# Advancements in Drug Discovery: The Role of Combinatorial Chemistry and High-Throughput Screening

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***Abstract*— Combinatorial chemistry and high-throughput screening (HTS) are transformative techniques in drug discovery, enabling the rapid synthesis and evaluation of large libraries of chemical compounds. Combinatorial chemistry allows for the generation of diverse chemical libraries by systematically combining sets of building blocks to create a vast array of potential drug candidates. This method significantly accelerates the drug discovery process by producing numerous analogues of a lead compound for biological evaluation. High-throughput screening complements combinatorial chemistry by automating the testing of these large compound libraries against biological targets, such as proteins or enzymes. HTS utilizes advanced robotic systems, miniaturized assays, and sensitive detection methods to evaluate thousands to millions of compounds in a relatively short period. This approach identifies active compounds, or "hits," that exhibit desired biological activity, which can then be optimized through further chemical modifications. Together, combinatorial chemistry and high-throughput screening have revolutionized the early stages of drug development, increasing the efficiency of identifying novel therapeutic agents. These techniques reduce the time and cost associated with traditional drug discovery methods, allowing for the rapid identification of promising drug candidates and facilitating the development of new treatments for various diseases.***

***Index Terms- Combinatorial Chemistry, Combinatorial Library, Drug Discovery, High-Throughput Screening, Computer-Assisted Drug Design, One-Bead One-Compound Library, DNA-Encoded Chemical Library***

## I. INTRODUCTION

Combinatorial chemistry involves the generation of a large array of structurally diverse compounds, called a chemical library, through systematic, repetitive and covalent linkage of various “building blocks”. Once prepared, the compounds in the chemical library can be screened, concurrently, for individual interactions with biological targets of interest. Positive compounds can then be identified, either directly (in position-addressable libraries) or via decoding (using genetic or chemical means).

The concept of combinatorial chemistry was developed in the mid 1980’s, with Geysen’s multi-pin technology [1] and Houghten’s tea-bag technology [2] to synthesize hundreds of thousands of peptides on solid support in parallel. In 1991, Lam *et al.* [3] introduced the one-bead one-compound (OBOC) combinatorial peptide libraries and Houghten *et al.* [4] described the solution-phase mixtures of combinatorial peptide libraries. In 1992, Bunin and Ellman reported the first example of a small-molecule combinatorial library [5]. In addition to being displayed on microbeads, peptides and other synthetic compounds can be displayed on planar surfaces or solid supports, such as glass, to form planar microarrays [6]. In 1985, Smith described the phage-display peptide library method [7]. Similar to OBOC libraries, each M13 phage displays one unique peptide entity (five copies); i.e., one-phage one-peptide. Positive phages can then be isolated for amplification,

re-panning, and eventually decoding with DNA sequencing. Unlike synthetic library methods, early biological libraries (phage-display, yeast-display, polysome-display peptide libraries) are restricted to the use of the 20 natural L-amino acids and simple cyclization with disulfide bonds. In the mid 2000's, Frankel *et al.* [8] Josephson *et al.* [9], and Murakami *et al.* [10] reported the mRNA-display macrocyclic peptide libraries using unnatural and D-amino acids as building blocks. In 2009, Heinis *et al.* introduced the method of post-translational chemical modification of phage-displayed peptide libraries [11]. The latter approaches enable the generation of libraries of conformationally constrained peptides with greater chemical diversity and resistance to proteolysis, and are, thus, potentially more useful as drugs. Recent advances in DNA-encoded chemical libraries (DECLs) have allowed investigators to create and decode huge diversity small-molecule organic, peptide or macrocyclic libraries.

Combinatorial chemistry has been used for both drug lead discovery and optimization [12,13,14•]. Figure 1 summarizes the various combinatorial library methods, the nature of the library compounds involved and the screening methods available to each of the technologies. As shown in Figure 1 (orange boxes), most of the combinatorial library methods have the ability to generate hugely diverse chemical libraries (e.g. >1 million). These include the phage-display, yeast-display, bacteria-display, mRNA-display, OBOC, DECL, and solution phase mixture libraries. In addition to generating a huge number of compounds, these combinatorial library methods also allow rapid concurrent screening against specific drug targets (see below). The parallel synthesis library and synthetic planar microarray library methods (black boxes, Figure 1) are much lower throughput, and the resultant libraries far more focused, than the aforementioned methods. The planar microarray method has mostly been used as a tool for peptide research; although, in theory, other types of compounds can be chemically prepared *in situ*, via automation. The highly focused parallel synthesis small-molecule libraries (hundreds to thousands of compounds), when developed in conjunction with computational chemistry, are particularly useful for optimization of drug leads (see below). The subject of combinatorial chemistry has been extensively documented and reviewed [14–16]; as such, this short review covers only recent advances in combinatorial library design, synthesis and high-throughput screening methods. Selected examples that utilize combinatorial library approaches for drug discovery will also be briefly discussed; however, nucleic acid-based combinatorial libraries (e.g. aptamer library [17]) will not be discussed here.

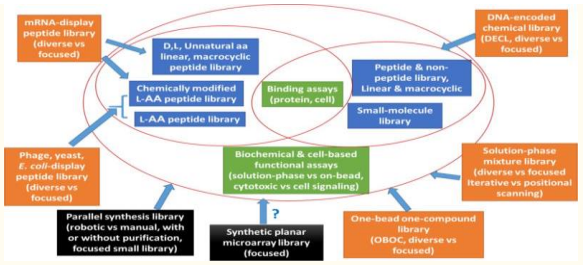


Figure 1
Computational Chemistry for Combinatorial Library Design

As the fields of combinatorial chemistry and computational chemistry began to mature, it became clear that combining the two would lead to higher hit rates. It is more cost-effective to design and screen virtual chemical libraries *in silico*, such that subsets of the chemical space of likely hits can be defined, prior to the actual synthesis and screening of the libraries. Computer-assisted drug design, such as generation of virtual libraries, analogue docking and *in silico* screening now becomes the standard procedure used in drug discovery programs. Fragment-based drug design (FBDD) involves the experimental screening of libraries of small chemical fragments, via nuclear magnetic resonance (NMR) spectroscopy or other biophysical technologies such as surface plasmon resonance (SPR) for low affinity hits (low mM to high μM), or *in silico* screening of virtual fragments if the structural information of the target is available. Proper linkers are then used to connect the fragment hits while maintaining their relative positions in the sub-pockets. High-affinity ligands have been found with these approaches [18,19]. Vemurafenib is the first drug discovered via FBDD to gain FDA approval [20]. To enhance the probability of obtaining hits that are more drug-like, ADMET (absorption, distribution, metabolism, excretion and toxicity) filters have also been included in the

algorithm for library design [21]. Examples of other library design methods include multi-objective optimization methods [22], the "adaptive" library approach with a simulated evolutionary process [23], and the multiple copy simultaneous search method which uses active site mapping and a *de novo* structure-based design tool [24]. A rapid and simple Python-based method for target-focused combinatorial library design was recently developed by Li *et al.* [25]. This method utilizes flexible SMILES strings, which are concatenated by Python language, to encode structures of molecules and create the library at a rate of approximately 70,000 molecules per second. The authors used the hybrid 3D similarity calculation software SHAFTS to help refine the size of the libraries and improve hit rates. Although the aforementioned computational methods can be applied to both diverse and focused library design, they are particularly important for the development of focused libraries of limited diversity, so that the hit rate can be increased.

Generation of Combinatorial Libraries

Parallel synthesis of combinatorial libraries can be achieved manually or robotically, in solution or on solid support. Diversity of these libraries tends to be small (hundred to a few thousands) but the choice of coupling chemistry is not limiting, and each library compound can be purified via automatic chromatography if needed. The intended structures of each of the library compounds are known. In contrast, the OBOC libraries are synthesized on microbeads using the split-pool synthesis strategy [3,4,26], resulting in greater diversity (thousands to millions) of bead-bound library compounds. However, these library compounds are non-addressable, and the positive bead isolated from screening must be decoded via a chemical or physical barcode, which can be constructed during library synthesis. Solution-phase positional scanning libraries can be prepared on solid support via split-pool synthesis, and later cleaved off the beads into a compound mixture in solution. Methods for the generation of biological peptide libraries such as phage-display, yeast-display, mRNA-display, and chemically modified phage-display libraries have been well described in the literature [14,27] and will not be discussed here. DECL libraries can be assembled via proximity ligation of DNA-tagged building blocks to form peptides, small-molecules or macrocycles. The available coupling chemistries for DECL; however, are more limited because they must be mild and compatible with the oligonucleotide tags. For reviews on the synthesis of chemical libraries, please refer to references [28–30] and the series of "Comprehensive Survey of Combinatorial Library Synthesis" in the *Journal of Combinatorial Chemistry* (currently *ACS Combinatorial Science*). Here, we would like to highlight several recently developed new chemical approaches and technologies in the preparation of combinatorial libraries.

Huang and Bode recently reported a "synthetic fermentation" method that does not require the use of organisms, enzymes or reagents to generate a combinatorial library of complex organic molecules "grown" from small building blocks in water [31••]. In this method, the authors adapted ketoacid ligation, which produces β-amino acid linkages. By adjusting the reaction conditions and the building blocks, products with different sequences, structures and compositions can be modulated. The authors prepared a 6,000-membered library from 23 simple building blocks and discovered a 1.0-μM inhibitor against hepatitis C virus NS3/4A protease.

Litovchick *et al.* developed a chemical ligation method for the construction of DECLs [32•]. The method relies on the ability of the Klenow fragment of DNA Polymerase I to translocate to a DNA backbone through triazole linkages via click cycloaddition. The authors have developed a strategy that allows for repetitive and specific installation of multiple oligonucleotide tags. Compared with previous DECL methods, this chemical ligation method represents an advance over, and could expand the scope and diversity of chemistry suitable for DECLs.

Many bioactive peptidic natural products contain macrocyclic structures. Suga and Bashiruddin recently published a review article [33] on the construction and screening of large libraries of natural product-like macrocyclic peptides using reconstituted translation systems where designated codons are made vacant and then reassigned to unnatural amino acids. Ribosomal synthesis of macrocyclic peptides can be achieved with a custom-made *in vitro* translation system containing flexizymes, amino acids (natural and

unnatural), as well as unnatural amino acid capable of crosslinking with other amino acids. Fasan *et al.* recently reported a novel and versatile method for generating side chain-to-tail cyclic peptide macrocycles from ribosomally derived polypeptides *in vitro* in a pH-triggered manner or directly in living bacterial cells [34••]. Unnatural amino acids bearing a side chain of 1,3-aminothiol (AmmF) or 1,2-aminothiol (MeaF) are first ribosomally inserted into intein-containing precursor proteins (Figure 2). Then spontaneous post-translational cyclization via a *C*-terminal ligation/ring contraction is achieved via an intein-catalyzed intramolecular transthioesterification, followed by ring closure through an irreversible *S, N* acyl transfer rearrangement. More recently, the Suga group reported a strategy for efficient post-translational modification of a library of ribosomally translated peptides by introducing exogenous free thiols, followed by ligation of carbohydrates to generate proteolytically stable thioglycopeptides [35].

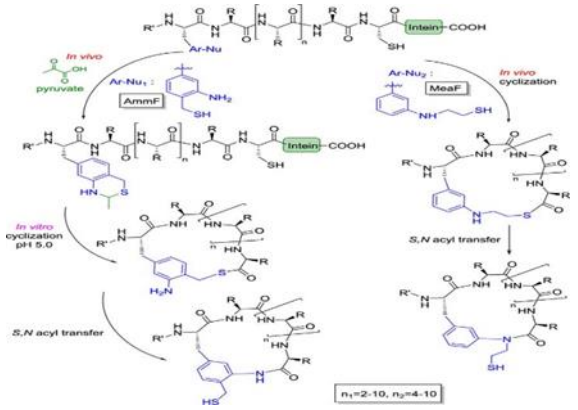


Figure 2

Screening of Combinatorial Libraries

The screening of a combinatorial library can be divided into two categories: virtual screening and experimental real screening. Virtual screening uses computational methods to predict or simulate how a particular compound interacts with a given target protein. The three virtual screening methods used in modern drug discovery include molecular docking, pharmacopoeia mapping, and quantitative structure-activity relationships. The disadvantages of virtual screening are that it cannot replace real screening, and generated hits may be very difficult to chemically synthesize. Real screening approaches, such as high-throughput screening (HTS), can test the activity of hundreds of thousands of compounds experimentally, providing real results; however, these methods are far more expensive and slower than virtual screening methods.

The most common assay to screen a combinatorial library is to determine the binding of the library compounds to the target protein. Other common assays are functional assays, such as biochemical and enzymatic assays, or cell-based assays. Cell-based assays can be direct cytotoxic assays, receptor-binding assays, or cell-signaling assays using cell lines with specific genetic reporter systems. Selection of screening methods greatly depends on the nature of the combinatorial libraries to be screened. Position-addressable soluble libraries prepared from parallel synthesis can be screened with automated HTS methods in 96-, 384-, and 1536-well plates. Libraries on solid supports (e.g. OBOC library) can be easily screened against a variety of biological targets (proteins, cells, viruses, etc.) for binding or functional activities [14], or released *in situ* for solution phase functional assays [36]. Phage-display peptide libraries can be screened with bio-panning [37] or limited cell-based functional assays, such as cell-binding and cellular uptake assays [37]. Structure-based virtual libraries are screened *in silico.* Several new screening approaches for combinatorial libraries have recently been developed; below are some examples.

Heusermann *et al.* recently reported the use of a standard wide-field fluorescence microscope, equipped with LED-based excitation and a modern CMOS camera [38] to detect signals associated with target proteins bound to beads in an OBOC library. The autofluorescence issue was overcome by an optical image subtraction approach. The screening system is ultra-high throughput and >200,000 bead-bound compounds can be screened in 1.5 h. Perez-Pineiro *et al.* reported a direct label-free ultra-fast method for the identification and spectroscopic classification of hits from OBOC peptide libraries [39]. They synthesized peptides directly on TentaGel beads decorated with bimetallic Au/Ag clusters on the surface, and subsequently use surface-enhanced Raman scattering analysis to detect the signals of the peptide on each bead. Because the Raman scattering intensity is closely associated with the distance to the

surface, this method is limited to short peptides with lengths of 7 to 10 amino acids. MacConnell *et al.* described a microfluidic circuit that enables automated and quantitative functional screening of DNA-encoded compound beads [40]. The device sequentially carries out the following steps: distribution of the library bead into picoliter-scale assay reagent droplets, photo-cleavage of compound from the bead, assay incubation, laser-induced fluorescence-based assay detection, and fluorescence-activated droplet sorting to isolate hits.

Agnew *et al.* reported the use of *in situ* click chemistry as a screening approach to assemble multi-ligand protein-capture agents on an OBOC library [41]. This method has several advantages, including: 1) the production of the capture agent does not require prior knowledge of affinity agents against the target protein; 2) the *in situ* click screening covers a very large chemical space; and 3) the process can be repeated until ligands with the desired affinity and specificity are identified. For example, once a bi-ligand has been identified, it can serve as the anchor ligand to click back to the same OBOC library for discovery of a tri-ligand, and so forth. Upon the addition of each ligand to the capture agent, the affinity and the selectivity of the capture agent for its target protein increase rapidly. We have recently developed a screening platform to identify death ligands (pro-apoptotic agents) via the screening of one-bead two-compound (OB2C) libraries [42–44]. In an OB2C library, a fixed cell-capturing ligand and a random library compound are co-displayed on each bead surface, and a coding tag resides inside the bead to exclude screening interference (Figure 3A). When live cells bind to the capturing ligand on the bead surface, the cells are forced to expose their cell membrane proteins to the OB2C library compounds (Figure 3B). After incubation, dead cells or cells undergoing apoptosis can be readily detected using propidium iodide (PI) or anti-cleaved caspase 3 antibody staining (Figure 3C). Peptide (LWK1) [42], peptidomimetic (S7-Y) [43] and small-molecule (LLS2) [44] death ligands have been identified through OB2C library approach (Figure 3D).

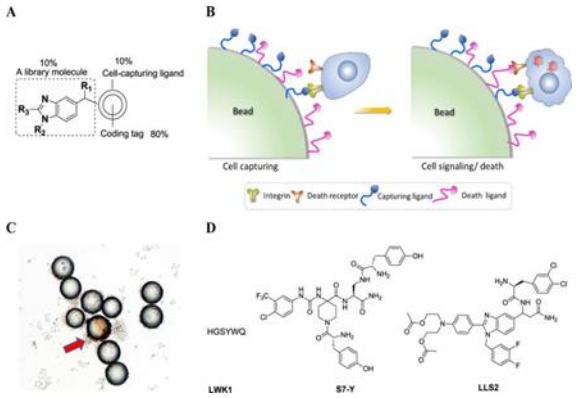


Figure 3

Several approaches have been used to generate DECLs with different library-encoding methods and assembly of chemical building blocks [45••,46••]. As all compounds in the library can be identified by their DNA tags, a very large number of compounds (up to billions of molecules) can be screened simultaneously in mixture in affinity-capture experiments on target proteins. The screening process involves three steps: 1) physical isolation of the binder using automated affinity selection; 2) PCR-amplification and sequencing of the DNA codes of the isolated binders; and, 3) evaluation of the obtained sequencing data using a computer program to eliminate false binders. DECL technology can yield specific binders to a variety of target proteins and is a very useful tool for hit discovery and lead expansion.

## Encoding and Decoding of Combinatorial Libraries

Since the chemical structure of individual compounds in conventional addressable combinatorial libraries or planar microarray libraries are known, there is no need to encode and decode the chemical hits. For mixture libraries in solution, such as positional-scanning libraries, deconvolution is needed to determine the identity of the hits. Biological-displayed peptide libraries (e.g., phage, yeast or mRNA-display) are genetically encoded and can be decoded with PCR and DNA sequencing. Similarly, DECL decoding can be easily achieved through PCR-amplification of the DNA barcode, followed by high-throughput DNA sequencing. Buller *et al.* reported another approach named “Illumina sequencing of DECLs” which can yield over 10 million DNA sequence tags per flow-lane [47]. This technology can be used in a multiplex format, allowing the encoding and subsequent

sequencing of multiple selections in the same experiment.

Many encoding and decoding strategies have previously been developed for OBOC libraries [48], with chemical barcodes usually decoded using automatic Edman microsequencing of bead-bound peptide tags [49] or mass spectroscopy of released coding tags [50,51]. Marcon *et al.* recently reported a fluorescence-based encoding method called "on-the-fly" encoding using colloidal barcoding [52]. In this method, 10–20 μm beads were encoded during a split-pool synthesis with smaller 0.6–0.8 μm silica colloids that contain specific and identifiable combinations of fluorescent dye. After screening, the colloidal barcode can be decoded with confocal microscopy. Recently, Lee *et al.* reported a simple and efficient surface-enhanced Raman spectroscopic (SERS) barcoding method using highly sensitive SERS nanoparticles (SERS ID) [53]. More than one million codes can be generated by using combinations of 44 different SERS IDs, which are highly stable and reliable under bioassay conditions.

Applications of Combinatorial Chemistry for Drug Discovery – Examples

Over the last decade, the combinatorial library approach has been applied successfully to various applications including drug discovery.

1. Lead Optimization in Cancer Therapeutics:
- Combinatorial chemistry has been extensively used in the development of cancer drugs. One notable example is the optimization of kinase inhibitors. By generating diverse libraries of compounds targeting specific kinases, researchers have identified highly selective and potent inhibitors. For instance, combinatorial chemistry played a key role in the development of Imatinib (Gleevec), a tyrosine kinase inhibitor used in the treatment of chronic myeloid leukemia (CML).
2. Development of Antiviral Agents:
- The emergence of viral diseases such as HIV and Hepatitis C has driven the use of combinatorial chemistry to develop antiviral drugs. Researchers created large libraries of nucleoside analogs and screened them for activity against viral polymerases. This approach contributed to the discovery of antiviral drugs like Zidovudine (AZT) for HIV treatment and Sofosbuvir for Hepatitis C.
3. Enzyme Inhibition for Metabolic Disorders:
- Combinatorial chemistry has been instrumental in identifying enzyme inhibitors for the treatment of metabolic disorders. For example, statins, which are used to lower cholesterol levels, were optimized through combinatorial approaches. By synthesizing and screening libraries of HMG-CoA reductase inhibitors, researchers identified potent compounds such as Atorvastatin (Lipitor), one of the best-selling drugs globally.
4. Discovery of Antibacterial Agents:
- The fight against antibiotic resistance has benefited from combinatorial chemistry. Libraries of novel antibiotics were synthesized and screened against bacterial targets. This approach led to the discovery of novel classes of antibiotics, such as Oxazolidinones, with Linezolid being the first to be approved for the treatment of resistant bacterial infections.
5. GPCR Ligand Development:
- G-Protein Coupled Receptors (GPCRs) are a major class of drug targets. Combinatorial chemistry has been used to generate libraries of small molecules targeting GPCRs, leading to the discovery of various agonists and antagonists. For instance, combinatorial approaches helped in developing drugs like Losartan, an angiotensin II receptor antagonist used to treat hypertension.
6. Peptide and Protein-Based Drug Discovery:
- Combinatorial chemistry is also applied in peptide and protein drug discovery. Phage display libraries, a form of combinatorial chemistry, have been used to identify peptides that bind specifically to target proteins. This technique contributed to the development of therapeutic antibodies such as Adalimumab (Humira), used to treat autoimmune diseases.

## CONCLUSION AND PERSPECTIVES

Combinatorial chemistry has accelerated the development of a whole set of combinatorial tools comprising combinatorial library design, efficient synthetic methods, reagents for library synthesis (including solid supported reagents), linkers, bilayer beads, library encoding and decoding strategies, HTS

methods and equipment, etc. The large diversity combinatorial bead and planar microarrays in the early 1990's had inspired investigators in fields beyond chemistry to think "combinatorially"; this change in thinking led to the development of oligonuleotide bead and planar microarrays, genomics and many other "-omics" technologies that involve the concurrent interrogation of thousands to hundreds of thousands of analytes or biomolecules. A recent report on single-cell RNAseq analysis with nanodroplet, indeed uses the "split-pool" synthesis approach to prepare sets of DNA barcodes on microbeads, for subsequent tracking of sequences derived from the same cell. Many investigators, particularly in the pharmaceutical industry, are now working on smaller target-focused solution-phase libraries of compounds with drug-like properties, and incorporating ADMET filters and structure-based drug design approaches into library development. However, for novel lead discovery against a large number of therapeutic targets, particularly for those targets with little structural information, the various high diversity library methods outlined in this mini-review will undoubtedly be invaluable.

Many macrocyclic natural products are non-peptides. Some of them are polyketide-based. There is a great need to develop novel and efficient chemistry for the generation of macrocycles that mimic such structures [33]. Incorporating chemical features of such molecules into the design of "easy-to-couple" building blocks will enable the development of large, diverse natural productlike macrocyclic libraries for the discovery of novel drug leads. Another promising method in combinatorial chemistry is the use of nature's highly stable peptides, such as cyclotides , as scaffolds for library design. Random peptide loops can be grafted, chemically or recombinantly , into cysteine knots to form cyclotide libraries.

Although the initial high expectations of combinatorial chemistry for drug discovery have yet to be realized, much has been learned over the last 30 years. Many new chemical, biological, computational, and screening tools have been developed. The limitations and strengths of combinatorial chemistry are better understood. We are now in a better position to truly leverage the power of combinatorial technologies for the discovery and development of next-generation drugs. The future of utilizing combinatorial chemistry for drug discovery is bright.

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