as has been used for soluble libraries[35,47]. This approach steadily decreases the number of compounds per mixture while steadily defining successive positions. The iterative deconvolution of soluble (i.e. not immobilized on a solid support) mixture-based combinatorial libraries is a successful approach for the identification of a wide range of compounds (reviewed in Ref. 34). The primary limitation of iterative deconvolution is the cost and time associated with the need for repetitive synthesis and screening steps, typically equal to the number of variable positions.

Positional scanning approach

A means to gather information rapidly concerning all possible variable positions in a library was presented in 1992 (Refs 36,37). A conceptual illustration of the positional scanning deconvolution approach for a simple tripeptide combinatorial library is shown in Fig. 1. Thus, if four different amino acids are incorporated at each of the three diversity positions, 64 (4^3) individual peptides will be generated. If the same diversity is arranged as a positional scanning synthetic combinatorial library (PS-SCL), only 12 peptide mixtures (four separate mixtures for each of the three positions) need to be synthesized. Each of the three

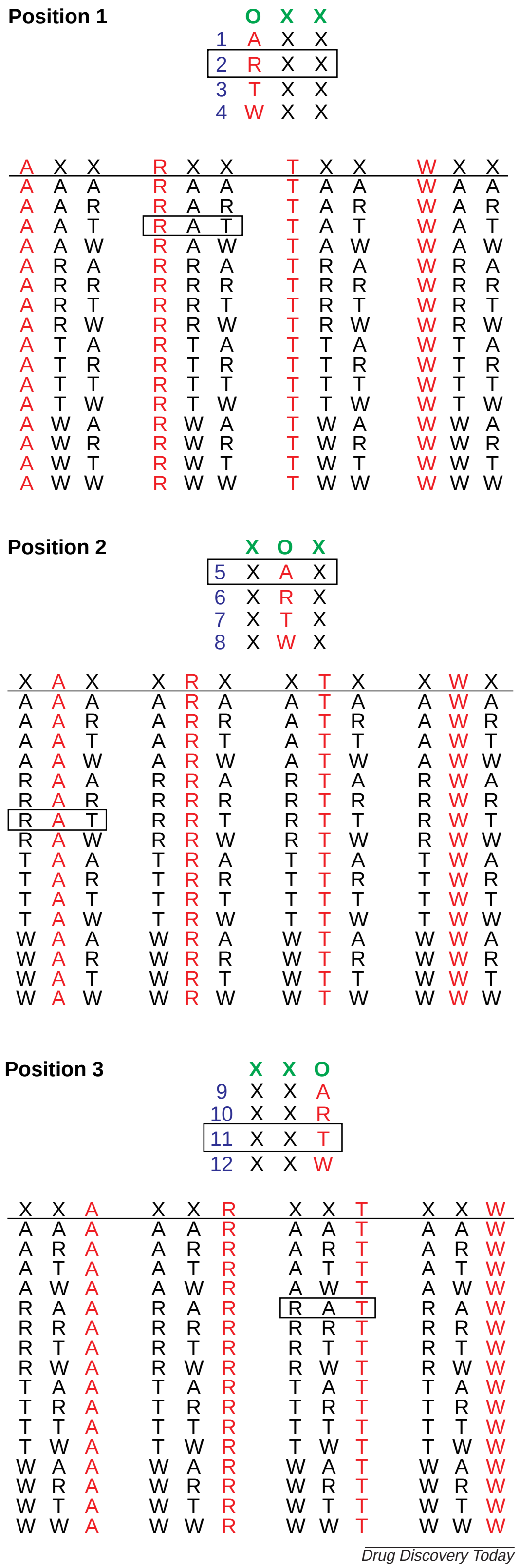

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Figure 1. *Illustration of the positional scanning format – tripeptide library. Abbreviations: O, single amino acid; X, equal mixture of A, R, T and W.*

separate positional sublibraries, namely OXX, XOX and XXO, contain exactly the same diversity of peptides, differing only in the location of the position defined. Each of the O positions are singularly and separately defined with one of the four amino acids, while the remaining two positions are mixtures (X) of the same four amino acids.

In this example, assume that the sequence RAT is the sole tripeptide that has activity. As each positional sublibrary contains the exact same diversity of peptides, the RAT tripeptide is present in all three positional sublibraries (Fig. 1). Thus, the only mixtures with activity will be RXX, XAX and XXT because the only active sequence, RAT, is present only in those mixtures. These three amino acids in their respective positions are the information resulting from the assay and indicate that in combination they should yield the tripeptide RAT, which can then be synthesized and tested for its individual activity. Note that the activity observed for each of the three mixtures (RXX, XAX and XXT) is due to the presence of the tripeptide RAT within each of these mixtures, and is not due to the individual amino acids (R, A and T) that occupy the defined positions. As expected, and found experimentally in more complex libraries, more than one mixture is typically found to have activity at each position. Selection of the building blocks for the synthesis of individual compounds is based first on the overall activity of the mixture and then on differences in the chemical character of the building block (to reduce the number of individual compounds to be made). Freier and coworkers have presented an excellent discussion of the theoretical and experimental aspects of iterative and positional scanning deconvolution[48,49].

When applicable, the use of mixtures of compounds in a PS-SCL format versus the use of individual compound arrays is clearly beneficial in terms of time and cost savings. This enables pharmaceutical and biotechnology companies, universities and research institutes to rapidly carry out basic research and the initial stages of the drug discovery process. Thus, previously unimagined numbers of compounds can be prepared and screened to yield highly active individual compounds.

To illustrate the power of mixture-based combinatorial libraries and deconvolution using the positional scanning approach, a published example will be summarized[50]. A mixture-based library composed of 200 mixtures, which represents 6.25 million tetrapeptides, was used to identify highly active and specific individual compounds in a κ-opioid radio-receptor binding assay. In this example, a library of tetrapeptides was prepared in which 50 different amino acids were incorporated at each of the four positions in the library (Fig. 2). This yields 6,250,000 tetrapeptides (50^4), but only 200 mixtures were prepared (50 separate mixtures were used to address each of the four positions) in which the 50 separate mixtures at each position were each made up of 125,000 different tetrapeptides (50^3). The inhibitory activities of each of the 50 mixtures for the four positions in a κ-opioid receptor-specific binding assay are shown in Fig. 3.

It can be seen that the profiles for each position are different, and clearly significant differences in activity exist between the active and inactive mixtures. After dose–response experiments in which IC_{50} values were determined for those mixtures that had a specific cutoff value (in this case 1/percent bound >0.15), the most active mixtures are selected. From these data, the combinations of the amino acids to be used at each position are chosen. This is typically limited by the importance of the project and the resources available. For example, if four amino acids are chosen for each position then 256 (4^4) different tetrapeptides would be prepared.

The individual activities of these 256 peptides are then determined. For the example presented here, 48 different peptides were synthesized and tested for κ receptor activity. The most active peptides are shown in Table 2. The most active tetrapeptides had an IC_{50} of 1 nM and were highly selective relative to μ and δ receptors (the other two primary opioid receptors). This series of tetrapeptides has been licensed to Ferring AG (San Diego, CA, USA) and is in development as a peripheral pain modulator and an anti-inflammatory drug. In addition, other highly active opioid compounds have been identified at the Torrey Pines Institute for Molecular Studies (San Diego, CA, USA) from small-molecule heterocyclic libraries (one example is described below in the peptidomimetic and heterocycle section). Examples of the use of mixture-based combinatorial libraries in a range of other studies have recently been compiled in an extensive review of mixture-based libraries[34].

O_1 X X X -NH_2
X O_2 X X -NH_2
X X O_3 X -NH_2
X X X O_4 -NH_2

(50 Different L-, D-, and unnatural amino acids)

O = individual X = mixture

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Figure 2. *Mixture-based combinatorial tetrapeptide library made up of 6,250,000 tetrapeptides.*

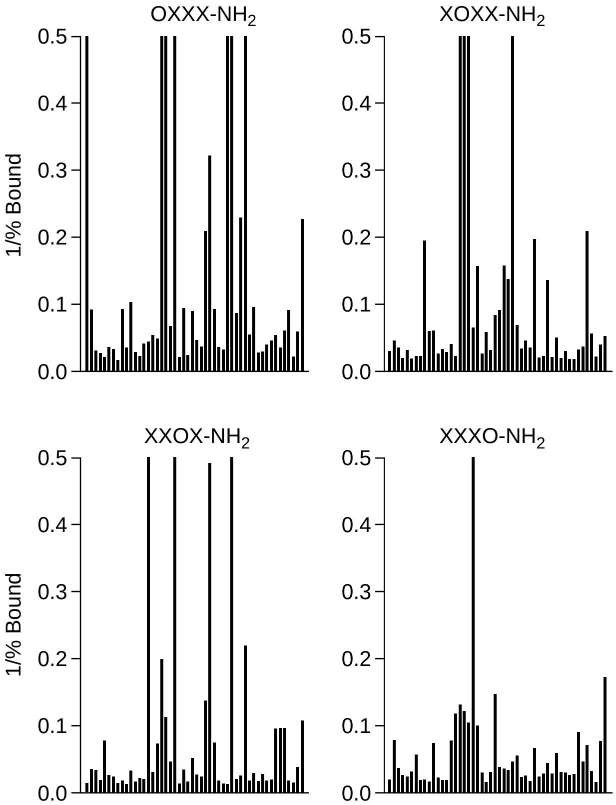

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Figure 3. *The use of the tetrapeptide library illustrated in Fig. 2 to inhibit binding in a κ-opioid radio-receptor specific binding assay. The κ receptor was labeled using [3H]U69593 and guinea pig brain homogenates. Each panel represents one of the four positional synthetic combinatorial libraries (SCLs; i.e. position one SCL is OXXX-NH_2). Each bar within a panel represents the activity (% bound^{-1}) of a peptide mixture defined in the O position with one of 50 amino acids.*

Identification of individual peptides, peptidomimetics and heterocycles from mixture-based combinatorial libraries

Peptide antigens and vaccine development

In immunology, the positional scanning approach was first applied to the study of antibody specificity and for the identification of cross-reactive epitopes. Many ligands for antibodies known to recognize peptides, proteins and carbohydrates have been identified using synthetic combinatorial libraries. A review of the studies carried out in the area of B-cell and T-cell epitope characterization was recently presented[51].

More recently, mixture-based combinatorial libraries made up of literally trillions of decapeptides have been

Table 2. Individual κ-opioid receptor-specific tetrapeptides identified from a mixture-based combinatorial library of 6,250,000 tetrapeptides

	κ U69593	μ DAMGO	δ DSLET
Sequence	**IC$_{50}$ (nM)**	**IC$_{50}$ (nM)**	**IC$_{50}$ (nM)**
DPhe - DNal - DNle - DArg -NH$_2$	1	2607	16,325
DPhe - DPhe - DNle - DArg -NH$_2$	2	42,963	>25,000
DNle - DNal - DIle - DArg -NH$_2$	2	3034	19,316
DPhe - DPhe - DIle - DArg -NH$_2$	2	>150,000	>25,000
DNle - DNal - DNle - DArg -NH$_2$	3	1709	>25,000
DPhe - DPhe - DNle - DCha -NH$_2$	6	15,000	28,932

used to identify and optimize ligands for T cells of clinical relevance in multiple sclerosis (MS)[52] and Lyme disease[53]. The T-cell clone TL3A6 was derived from an individual with MS and was known to recognize residues 89–98 of myelin basic protein (FFKNIVTPRT). The proliferative response of the clone to a decapeptide positional scanning library is shown in Fig. 4. It can be seen that several mixtures are active while other mixtures are not. Additionally, for eight out of the ten positions, the defined amino acid of one of the top three most active mixtures corresponded to an amino acid in the known antigen. These screening results were used to design 36 different peptides, and the most active are shown in Table 3. Fourteen of these peptides, despite having multiple amino acid differences from the native antigen, were able to stimulate the original T-cell clone (TL3A6) ten- to 1000-fold more effectively than the native ligand. Furthermore, several of these peptides were immunogenic and

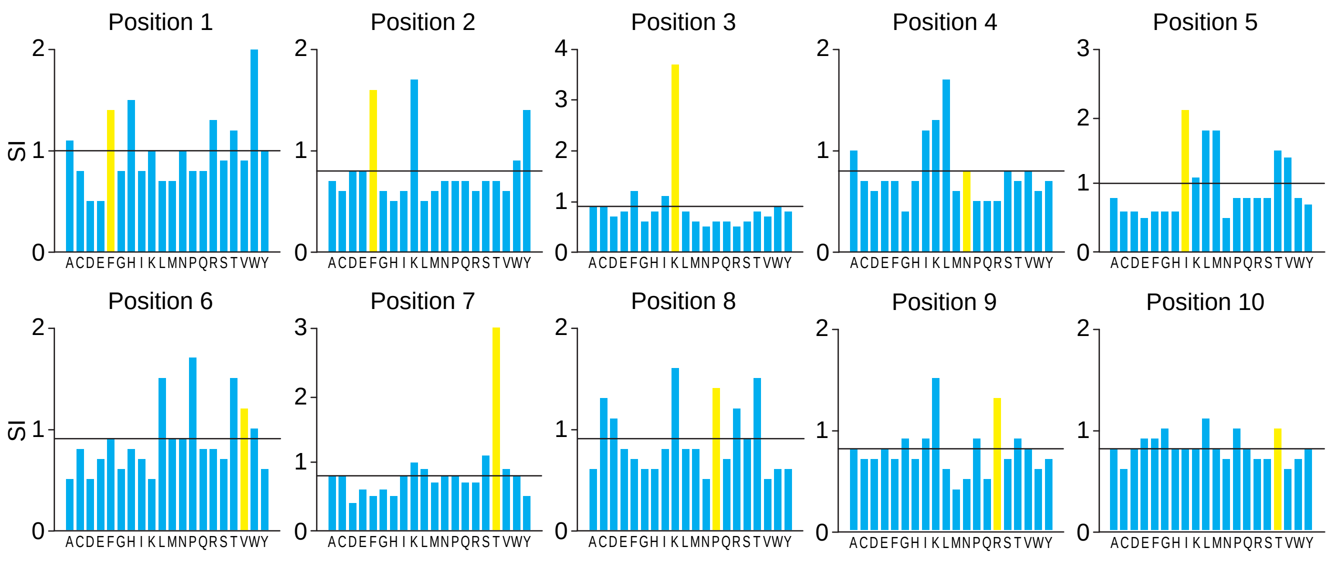

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Figure 4. *T-cell proliferation by the 200 mixtures that make up a decapeptide positional scanning library of that in total represent 6,000,000,000,000 decapeptides. Each panel represents one of the ten positional synthetic combinatorial libraries (SCLs; i.e. position one SCL is OXXXXXXXXX-NH$_2$). Each bar within a panel represents the stimulation index (SI) of a peptide mixture defined in the O position with one of 20 amino acids, which are indicated on the x-axis using their single-letter abbreviation. The SI is determined from the proliferation in counts-per-minute of the background in the screening assay. The horizontal line within each panel represents the average SI of the 20 peptide mixtures for that position.*

Table 3. Proliferative response by TL3A6 T-cell clone to peptides derived from screening of the decapeptide positional scanning synthetic combinatorial library (PS-SCL)

[a]Peptide sequence	EC_{50} pg ml^{-1}	Relative to native
Ac-**W** F K **L** I **T** T **T** **K** **L** -NH_2	3	9932
Ac-**W** F K **L** I **T** T P **K** **L** -NH_2	115	292
Ac-**W** F K **L** I **L** T **K** **K** **G** -NH_2	128	264
Ac-**W** F K **L** I **P** T **K** **K** **L** -NH_2	174	194
Ac-**W** F K **L** I **T** T **T** **K** **G** -NH_2	517	65
Ac-**W** F K **L** I **L** T P **K** **L** -NH_2	595	57
Ac-**W** F K **L** I **T** T P **K** **G** -NH_2	1233	27
Ac-**W** F K **L** I **L** T **T** **K** **L** -NH_2	1303	26
Ac-**W** F K **L** I **T** T **K** **K** **L** -NH_2	1378	24
Ac-**W** F K **L** I **P** T P **K** **L** -NH_2	1766	19
Ac-**W** F K **L** I **T** T **K** **K** **G** -NH_2	2646	13
Ac-**W** F K **L** I **P** T **T** **K** **L** -NH_2	2829	12
Ac-**W** F K **L** I **L** T **T** **K** **G** -NH_2	3285	10
Ac-**W** F K **L** I **P** T **K** **K** **G** -NH_2	3716	9
Ac- F F K N I V T P R T -NH_2	33,720	1

[a] The last sequence is the native sequence. Bold letters refer to residues that differ from the native sequence.

pathogenic in an animal model for MS (Ref. 54). It is important to note that to be pathogenic the immune response generated to these peptides must also be directed to the native autoantigen peptide. These results have clear implications for the use of such 'super antigens' in the development of synthetic vaccines.

In a separate study, the results obtained from a positional scanning library in combination with biometric data analysis were used to elucidate the specificity of a T-cell clone (CSF-3) of unknown specificity[53]. This clone was generated by stimulating cells from the cerebrospinal fluid of an individual with Lyme disease with the complete lysate of *Borrelia burgdorferi*. This strategy led to the identification of several specific *B. burgdorferi* peptides, as well as candidate autoantigens recognized by this clone. The biological importance of the peptides identified for other individuals with Lyme disease is under investigation.

Peptidomimetics and heterocycles

A wide variety of classic heterocycles and other acyclic organic compounds have now also been prepared using solid-phase parallel synthesis (reviewed in Refs 28,30,55). Existing libraries can be used to generate a diverse range of mixture-based heterocyclic libraries through the transformation of peptide, peptidomimetic, and small-molecule–heterocycle libraries using the 'libraries from libraries' approach[34,56,57]. The resulting heterocyclic compounds and libraries have dramatically different physical and biological properties from the compound types and libraries used as starting materials. Figure 5 illustrates some of the transformations that have been successfully carried out at the Torrey Pines Institute for Molecular Studies. The average library generated for the compound classes shown contains >50,000 compounds.

Highly active antimicrobial compounds[57] (Fig. 6) and opioid compounds that have potential as pain modulators (Fig. 7) have been identified from such libraries[34]. The synthetic approaches used in the transformation of *N*-acylated dipeptides to tri-substituted bicyclic guanidines is shown in Fig. 8. In the generation of every mixture-based combinatorial library, hundreds of individual controls are prepared to establish the stability, yield and purity of the resulting individual compound with the given side-chain group being examined. If the yield or purity of the given individual side-chain group in these control compounds is unacceptable, then this side-chain group is left out of the final preparation of the mixture-based library.

The early unsuccessful results obtained when other groups used mixture-based methods could be explained

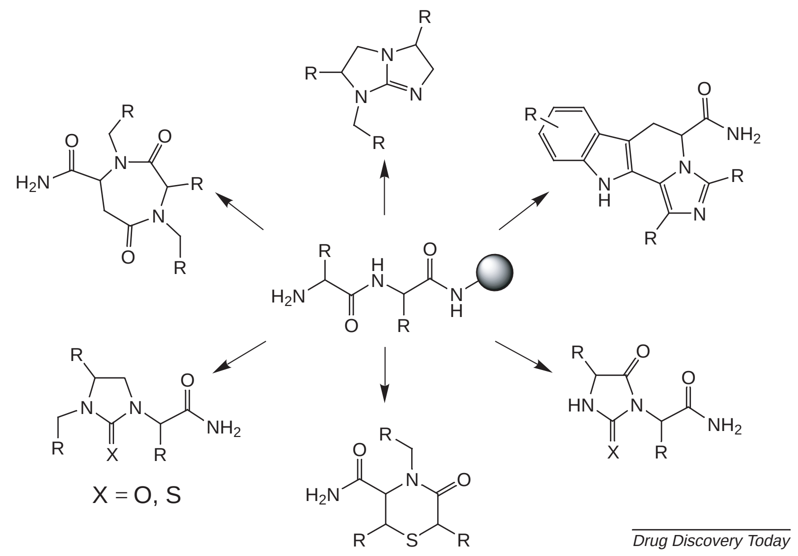

Figure 5. *Solid-phase synthesis of heterocyclic compounds using dipeptides and* N*-acylated dipeptides as starting materials.*

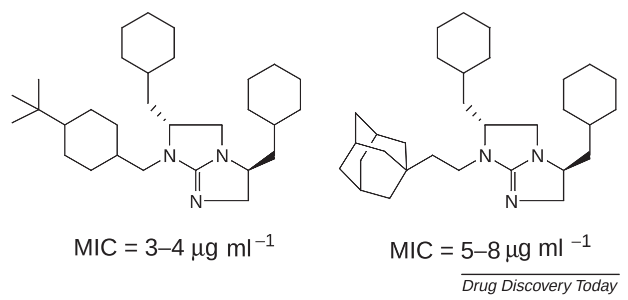

Figure 6. *Antibacterial compounds identified through the use of mixture-based combinatorial libraries. Abbreviation: MIC, minimum inhibitory concentration.*

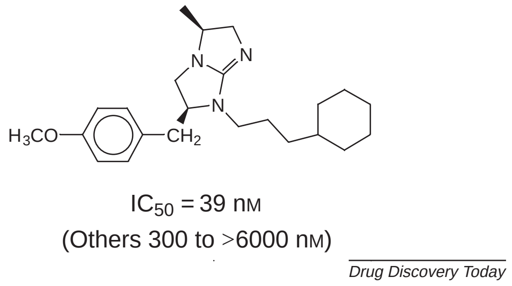

Figure 7. *κ-Receptor-specific opioid bicyclic guanidines identified from mixture-based combinatorial libraries.*

by their lack of experience in the exacting nature of each step required for the synthesis of mixture-based libraries, coupled with the need to optimize and ensure reproducibility of the chemistries used. In many cases, false positives are the result of poorly developed synthetic approaches that are not reproducible. Such 'false positives' appear to result from compounds, probably side reactions, that are not reproducibly formed in one library relative to its re-synthesis. Workers at the Torrey Pines Institute for Molecular Studies have generated >4,000,000 classical heterocyclic compounds in mixture-based positional scanning formats. It should be noted that each of the pharmacophores illustrated in Fig. 5 is able to be further transformed to generate additional compound types and therefore additional library diversities.

Conclusions

Extensive studies on the use of mixture-based combinatorial libraries carried out by this and other laboratories (reviewed in Ref. 34) have enabled the rapid, cost-effective identification of highly active and specific individual compounds. These methods are extremely effective and broadly applicable. Furthermore, it has been shown that there is nothing inherently unique about peptides or other oligomers that permits their successful use in mixture-based library formats, when compared with heterocycles. The limitations of mixture-based libraries are primarily:

- The need for reproducible synthetic approaches (this was not appreciated by others initially using this approach)
- The need for assay systems in which signal-to-noise levels are small and for which the assay reproducibility has been clearly established.

Although a single library cannot be expected to yield successful results in every assay, we have repeatedly found that when poor results are achieved for a given mixture-based library the most effective strategy is to simply test a different mixture-based library. It is obvious that if all compounds that make up a mixture-based library are available as individual compounds, and can be screened in a cost effective and timely manner, then the resulting data are inherently more complete and useful than can possibly be obtained by mixture-based libraries. The generation and testing of the sheer numbers of compounds (hundreds of thousands to trillions) that make up mixture-based combinatorial libraries is clearly impossible in all but the most

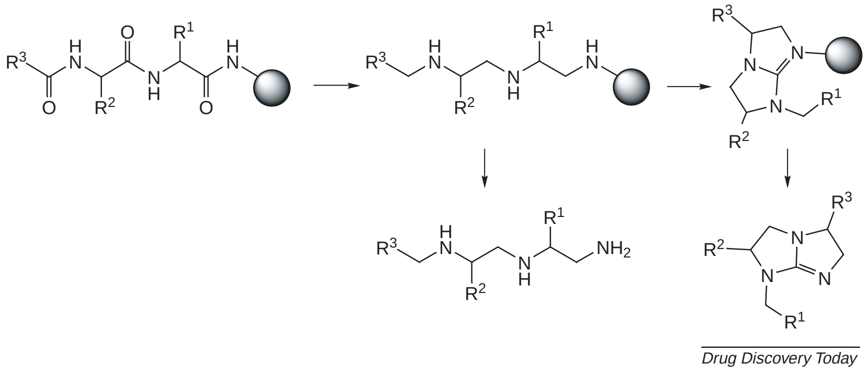

Figure 8. *The chemical transformation of* N*-acylated dipeptides to tri-substituted bicyclic guanidines.*

extremely well-funded facilities. Thus, the use of massive parallel-array synthesis in conjunction with classical HTS methods, relative to the use of mixture-based combinatorial libraries, is tied to the balance between the need for complete data acquisition, and the pragmatic and rapid gathering of compound information for lead development.

The use of mixture-based combinatorial libraries made up of large numbers of compounds offers unique advantages that are simply not possible with other approaches. For example, mixture-based libraries can be used in *in vivo* studies and whole tissue assays where very large numbers of individual compounds are impractical. Combinatorial chemistry has fundamentally changed synthetic chemistry in all areas of basic research and drug discovery. When coupled with methods such as computer-assisted design and genomic identification of new targets, combinatorial chemistry can be expected to enhance and continually increase the speed and thoroughness of drug discovery and vaccine development.

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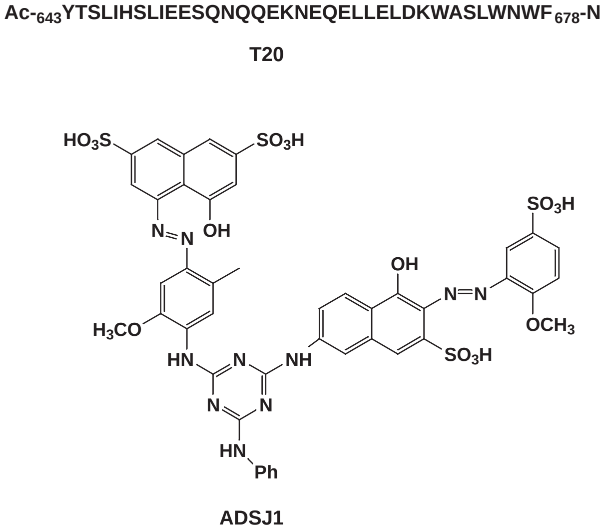
Corrigendum

Please note a correction to Fig. 5 in the article *HIV-1 entry – an expanding portal for drug discovery* by Wade S. Blair, Pin-Fang Lin, Nicholas A. Meanwell and Owen B. Wallace published in *Drug Discovery Today* (2000) 5(5), 183–193.

The corrected figure is shown below. The author would like to apologize for this inaccuracy and for any misunderstandings that this might have caused.



Drug Discovery Today

Figure 5. *The structure of ADSJ1, an inhibitor of gp41 function.*