Phage display for target-based antibacterial drug discovery

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Increasing bacterial drug resistance and hard-to-eradicate opportunistic infections have created a need for new antibiotics. Sequencing of microbial genomes has yielded many new potential targets for antibacterial drug discovery. However, little is known about the biochemical activities of many of these targets, making it difficult to develop HTS assays for them. Peptides isolated by phage display can be used as 'surrogate ligands' in competition assays for screening of targets of unknown function with small-molecule libraries. These screening assays can be adapted into a variety of high-throughput formats, including those based on radioactive, luminescence or fluorescence detection.

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▼ Since the discovery of penicillin almost 60 years ago, numerous antibacterial agents have been produced to treat infectious diseases; this has resulted in dramatic reductions in illness and death. By the 1980s, it was believed that industrialized nations had developed all of the tools necessary to control microbial pathogens[1]. Widespread use of antibiotics, however, provided powerful selective pressure for mutations that conferred resistance to these antibacterial agents. These resistance mutations have spread through bacterial populations so pervasively that antibiotic-resistant strains have seriously compromised the ability to treat many infectious diseases. When penicillin was first introduced, for example, almost all isolates of *Staphylococcus aureus* were susceptible. By 1990, approximately 90% of *S. aureus* isolates were resistant to penicillin[2]. New β-lactam antibiotics, such as methicillin, were developed to combat resistance in *S. aureus*. However, methicillin-resistant *S. aureus* (MRSA) strains now represent ~50% of the clinical isolates in the USA[3]. Originally seen only in hospitals and other long-term-care facilities, community-acquired cases of MRSA are now being reported[4]. Many MRSA strains have accumulated resistance determinants to other antibiotics, such as erythromycin, gentamycin and quinolones[5]. This often leaves glycopeptide antibiotics, such as vancomycin, as the treatment of 'last resort'. Recently, however, clinical isolates of MRSA with reduced susceptibility to vancomycin were reported in Japan and the USA[6–8]. This is consistent with recent evidence for the transfer of genetic material between bacteria, and even between distinct bacterial species[9,10]. These findings indicate the potential emergence of strains of *S. aureus* that are completely resistant to antibacterial chemotherapy.

In addition to *S. aureus* and other Gram-positive pathogens, Gram-negative pathogens are becoming increasingly resistant to antibiotics. Farm animals that are fed antibiotics can be a reservoir for antibiotic-resistant bacteria, and resistant pathogens can contaminate food products at the time of slaughter and be transmitted to humans[11,12]. In 1999, a survey by the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) found that 49% of *Salmonella typhimurium*, 91% of *Shigella* species, 53% of *Campylobacter* species and 10% of *Escherichia coli* O157 clinical isolates were resistant to one or more commonly used antibiotics (www.cdc.gov./ncidod/dbmd/narms). Of the *S. typhimurium* isolates, 28% were resistant to five or more antibiotics.

Overcoming antibacterial resistance requires several approaches. These include changes in healthcare practices, such as prudent use of antibacterial agents and control of transmission[13,14], as well as a reduction in the use of antibiotics in animals. One of the most effective approaches, however, is the development

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of novel antibacterial agents with unique modes of action[14–16]. Existing antibiotics fall into a relatively small number of classes with respect to mechanism of action. To date, bacteria have been able to subvert every one of these drugs, making the discovery of new antibacterials an important goal.

Opportunistic and emerging infections

Opportunistic pathogens, some of which might also be multidrug-resistant, present another challenge for the development of novel antibacterial therapeutics. These organisms are often hard to treat with existing antibiotics because of immune dysfunction or other conditions in the host, which promote the growth of the pathogen. The population of immunocompromised patients comprises organ transplant recipients, chemotherapy patients and people with AIDS. Patients who are immunocompromised because of HIV are infected by many pathogens, including bacteria such as *Mycobacterium tuberculosis*, viruses, fungi and protozoa. Although highly active antiretroviral therapy that restores the CD4 cell count is generally protective against these opportunistic infections, anti-infective therapy remains an important consideration for treatment of these patients[17]. However, current treatments are hampered by drug interactions, toxicity and the development of resistance. For example, the antituberculosis drug isoniazid, and HIV protease inhibitors are hepatotoxic, and rifampicin interacts with protease inhibitors, reducing their efficacy[17,18].

Another example of a disease complicated by opportunistic pathogens is cystic fibrosis (CF). In CF, mucociliary clearance of bacteria from the lungs is impaired by the viscous nature of airway secretions that is characteristic of the disease[19,20]. As a result, the lungs of CF patients are colonized by a succession of opportunistic pathogens. Susceptibility to a particular organism is related to age, with *S. aureus*, *Haemophilus influenzae* and *Streptococcus pneumoniae* colonization occurring in early childhood and *Pseudomonas aeruginosa* infections occurring in late childhood and into adulthood[21]. In the 1980s, clinical centers reported the emergence of *Burkholderia cepacia*, a multidrug-resistant organism, as an important pathogen in CF patients[20]. The deleterious consequences of infection by these organisms include bronchiolitis, atelactasis, hemoptysis, pneumothorax, fibrosis and, finally, respiratory failure; these constitute the major causes of morbidity and mortality in CF patients[19]. Overall, *P. aeruginosa* remains the most frequently reported pathogen in CF patients; in one study, >80% of CF patients were found to be infected by the age of 26 (Ref. 22). *P. aeruginosa* is difficult to eradicate because of its intrinsic resistance to existing antibiotics[23,24]. Therefore, the development of more-effective drugs is urgently needed.

The development of new antibiotics would also be useful to treat emerging diseases that are noted to be on the increase or whose causes have only recently been discovered. Existing antibiotics might not be useful against such emerging organisms, creating the need for rapid development of narrow-spectrum drugs. Knowledge of the biology of individual pathogens, in conjunction with genomic sequence information, should result in the development of highly specific antibiotics. Genomic information could also be used to identify common targets in many species of bacteria for the design of broad-spectrum antibiotics.

Bacterial genomics and target identification

The recent development of technology for efficient genomic sequencing has resulted in the complete sequencing of numerous bacterial genomes[25–30]. Currently, the DNA sequences of >30 bacterial genomes have been determined, and the sequencing of another 100 bacterial genomes is in progress. Because many of these genomes are from human pathogens, a wide range of targets involved in different microbial pathways is now accessible for antibacterial drug discovery.

The basic criterion for the selection of a gene product as an antibacterial target is that it is essential for the survival of the pathogen in the host[1,31]. Thus, several different methods have been used to analyze bacterial genomes and to estimate the minimal gene-set required for viability of a microbe. Comparative computer analysis of the *H. influenzae* and *Mycoplasma genitalium* genomes led to the estimation of 256 genes as the minimal gene-set that is necessary and sufficient to sustain the existence of a bacterial cell[32]. Hutchinson and coworkers used transposon mutagenesis to identify non-essential genes from the *M. genitalium* genome and estimated that there are between 265 and 350 essential protein-coding genes[33]. Both methods gave estimates that are considerably larger than the number of known targets for currently existing antibiotics[1].

Whereas many of the genes share sequence similarity among bacteria, and presumably have similar functions to known gene products, a significant number of genes have unknown functions. For example, approximately 40% of the genes in the *E. coli* genome sequence have no known function[26]. Many of these genes of unknown function could be shown to be essential for cell growth and could, therefore, be good targets for antibacterial drug discovery[31]. However, many of these targets will be impractical to screen using conventional HTS-compatible assay methods because most current target-based antibacterial drug discovery strategies rely on biochemical activity to screen for

inhibitors. Many of the gene products known to be essential for bacteria do not possess enzymatic activity. There is a clear need for technologies to accelerate the antibacterial drug discovery process that allow for screening of previously unscreenable targets. Phage display techniques provide one approach to solving this problem.

Technological advances in M13 phage display

Phage display of combinatorial peptide libraries and affinity selection technologies were pioneered by George P. Smith[34,35]. Phage-display technology has proved to be a powerful tool for isolating ligands for drug discovery[36,37], affinity chromatography[38,39], studying protein–protein interactions[40], epitope mapping of antibodies[41–43], isolating antibody fragments[44,45], engineering the binding affinity of displayed proteins[46–48] and identifying peptides that 'home' to organs or tissues[49,50].

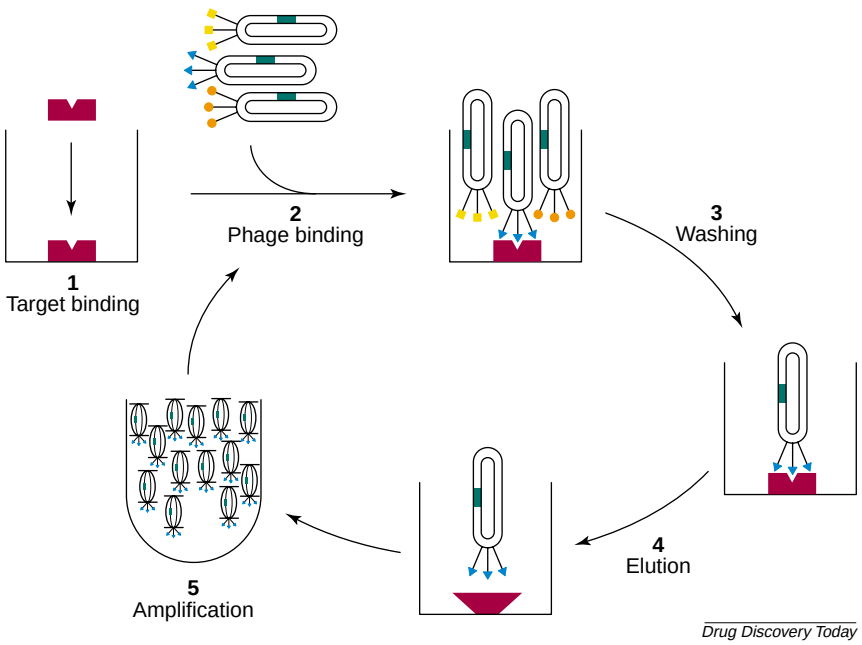

Figure 1. The process for affinity selection of phage-displayed peptides. Step 1, the target protein is immobilized; Step 2, a phage library is added and allowed to bind to the target protein; Step 3, the plate is washed to remove unbound phage; Step 4, the target protein is denatured to elute the bound phage; and Step 5, the eluted phage are amplified by infection of bacterial cells and the process is repeated with the enriched phage.

The foundation of the technology is the construction of a library of variants of polypeptides fused to a bacteriophage coat protein; the DNA encoding the polypeptide is contained within the phage particle. This creates a physical linkage between phenotype and genotype that allows for affinity selection from the combinatorial library of those phage particles displaying peptides that bind to a target of interest, while retaining the genetic identity. Phage isolated in one cycle of affinity selection can be amplified by infection of *E. coli* host cells and used for additional cycles of affinity selection.

The typical protocol for affinity selection, summarized in Figure 1, begins with immobilization of the target on the surface of beads or the wells of a microtiter plate (Step 1). Phage from the library are then incubated with the immobilized target (Step 2) to allow the phage particles that display peptides with affinity for the target protein to bind. The plate is washed to remove unbound phage (Step 3) and the bound phage are eluted from the plate by denaturation of the target protein (Step 4). The eluted phage are incubated with *E. coli* cells to allow for infection and amplification of the subpopulation of phage that display peptides with affinity for the target (Step 5). The amplified phage are used for additional cycles of selection until only phage displaying specific, tight-binding peptides remain. At this point, individual phage are isolated, the DNA sequence encoding the displayed peptide is determined and the peptide sequence is deduced. The affinity selection process has been performed on numerous target classes and also using many methods for immobilization of the target, as reviewed elsewhere[39,51].

Phage display was originally developed with peptides fused to the N-terminus of the phage coat protein PIII (Refs 34,52). This context allows for pentavalent presentation of each unique peptide, with a lack of steric hindrance at the N-terminus. Short peptides (<10 amino acids) can also be displayed at the N-terminus of the major coat protein pVIII (Ref. 53). These simple phage-display systems are very useful for the identification of peptide ligands. However, they are not very useful for larger polypeptides, where the fusions often have a detrimental effect on the function of the coat protein. This problem can be overcome by the use of two-gene phagemid systems, where the polypeptide is fused to a phage coat protein in the phagemid vector and wild-type coat protein is supplied by a helper phage.

Although pIII or pVIII display systems remain the most popular, other M13 coat proteins have been shown to be useful for display and selection. Jespers and colleagues[54] demonstrated that a cDNA expression library can be fused to the C-terminus of the pVI protein. Phage coat proteins pVII and pIX have been used to display antibody fragments[55].

Table 1. Detection of small molecules with peptides

Assay format and detection method	Detection label	Target modification	Peptide modification
Target-on-plate–scintillation proximity assay (TOP–SPA)	^{35}S–SA	Immobilized	^{35}S–SA
Target-on-plate–time-resolved fluorescence (TOP–TRF)	SA–Eu	Immobilized	SA–Eu
Peptide-on-plate–time-resolved fluorescence (POP–TRF)	SA–Eu	SA–Eu	Immobilized
Fluorescence polarization (FP)	Fluorescein	–	Fluorescein
Fluorescence resonance energy transfer (FRET)	SA–Eu or SA–APC	SA–APC	SA–Eu

Abbreviations: APC, allophycocyanin; Eu, europium chelate or europium cryptate; SA, streptavidin.

In addition, the utility of pIII and pVIII systems has been extended by the development of C-terminal display systems. Fuh and coworkers[56] described the addition of a linker to the C-terminus of pVIII, thereby allowing the display of peptides fused to the end of the pVIII protein. This C-terminal pVIII phagemid system was used for the successful identification of peptides that interact with PDZ domains. Modifications to the pIII protein have also allowed the display of polypeptides fused to the C-terminus of pIII (Ref. 57). These modifications make it possible to perform phage display of both N-terminal- and C-terminal-fused polypeptides on the surface of the phage, and to establish display systems for every coat protein of the M13 phage.

Phage display in antibacterial drug discovery

The use of peptides derived from phage display for drug discovery has most often been associated with attempts to use peptides as drugs or with peptidomimetic chemistry[58,59]. However, peptides directed to key functional sites on the target protein can be used as surrogate ligands in HTS. Ligand-displacement assays are common for cytokine receptors[60], nuclear hormone receptors[61] and G-protein-coupled receptors[62], and numerous detection technologies and screening formats have been developed for ligand-displacement assays[63,64]. Therefore, if the peptides isolated from phage display are useful as surrogate ligands for the detection of small molecules, this technology would provide a unique approach to screening a wide variety of antibacterial targets.

An evaluation of phage-display-derived peptides as surrogate ligands for the detection of small molecules was recently reported[36]. Interestingly, these peptides were shown to be potent inhibitors of enzyme function and to be effective as surrogate ligands for the detection of small-molecule inhibitors of enzymatic function in ligand-binding-type HTS assays[36,37,65,66]. This validated the use of peptides derived from phage display for the discovery of small-molecule leads. Enzyme active sites are not the only binding site of these peptides; indeed, it is clear that exosite binding by peptides can also inhibit the biological activity of a given target protein[67]. Whereas peptide binding directly to active sites explains the inhibition of target protein activities in some instances, allosteric binding plays a role in other cases[67]. Crucial sites of protein–protein interaction can also be targeted by this approach[68,69]. All of these interaction possibilities are supported by crystallographic studies that have shown the specificity and affinity of such peptides for protein functional sites[70–73].

The extension of this technology to targets of unknown function, however, requires either reliance on evidence gathered from targets with known function or the development of technologies for the validation of peptides that bind to targets of unknown function. Intracellular expression of the peptide surrogate ligand has proved to be a suitable method for validation of peptides.

Regulated expression of peptides inside cells has been demonstrated by many approaches. In an intracellular peptide-selection scheme, Norman and coworkers expressed peptides fused to an inactive variant of *Staphylococcus* nuclease in yeast cells[74]. Selection was then performed to identify intracellular peptide inhibitors of pheromone signalling, transcriptional silencing and the spindle checkpoint. Norris and coworkers demonstrated that peptides identified by phage display, which bind to specific ligand-bound forms of the human estrogen receptor, disrupt estrogen-receptor-mediated transcription when expressed inside cells[75].

The use of peptides identified by phage display to inhibit the function of target proteins inside a bacterial cell has also been reported[76,77]. Peptides that bind to essential targets of known function were selected using phage display. These peptides were then expressed as fusions to glutathione-*S*-transferase (GST) under the control of a tightly regulated promoter. Induced expression of the peptide–GST fusion inhibited growth of the bacterial cells. Growth inhibition caused by inactivation of the specific target protein was demonstrated by coexpression of the target protein, which resulted in rescue from the inhibitory

effect of the expressed peptide[77]. Further, intracellular expression can be used to validate peptide binding at a crucial site for the biological activity of a target of unknown function. If a target is essential for growth of the bacteria, peptides that bind to a functional site will inhibit the function of the target and inhibit bacterial growth.

Direct binding of compounds to a target protein is another method that has been developed to assay targets of unknown function. When a compound binds to a protein, the thermal melting temperature of the protein changes and can be detected by microcalorimetry or binding of other fluorescent probes[78,79]. However, these assays cannot be validated for use as HTS assays because compound binding is detected for binding at any site on the target protein. This includes binding at sites that are not crucial for the function of the target protein. Therefore, the use of peptides as surrogate ligands provides the only available method for HTS with targets of unknown function that allows for validation of the assay before screening.

Surrogate ligand HTS assays

Assays for HTS using peptides as surrogate ligands can be adapted to nearly any available detection format. Peptides have been used extensively for ligand-binding assays with radioactivity, luminescence or fluorescence for detection[60–64]. HTS assays using peptides from phage display have been reported using each of these methods, as well as homogeneous methods such as fluorescence polarization (FP) and fluorescence resonance energy transfer (FRET). Finn and coworkers recently disclosed an additional assay where complex formation between a target protein and a fluorescently labelled peptide is monitored by capillary electrophoresis using fluorescence detection[66].

In another study, the ability to detect a series of four inhibitors of tyrosyl-tRNA synthetase was determined for assays using many detection methods[36]. With the exception of FP, each method requires the protein and peptide to be labelled or immobilized. The detection methods and labelling groups used for labelling or immobilization of the peptide and protein in these assays are summarized in Table 1.

Each assay format was tested for the ability to detect known inhibitors and to determine the potency of each inhibitor. The concentration of compound required to reduce the signal by 50% (IC_{50}) was determined for each inhibitor using the functional assay and each peptide-based assay format. The results, summarized in Table 2, show that the observed IC_{50} values for each compound remained fairly consistent between assay formats. This versatility indicates that the use of phage-displayed peptides as surrogate ligands for HTS will allow the user to customize the assay format and detection technology for their instrumentation, rather than requiring costly new equipment.

Table 2. Comparison of assay methods for inhibitor detection

	Inhibitor IC_{50} (μM)			
Assay format	**NPC0101**	**NPC0102**	**NPC0103**	**NPC0104**
Biochemical	0.04	0.20	3.0	1.6
TOP–SPA	0.03	0 .19	7.0	18
TOP–TRF	0.01	0.12	6.0	10.0
POP–TRF	0.02	0.24	3.7	10.0
FP	0.02	0.22	2.1	3.6
FRET	0.03	0.45	6.8	12.2

Abbreviations: FP, fluorescence polarization; FRET, fluorescence resonance energy transfer; POP, peptide-on-plate; SPA, scintillation proximity assay; TOP, target-on-plate; TRF, time-resolved fluorescence.

Conclusion

During the 1990s it became clear that, if drug resistance is to be combated successfully, new antibacterial compounds must be developed continually. The whole-genome sequencing of bacterial species has progressed rapidly during the past decade, producing a large number of new targets for antibacterial drug discovery. However, many of the genes, even in well-studied organisms such as *E. coli*, encode proteins with no known biological function, thus complicating drug discovery efforts. A novel approach, which is especially useful for drug discovery efforts aimed at targets of unknown function, uses peptides identified using phage-display methods as surrogate ligands for HTS and target validation. Although this technology is rather recent, its use to discover new antibacterial leads has been reported[65,66,80] and the number of such discoveries is expected to increase as the technique becomes more widely used.

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