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Finding a New Type of Drug Candidate Using Combinatorial Library Approaches

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Abstract

As the consequence of an ever-increasing trend toward bacterial antibiotic drug resistance, scientists are compelled to constantly develop a great diversity of molecules that can function as potent antibiotics. Using combinatorial library methods, a number of potential drugs can be selected from a sizeable pool of antibiotics. The several molecules selected and described herein, which generally inhibit specific enzymatic activities, show promise in medical applications, including inhibitors of metallo-β-lactamase in *B. cereus* and of acetohydroxyacid synthase in *M. tuberculosis*. These potential antibiotics evidence different structures than are exhibited by the currently-used inhibitors, thus raising the possibility that an entirely new class of antibiotics has been found. Combinatorial methods may also be utilized to explore inhibitor specificity, as was previously done using both metallo-β-lactamase and acetohydroxyacid synthase. The isolation of these molecules and the exploration of their specificity underlines the importance and potential of combinatorial methods in the discovery of future antibiotics to combat changing bacterial antibiotic resistance.

Keywords: SELEX, Combinatorial library, Inhibitors

Introduction

Research into effective antibiotics is a ceaseless process, owing principally to the great multitude of mutated pathogenic bacteria. The first antibiotic was discovered in 1928 by the Scottish scientist, Sir Alexander Fleming, in his work with *Penicillium* mold, after noting that bacterial growth inhibition occurred in a circular region surrounding the *Penicillium*. Despite noting its antibacterial effects, Fleming did not continue to research penicillin, incorrectly concluding that the antibiotic would be incapable of surviving in the body long enough for the treatment of bacterial infections. Not until 1942 was a patient treated successfully with penicillin, resulting in the development of a pathway three years later, which was suitable for the mass production of the compound. Owing to penicillin's relatively narrow activity spectrum, several other antibiotics were later developed: ampicillin in 1951, methicillin in 1959, amoxicillin in 1972, and many more in later years, some of which extended the spectrum of antibiotic treatment to Gram-negative bacteria.

Penicillin and penicillin-derivatives are known as effective antibiotics, due primarily to their ability to interrupt cell wall synthesis, which they accomplish by binding irreversibly to transpeptidases, which are critical to the formation of the cell wall[1]. These enzymes, which are also referred to as penicillin-binding proteins (PBPs), catalyze the linkage of peptidoglycan subunits at the carboxyl terminal (D-alanyl-D-alanine end) of the subunits[2]. The antibiotics inhibit this process via the acylation of the active sites of PBPs, thereby preventing the catalytic activity that binds the peptidoglycan subunits together; the non-bound subunits, however, are still incorporated into the cell wall of the organism. At sufficiently high antibiotic concentrations, the peptidoglycan wall becomes too weak to maintain its structural integrity due to internal mechanical and osmotic pressures, thereby causing cell lysis. Although not all bacteria that are treated with penicillin or penicillin-derivatives evidence lysis, morphological alterations in treated bacteria indicate involvement in cell wall synthesis[2]. In one recent report, it has been suggested that β-lactam antibiotics, along with other classes of antibiotics, including quinolones which target DNA replication and repair by binding to DNA gyrase[3] and aminoglycosides which inhibit ribosome function[4-6], ultimately induce cell death via the production of hydroxyl radicals, although the specific method of hydroxyl radical formation varies from class to class[7]. In either case, the penicillin-type antibiotics destroy the viability of a bacterium, rendering it harmless

within a clinical setting[2].

As a response to the widespread use of β-lactam antibiotics, a significant number of bacterial species have currently been shown to be resistant to β-lactam antibiotic treatment via the hydrolysis of the β-lactam ring by penicillinase, synthesized by the resistant bacteria, thereby converting the antibiotic to penicilloic acid. With a structure that no longer resembles that of the peptidoglycan subunits, and is thus unable to function as a pseudosubstrate, the β-lactam antibiotic is, for antimicrobial purposes, inactivated. Due to the rate at which penicillinase can be generated in penicillin-resistant bacteria, the antibiotics have proved entirely ineffective at killing infectious bacteria that have developed resistance to antibiotic treatments[1].

If isolated among only a few bacterial strains, the antibiotic resistance would prove relatively trivial; a variety of bacterial pathways can be attacked with bactericidal agents[3]. However, antibiotic resistance is not limited to a small number of bacterial species, and clinically notable antibiotic resistance usually occurs relatively quickly, thereby rendering the typical antibiotic useless within a number of years (vancomycin representing a noteworthy exception). Cultures of *S. aureus* have been observed to develop resistance to methicillin in as few as five days. Given the large number of bacteria involved in an infection cycle and the high rate of mutation (1 in 10^7 loci) inherent to bacteria, mutations are relatively commonplace. If one bacterium is mutated in a manner that confers antibiotic resistance and the infection is subsequently treated with that antibiotic, natural selection quickly results in a population in which all individuals are antibiotic-resistant. The ability of bacterial DNA to be stored in plasmids that independently replicate further increases the rate at which antibiotic resistance occurs in bacteria, giving bacterial cells the ability to readily duplicate and transfer antibiotic-resistance genes between both individuals and species[1]. With these facts in mind, antibiotic resistance clearly presents a serious obstacle to the continuation of antibacterial treatment, thereby creating a race between the evolution of antibiotic-drug resistance in bacteria and the ability of biochemists to develop a variety of antibiotics. Here, two combinatorial technologies have been introduced to discover new types of drug candidates, namely, oligonucleotide library and chemical library approaches.

Combinatorial Oligonucleotide Library: Selex Technology

Via combinatorial chemistry, a wide variety of compounds can be tested effectively for promising drug activity. Combinatorial techniques have been widely adopted by biotechnology and pharmaceutical companies over the previous 10 years. One of the most promising combinatorial chemistry techniques is referred to as SELEX (Systematic Evolution of Ligands by EXponential enrichment)[8]. This technique is also referred to as *in vitro* selection or as *in vitro* evolution[9-11]. During the 1990s, the laboratories of G.F. Joyce, J.W. Szostak, and L. Gold developed this technique, which allows for the simultaneous screening of a large number of nucleic acid molecules for possible functional drug use. Functional molecules are selected from the mainly non-functional pool of oligonucleotides via column chromatography or by other selection techniques, including gel shifting assays, which are suitable for the enrichment of any particular desired property.

One common approach is conducted as follows: a starting, degenerate oligonucleotide pool is generated with a standard DNA-oligonucleotide synthesizer. The instrument synthesizes an oligonucleotide with a completely random base-sequence flanked by defined primer binding sites. The immense complexity of the generated pool justifies the assumption that the pool may harbor a few molecules, referred to as aptamers, with the correct secondary and/or tertiary structures that bind tightly and specifically to a target material. These are selected, for example, via affinity chromatography or filter binding. As a pool of such profound complexity can be expected to harbor only a very small fraction of functional molecules, several purification steps are generally required. Therefore, the very rare "active" molecules are amplified via polymerase chain reaction (PCR), which allows for iterative cycles to be carried out, thus resulting in an exponential increase in the abundance of functional sequences until they dominate the population.

Application of Selex Technology

A practical example of an oligonucleotide inhibitor is the metallo-β-lactamase inhibitor[12], in which the oligonucleotide specifically inhibits the enzyme. The general method by which enzyme inhibitors are discovered is illustrated in Figure 1. According to these results, single-stranded DNA aptamers were developed using SELEX combinatorial chemistry technology. A 61-mer harboring a random 30-mer (comprising over 10^{18} sequences) was synthesized. After 21 rounds of SELEX, one sequence was found, which evidenced a noncompetitive inhibition for *Bacillus cereus* 5/B/6 metallo-β-lactamase with an

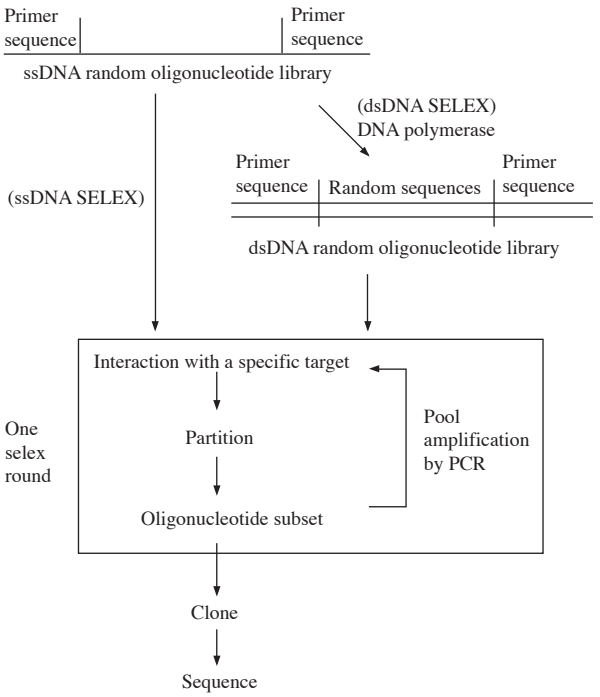

Figure 1. Diagrammatic representation of the SELEX procedure. Primer sequences permit amplification (modified from ref [11]).

IC_{50} value of 1.2 nM. In addition to the 30-mer, a 10-mer predicated on a structure developed using the computer program, MFold[13], which predicts possible usable secondary structures (usually stem-and-loop structures), evidenced an identical IC_{50} value. In an effort to assess the inhibition specificity, the inhibitors were applied to *Bacillus cereus* 569/H/9 β-lactamase I and bovine carboxypeptidase A, resulting in no inhibitory effects on either enzyme. β-lactamase I has no metal ion in or proximal to the active cleft, and the bovine carboxypeptidase A has one zinc ion near the active site, thereby suggesting that the inhibition most probably occurs in the presence of a metal ion of the metallo-β-lactamase, and appears to bind specifically to the zinc ion(s) of metallo-β-lactamase.

One example of the successful application of SELEX technology is the creation of an aptamer which inhibits vascular endothelial growth factor (VEGF)[14]. This anti-VEGF aptamer blocks vessel growth and inhibits neovascularization in pre-clinical models, binding specifically to the VEFG isoform, a dimeric protein featuring a receptor-binding domain and a heparin-binding domain. Interestingly, no binding to the heparin-binding domain was detected in the absence of Ca^{2+}[14,15]. In order to assess practicality, a tissue culture-based competition assay was conducted *in vivo*. The heparin-binding domain effectively competed with VEFG for aptamer binding, thereby illustrating the feasibility of oligonucleotide inhibitors using SELEX *in vivo*.

Table 1. DNA aptamers for possible therapeutic applications.

Target	Application	Reference
Metallo-β-lactamase	Antibacterial	[12]
Thrombin	Anticoagulant	[16]
L-selectin	Anti-inflammatory agent	[18]
Hemagglutinin of influenza virus	Viral infection prevention	[29]
Platelet-derived growth factor	Progressive renal disease	[30]

Several other potential drug candidates have been detected using SELEX technology. A DNA aptamer for thrombin, which performs an important function as an enzyme in the regulation of the coagulation pathway, has been identified as an extracellular target with a binding affinity value of 25-200 nM[16]. The aptamer has been recognized as a very promising anticoagulant drug candidate *in vivo*[17]. Another example, L-selectin, is a receptor which allows leukocytes to enter tissues at the inflammation site. The DNA aptamer of L-selectin has a dissociation constant value of 1.2 nM, thereby indicating that the aptamer may be a drug candidate[18]. Single-stranded DNA aptamers that can be employed for the prevention of viral infection and progressive renal disease have also been identified (Table 1).

It should be mentioned that, in order to acquire good drug candidates with aptamers, the resultant aptamers may require chemical modification to prevent nuclease activity. RNA aptamers frequently require chemical modifications in order to enhance stability, as RNA is quite susceptible to RNase activities, whereas DNA aptamers are frequently not modified for further stabilization. However, resistance against nuclease activity can be enhanced via phosphorothioate modification for DNA aptamers (Figure 2).

Combinatorial Chemical Library

Combinatorial library chemistry involves a sizeable number of structurally related synthetic organic chemical compounds and is utilized, particularly in the pharmaceutical industry, for the isolation of possible drug candidates. This approach has been employed to find inhibitors of enzymes in the biosynthesis path-

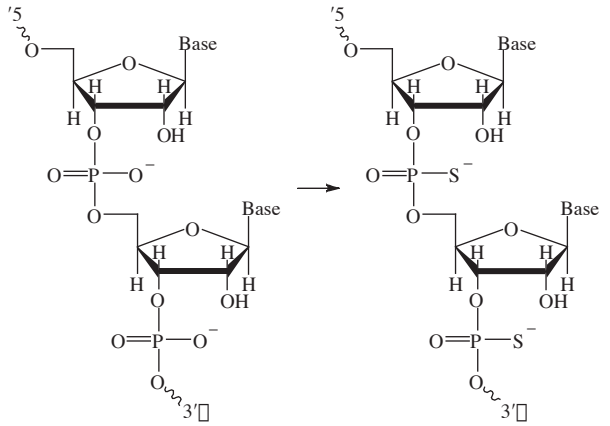

Figure 2. Phosphorothioate modification to achieve nuclease resistance for DNA aptamers.

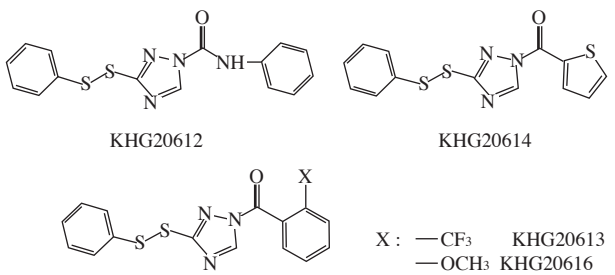

Figure 3. Chemical structure of hit compounds obtained from chemical library screening (adapted from ref [27]).

way, including the detection of possible inhibitors of acetohydroxyacid synthase (AHAS) from pathogenic bacteria, an anabolic enzyme which catalyzes the biosynthesis of branched-chain amino acids[19-21]. AHAS is an essential enzyme within plants in which specific AHAS inhibitors can potentially be used as herbicides[22,23]. AHAS is comprised of a larger catalytic subunit and a smaller regulatory subunit[24]. The larger harbors a conserved thiamine diphosphate (ThDP) binding site, requiring Mg^{2+} to attach to the diphosphate moiety of ThDP to the active site. The catalytic subunit of AHAS has one flavin adenine dinucleotide (FAD) molecule per active site, which is necessary for catalytic activity, because FAD contributes to structural maintenance and protects the reaction intermediate in the catalytic cycle[21,24,25].

AHAS from *Mycobacterium tuberculosis* has proven particularly useful in the pursuit of new inhibitors. Several multi-drug resistant strains of *M. tuberculosis* have been developed, requiring the development of novel drug target proteins. Recently, branched amino acids in the auxotrophic strain of *Mycobacterium* have failed to propagate as the result of their inability to utilize their hosts' amino acids, which indicates that inhibitors for branched-chain amino acid biosynthesis may be employed as anti-*Mycobacterium* agents[26]. AHAS appears to be a probable target of novel anti-tuberculosis (TB) drugs.

Choi *et al*. reported a successful attempt at the cloning, expression, and purification of the catalytic and regulatory subunits of AHAS from *M. tuberculosis*[27]. A colorimetric assay technique was utilized for the screening of 5,600 compounds constructed from a combinatorial chemical library in order to recognize molecular inhibitors for *M. tuberculosis* AHAS[27]. Four structurally related compounds were identified, all of which could inhibit the catalytic subunit of AHAS. These compounds were designated as KHG-20612, KHG20614, KHG20613, and KHG20613, and can be distinguished by a disulfide bond harboring phenyl and 1-substituted triazolyl derivatives (Figure 3). This dose-dependent inhibition evidences IC_{50} values in a range of 1.8–2.6 μM. These novel compounds appear to be very promising, potent inhibitors of *M. tuberculosis* AHAS.

Conclusion

SELEX is currently emerging as a reliable method for inferring and quantifying protein-nucleic acid interactions. The overwhelming experimental and theoretical evidence indicates that SELEX can efficiently select the strongest binders from large nucleic acid libraries, which in most cases saturate the relevant sequence space. The most potent affinity binders acquired via the SELEX technique have important research, diagnostic, and therapeutic applications; however, the standard SELEX procedure is generally not suitable for the correct determination of protein-nucleic acid binding parameters, from which the binding affinity of any sequence specific binder can be identified.

On the other hand, recent advances in the SELEX protocol allow for the determination of protein-DNA interaction parameters with unprecedented accuracy. In particular, the fixed stringency/high-throughput SELEX procedure allows for the robust sequencing of a large number of medium-to lower-affinity sequence specific binders, under controlled condi-tions. The interaction parameters can be determined with a high degree of accuracy from such a dataset using a quantitative physical understanding of SELEX experiments, along with the developed bioinformatics techniques. The determined parameters, in turn, allow for the reliable detection of putative protein binding

sites in genomic DNA. Therefore, such methodologies can be applied to a large number of different DNA binding proteins, which would facilitate a comprehensive understanding of gene regulation.

As an alternative approach, genomic SELEX can be employed to determine genome-derived nucleic acid sequences with high degrees of binding affinity to a given protein target. Genomic SELEX has been successfully utilized to isolate profound genomic binding sequences in a number of organisms[28], but this method is generally biased against weaker interactions, which may prove to be highly important for drug function. While not yet realized in practice, the combination of genomic SELEX with the fixed stringency/high-throughput SELEX procedure should help to alleviate this bias.

The possible inhibition of these compounds as antibiotics has been previously speculated, since the infected bacteria could take amino acids up from their host, although these compounds block the biosynthetic pathways of infected bacteria. According to the results of a test of recombinant *M. tuberculosis*, the AHAS large subunit evidenced differing sensitivities to the tested compounds, particularly imidazolinones, thereby indicating that the active site of *M. tuberculosis* probably differs from the active sites of the AHAS enzymes observed in other organisms[22,23]. Sulfonylurea herbicides consist of a sulfonyl group containing two rings[22,23]. An additional CH_2 group within the sulfonylurea of bensulfuron methyl reduced the inhibition effects of *M. tuberculosis* AHAS. From the analysis of the structure for active sulfonylureas herbicides, the 1-substituted group within the benzene ring exerts a critical effect on the inhibition of *M. tuberculosis* AHAS.

The newly identified compounds from the screening of the combinatorial chemistry library were shown to efficiently inhibit the activity of the *M. tuberculosis* AHAS enzyme, and evidenced completely different structural features than were associated with sulfonylureas or imidazolinones. KHG 20612 also evidenced inhibitory activity against the growth of several *M. tuberculosis* strains, including the drug-resistant strains. The screening method used for inhibitors of *M. tuberculosis* AHAS, and the utilization of newly identified inhibitory compounds may contribute to the development of more potent and novel anti-TB agents.

In summary, different methodologies predicated on SELEX have the potential to reliably infer the nucleic acid binding specificity of molecules, including proteins, peptides, and organic compounds. The combinatorial chemical library approach is also a very promising method in the development of new types of inhibitors. These two potential methods will likely result in the novel, routine discovery of drug candidates in the future.

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Abbreviations

AHAS, acetohydroxyacid synthase; FAD, flavin adenine dinucleotide; IC_{50}, 50% inhibition concentration; SELEX, Systematic Evolution of Ligands by EXponetial enrichment; TB, Tuberculosis, ThDP, thiamine diphosphate; VEGF, vascular endothelial growth factor.

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