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Targeting Beta-Lactamase Enzymes with Phage Display: Challenges, Advances, and Future Prospects

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Abstract

The emergence of beta-lactamases has rendered antibiotics resistance, a major public health threat worldwide, which is accompanied by an enhanced capacity for morbidity and mortality due to bacterial infections. This review has identified the mechanisms of beta-lactam resistance and stressed the need to develop new therapeutic strategies and drugs. Phage display technology has been identified as a potential technique for the discovery of novel beta-lactamase inhibitors, thereby enabling the rapid and systematic analysis of peptide and protein libraries. We have discussed the theory of phage display, the processes involved in identifying inhibitors through it, and the difficulties in moving the discoveries to clinical practice. The review of the literature has identified some critical weaknesses, including the complexity of the protein interactions, the rapid bacterial resistance mechanisms, and the challenges in the drug development pipeline. From the available information and the latest developments, this review aims to discuss how phage display technology can be used to fight antibiotic resistance and develop new therapeutic strategies for bacterial strains that are resistant to antibiotics.

Keywords: Phage Display, Beta-lactamase Inhibitors, Antibiotic Resistance, Peptide Screening, Drug Discovery

1. Introduction

Antibiotic resistance (ABR) is currently a major global health crisis that is varying across regions and is causing many complications. This is a major threat to public health resulting in increased morbidity, mortality and healthcare costs. First, we will look at the current state of antibiotic resistance, specifically beta-lactamase resistance. This makes it a big threat to public health and results in more sickness, death, and healthcare costs. Here, we will review today's antibiotic resistance, focusing on beta-lactamase resistance. We will also examine public health policies designed to tackle this issue. In 2019, antibiotic resistance directly caused around 1.27 million deaths from bacterial infections. Another 4.95 million deaths were related to drug-resistant infections worldwide [1]. The World Health Organization (WHO) sees AMR as a critical public health threat, and it needs action now. A recent study showed that 569,000 deaths in the Americas were related to bacterial AMR in 2019. This is over 43% of all infection-related deaths in that region. The most dangerous bacteria included *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* ([2]). Experts think AMR could cause about 5.2 million deaths in the Western Pacific by 2030. They also estimate an economic impact of US$ 148 billion because of decreased productivity and increased healthcare costs [3].

1.1 Beta-Lactamase Resistance

Beta-lactam antibiotics are among the most used antibiotics worldwide. Unfortunately, bacteria that produce beta-lactamase have greatly reduced their effectiveness. For instance, *Escherichia coli* strains producing extended-spectrum beta-lactamases (ESBLs) are now very common. This complicates treatment options in many countries. In places like the United States, antibiotic resistance is still a big worry. The CDC reported that resistant infections lead to about 35,000 deaths each year. This highlights the need for better programs for using antibiotics wisely [4]. In areas like South Asia and Sub-Saharan Africa, the problem is even worse. This is due to poor healthcare systems and antibiotics being readily available over the counter. For instance, countries with such problems include India which uses antibiotics extensively, thus leading to the development of resistant infections. The WHO has come up with a plan to address AMR through the implementation of NAPs. More than 170 countries have put in place the NAPs in order to enhance the capacity of surveillance, promote the appropriate use of antibiotics and enhance IPC [1]. WHO has also listed 13 key actions that countries should consider in the fight against AMR. These include enhancing the coverage and quality of health services, increasing the understanding of policymakers and the public, and integrating AMR management into primary healthcare [1].

The US National Action Plan for Combating Antibiotic-Resistant Bacteria has been reissued to focus on prevention of infections and appropriate use of antibiotics in population healthcare including patients and food-producing animals. This applies to both human and animal medicine [4]. For instance, the PASTEUR Act is an example of the laws that seek to encourage the development of new antibiotics. They also seek to ensure that current antibiotics are used correctly. The EU has adopted the One Health approach in its AMR plan which incorporates the human, animal and environmental dimensions. This includes ban on the use of antibiotics in farming and improvement of the surveillance systems [4]. For instance, countries that have published their NAPs and funded AMR efforts have lower mortality rates from drug-resistant infections than countries that have not done so. On the other hand, MDR infections cause high

mortality rates in countries like Haiti and Venezuela. This is partly because these countries have not adopted effective national policies or have the necessary resources to fight AMR. Beta-lactamase resistance is one of the biggest global problems that has different manifestations in various regions. Despite the progress being made through international partnerships and the development of the national action plans, more needs to be done. More surveillance, education and cooperation are very important to address this issue. If there is no action from all the groups that include governments, healthcare providers, researchers and the public then the effects of antibiotic resistance may increase significantly in the next few years.

Antibiotics are also used correctly and improperly in healthcare, animal farming, and aquaculture, which have led to the transfer of resistance genes in the environment [5,6]. There are many ways by which bacteria acquire resistance; these include: by reducing the permeability of the cell to the antibiotic, by altering the cellular targets of the antibiotic, by inactivating the antibiotic and by ejecting the antibiotic from the cell [7]. These resistance genes can be as old as the ancient microbes that produce antibiotics. Therefore, as the populations of the pathogenic microbes that are resistant to the available antibiotics increase, new drugs are required and other means of controlling the bacteria [6]. Other potential strategies that may be useful include the use of antibiotic synergism, adjuvants and hybrid antibiotics [6]. Synergistic combinations are emerging. This approach entails the use of two or more antibiotics to enhance the efficacy of the treatment compared to the use of each antibiotic alone. For instance, the combination of beta-lactams with aminoglycosides is known to improve bacterial killing using different pathways and therefore reducing the chances of resistance development [8,9]. Adjuvants are another approach. These are substances that increase the potency of antibiotics but are not antibacterial themselves. They can act as inhibitors of bacterial pumps or as agents that disrupt biofilms. Biofilms can protect bacteria from antibiotics [10]. For example, using efflux pump inhibitors along with standard antibiotics has been shown to help restore antibiotic sensitivity in resistant *E. coli* strains. Also, hybrid antibiotics are a new group of antimicrobials designed to overcome resistance. These are made by linking two pharmacophores. Usually, this means linking an antibiotic with another antibiotic or adjuvant to create a single molecule that has the benefits of both. A good example is ciprofloxacin and neomycin which has better antibacterial activity against Gram-positive and Gram-negative bacteria and also slows resistance development [9].

It's estimated that antibiotic-resistant infections lead to around 700,000 deaths per year worldwide. This number could significantly rise if we don't act. So, it is very important to understand synergistic combinations, adjuvants, and hybrid antibiotics to help protect public health from antibiotic resistance. Beta-lactam antibiotics, such as penicillin and cephalosporins, are widely used to treat infections. They function by recognizing penicillin-binding proteins and disrupting the bacterial cell wall [11]. However, there is a challenge of resistance to these antibiotics by the bacteria. They achieve this primarily through the production of beta-lactamase enzymes that inactivate the antibiotics [12]. To combat resistance, combination therapy has been shown to be effective [13]. This includes the use of beta-lactam antibiotics with beta-lactamase inhibitors. This enables us to recycle existing antibiotics that are known to be safe and effective [13]. Recent study is aimed at finding new beta-lactamase inhibitors to counteract the effects of these enzymes and enable beta-lactam antibiotics to be effective against resistant bacteria [14]. It is crucial to know how antibiotics work and how resistance develops to design ways of combating this emerging threat [11].

1.2 Importance of Beta-lactam Antibiotics and Mechanisms of Resistance (Beta-lactamases)

Bacterial infections are usually treated with beta-lactams, penicillin and cephalosporin. They do this by attacking penicillin binding proteins and hence leading to the destruction of the cell walls [11]. However, there is a challenge of resistance to these antibiotics, and this has been noted in bacteria through the production of beta-lactamases [15]. Serine beta-lactamases are derived from penicillin-binding proteins and can bind and break down beta-lactams, rendering them inactive [16]. To combat this resistance, researchers have created beta-lactamase inhibitors. They have employed different tactics to inhibit these enzymes [16]. Gram negative bacteria also manifest resistance through changes in permeability, efflux pumps and alterations in the targets[17]. The rapid rise in beta-lactam resistance requires a coordinated effort to deal with this global threat [15].The purpose of this review is to discuss the available literature on the application of phage display technology to overcome antibiotic resistance through beta lactamases. Antibiotic resistance, particularly by way of beta lactamase production, has caused a problem with antibiotics. We will see how phage display can be used to identify new inhibitors. It is our aim to present the challenges, results and prospects of this approach for combating the beta-lactamase resistance.

2. Introduction to Phage Display Technology

Phage display is the process of genetically incorporating peptides on the surface of bacteriophages. This has changed biological research [18]. It has a number of applications in cancer research, cell biology, pharmacology and immunology [18,19] Jaroszewicz et al., 2021). With it, we can explore protein interactions, discover bioactive peptides, antigens of disease, and cell-specific peptides [18]. The phage display systems have been continually developed and have found many applications in biotechnological processes, including generation of new materials [19]. The relevance of this technology was established when the 2018 Nobel Prize was awarded for it [19]. In addition to phage display, other peptide display methods have also been developed using bacteria, yeast, and mammalian cells, as well as eukaryotic viruses and cell-free systems [19]. These new techniques in microbiology have led to the creation of sophisticated tools that find application in science, medicine, and biotechnology [20].

Phage display technology has been adopted as an important strategy in the development of drugs. It is particularly valuable for the design of peptide drugs and monoclonal antibodies [21,22].. This method allows us to identify peptides and antibodies that can recognize their target compounds with high sensitivity and specificity [22,23]. It has also helped to solve some issues inherent to initial antibody drugs, such as immunogenicity. This has made it possible to produce human monoclonal antibodies [21]. The recent advances in phage display include the application of next-generation sequencing and microfluidics. This has enhanced the throughput and productivity of the screening process [22]. New strategies for antigen presentation and selection have also been described. These assist in identifying antibodies that have unique recognition requirements, including pH-sensitive binding or cross-reactivity [24]. Therefore, phage display remains an

important strategy in the production of protein-based therapeutics for the treatment of various diseases [23].

2.1 Basics of Phage Display Technology

Phage display is the process of genetically engineering phage DNA to express a peptide, protein or antibody fragment on the phage surface. The molecule is physically attached to its DNA sequence. This enables us to pick out certain clones and determine their amino acid compositions. It is capable of presenting more than one billion random sequences of variants on the phage surface [25]. In order to generate a random peptide library, scientists first produce random DNA sequences that encode for different peptides. To do this, the codons that are randomized are made of synthetic DNA (oligonucleotides). These randomized oligonucleotides contain a mix of nucleotides at certain positions. This creates a variety of amino acid sequences [26]. The DNA can be completely randomized (allowing any of the 20 amino acids). They can also be biased toward certain amino acids by changing the ratio of nucleotides. This helps fine-tune the library's diversity [27]. Once the DNA sequences are made, they are put into a bacteriophage genome to create a library. This randomized DNA is typically inserted in-frame into a coat protein gene, usually the pIII or pVIII genes in M13 phages. This allows the encoded peptide to be displayed on the surface of the phage particle (Hoess, 2001). Specialized phage vectors or phagemids are used for this. Phagemids have both phage and bacterial origins of replication. This allows the fusion protein to be efficiently displayed on the phage surface[28]. The recombinant phage DNA is then put into *E. coli* cells to help the phage multiply. The DNA with random peptide-coding inserts is changed in *E. coli* cells where they are packaged into phage particles. Each transformed cell produces phages that display a unique peptide sequence. Collectively, a large and diverse library of phage particles is created. Large-scale transformations (millions to billions of clones) make sure there is a wide variety of peptides [29]. The quality of the library is then verified to ensure it represents diverse and functional peptides. The library's diversity is evaluated by sequencing a subset of phage clones. Libraries with around 10^9 distinct clones are considered robust, offering a good chance of identifying strong binders [30]. The library is tested by screening against known targets to confirm functional peptides are displayed and accessible for binding.

2.2 Mechanisms of Selection and Enrichment

The steps of selection and enrichment involve creating a diverse library of phage clones displaying different peptides or proteins (see Fig. 1). The library is exposed to an immobilized target molecule to allow binding. The unbound phages are washed away, as well as the weakly bound phages. The bound phages are eluted, often by enzymatic cleavage or shift in pH. The collected phages are then propagated on *E. coli*. The binding elusion-amplification process is then repeated 3-5 times to enrich for phages that are specific to the target significantly.

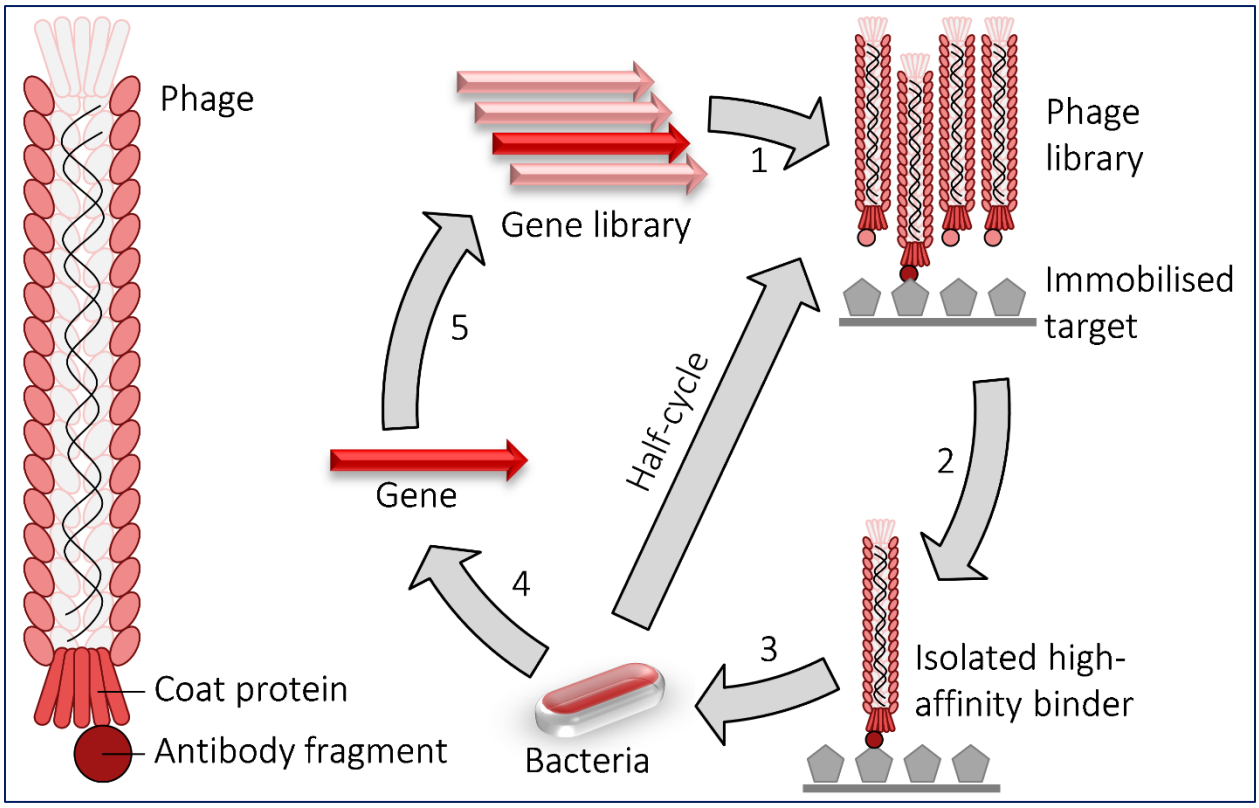

Figure 1: Schematic Representation of the Phage Display Technique (Reproduced from https://upload.wikimedia.org/wikipedia/commons/4/49/Phage_display.png)

3. Applications of Phage Display in Drug Discovery

3.1 Identifying interaction partners of organic and inorganic compounds for in vitro protein engineering

Phage display is a very valuable tool for studying protein-protein interactions. It can also indicate the binding partners for different targets including organic and inorganic molecules [31]. This method is employed for identifying high affinity peptide ligands that recognize proteins, polymers and small organic molecules [31]. Specifically, it is useful for the analysis of domain-motif interactions and the mapping of antibody epitopes [32]. The phage display libraries can be made using genomic DNA, cDNA or oligonucleotides which are designed to represent particular proteome regions [32]. The technique has been used to incorporate in vivo tight binding protein variant enrichment. This eliminates the need for target protein purification and immobilization [33]. This method employs both receptor and ligand genes on the same plasmid to select the tight binding complexes from the bacterial periplasm [33]. Phage display is a very important tool in chemical biology, and this technique can also be used to develop and improve monoclonal antibodies and to identify interacting protein partners [34].

3.2 Designing humanized antibodies or peptides for various diseases like cancer, autoimmune disorders, etc.

An effective way of producing therapeutic antibodies and peptides for various diseases is phage display. It has been used to produce fully human antibodies that are more effective clinically and less immunogenic [35]. It has been employed in the identification of immunodominant epitopes in autoimmune diseases such as Goodpasture's syndrome and systemic lupus erythematosus [36]. Of importance, phage display enables us to select autoantibody repertoires and design antibody fragments that may prevent pathogenic autoantibodies [36,37]). This method has also been employed to investigate protein-ligand interactions, enhance antibody affinity, and determine epitopes and mimotopes [38]. By 2016, six human antibodies produced by the phage display technique have been approved for treatment, and adalimumab (Humira®) was the first and the most successful antibody drug on the market [35].

3.3 Validating drug targets and screening for inhibition strategies using small molecules, antibodies, and peptides

Phage display is currently used for the identification of new drugs and discovering the targets for such drugs ([39,40],. It facilitates the detection and selection of ligands with high affinity to their targets, be it peptides, antibodies or other proteins [41]. New strategies have been introduced to broaden its impact on biosensing, molecular imaging and vaccine production [40]. More than 20 antibodies and peptides are in the last stage of clinical trials or are already on the market due to phage display [41]. A new way of applying phage display for identifying covalent inhibitors has been reported. This enables the identification of highly selective cyclic peptides that can permanently block the activity of a given enzyme with nanomolar efficacy [42]. Thus, as the technology is continuously being improved, phage display is set to play an increasingly important role in opening new regions of the proteome for drug discovery and generation of small-molecule drugs [39].

3.4 Using phages as drug and gene delivery vehicles to specific tissues

Bacteriophages are currently investigated as potential carriers of drugs and genes to particular organs and tissues. They are better than eukaryotic viral vectors as vectors [43]. They are safe, long-lasting and genetically versatile and thus suitable for use as delivery systems [44]. Phages can be engineered with the help of phage display technique in order to overcome the barriers to cell entry and intracellular transport [45]. These bios inspired nanocarriers can be deposited with inorganic substances in order to improve their effectiveness as the delivery systems [46]. Phage based systems have found their application in different treatments, including cancer therapy and gene therapy [45]. But there are still some problems, for example, internalization, endosomal release, and nuclear targeting [44]. Despite all the difficulties, the current studies are aimed at further innovation and enhancement of the phage-based delivery systems for the generation of novel and improved therapeutic strategies [43,45].

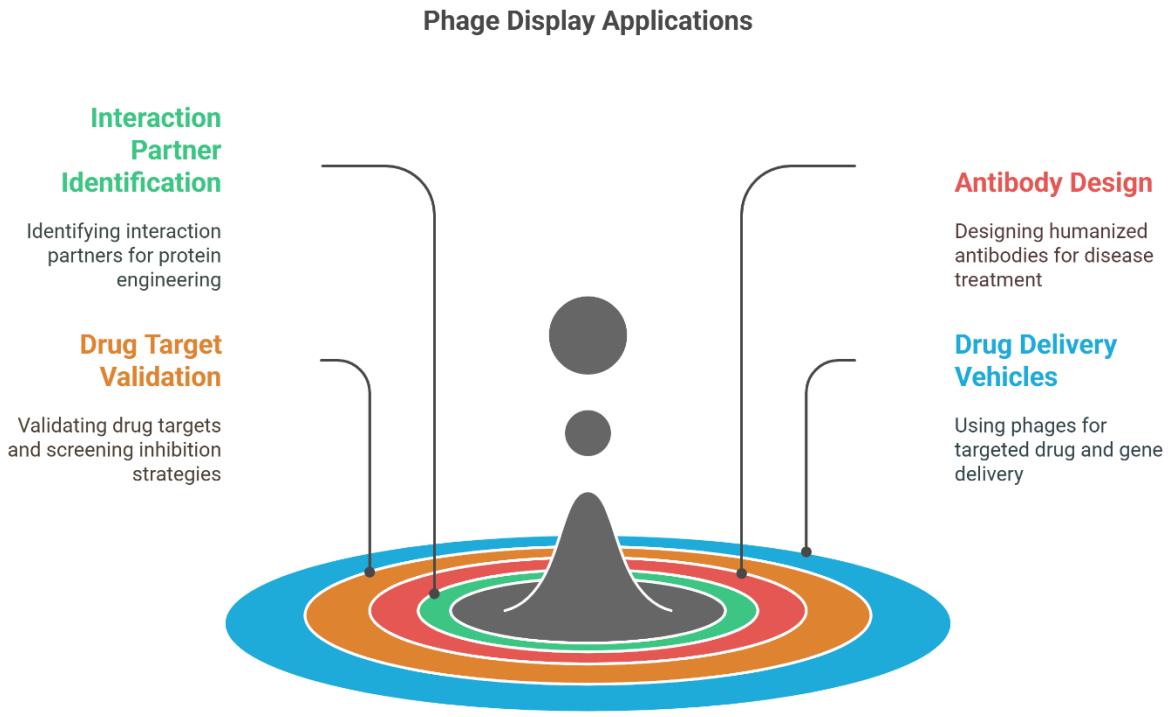

Figure 2: Schematic showing applications of phage display in drug discovery

4. Applications of Phage Display in Antibiotic Resistance Research: Targeting Beta-Lactamase Resistance

Phage display is an effective system of producing combinatorial peptide libraries for the identification of new beta-lactamase inhibitors in the fight against antibiotic resistance. Muteeb et al. [47] was also able to isolate catalytic antibodies with beta-lactamase activity. This showed the potential of the method in the understanding of enzyme mechanisms. Zou & Yang [48] reported the use of phage display to identify peptide inhibitors of penicillinase. One of the peptides had a good inhibitory effect. Muteeb et al [47] compared the merits of peptide inhibitors obtained by phage display. They pointed out that because the inhibitors are peptide in nature, the bacteria may not be able to develop resistance against them. Fragment based approaches have been shown to be successful in the identification of new beta-lactamase inhibitors [49]. However, phage display has distinct advantages in screening large peptide libraries. These studies reveal the possibility of phage display in the identification of various inhibitors of beta-lactamases. This may open new possibilities in the fight against bacterial resistance to antibiotics. Antibiotic resistance, especially to beta-lactam antibiotics, is increasing at an alarming rate and this is a major world health issue. Beta-lactamases are enzymes produced by bacteria that break down beta-lactam antibiotics, rendering them inactive. Phage display technology has been useful in the identification of inhibitors, antibodies and novel peptides that can be used to block beta-lactamases. This returns

the effectiveness of the antibiotics. I will now describe how phage display has been adapted to confront beta-lactamase resistance in antibiotic resistant bacteria.

4.1 Screening for Peptide Inhibitors of Beta-Lactamases

To develop peptide inhibitors against beta lactamases, phage display has been employed effectively. To this end, diverse peptide libraries are generated and screened for sequences that bind to the active sites or key functional areas of beta lactamase enzymes. Peptides that bind strongly can act as inhibitors. They prevent enzymes from decomposing beta-lactam antibiotics. In addition, researchers have employed phage display to sort through an M13 phage library to identify peptide sequences that will adhere to extended-spectrum beta-lactamases (ESBLs). ESBLs is a strain that is responsible for a large number of the multi-drug-resistant strains of *E. coli* and *Klebsiella pneumoniae*. These peptides exhibited a strong suppression of beta-lactamase activity, which proves that phage display can be employed effectively for recognizing these resistant enzymes.

4.2 Development of Antibody-Based Beta-Lactamase Inhibitors

Another fine application of phage display in handling beta-lactamase resistance is the generation of monoclonal antibodies that target beta-lactamase. These antibodies recognize only beta-lactamase enzymes. Thus, random peptide libraries phage display can be employed for the selection of high affinity antibodies. These antibodies stick to and inhibit the function of beta-lactamase. This method may be more specific, and the duration of action may be more prolonged than that of the above-mentioned methods. For example, monoclonal antibodies against Class A and Class C beta-lactamases were purified using phage display. The antibodies displayed high affinity and specificity towards the targets. These antibodies are designed to stick to the conserved regions of the beta-lactamases, thus reducing the likelihood of the pathogen developing resistance. These antibodies could be used clinically to block the activity of beta-lactamase in conjunction with beta-lactam antibiotics.

4.3 Creating and Screening Small-Molecule Mimetics

Phage display can also be used in the identification of small molecule mimetics that can block beta lactamases. This involves identifying peptides that are strongly affiliated with the beta lactamase. These peptides are then used as a reference to develop small molecules with the same affinity. Small molecule inhibitors may have better pharmacokinetic properties compared to peptides or proteins, for instance, they are more stable and have higher bioavailability. A study employed phage display to identify peptide ligands that interact with a metallo-beta-lactamase and used them as a reference to design small molecule inhibitors [50]. This work illustrates how phage display can connect peptide discovery with small molecule drug discovery to address the problem of beta lactamase activity.

4.4 Dual-Target Strategies for Overcoming Multi-Mechanism Resistance

Bacteria are known to possess multiple mechanisms of resistance and therefore cannot be easily treated. However, to overcome this challenge, phage display technology has been modified to identify compounds or antibodies that are able to interact with beta-lactamase and other resistance factors simultaneously. Examples of other mechanisms include efflux pumps or alterations in membrane permeability. A novel approach is based on a dual-targeting strategy where phage

display libraries are used for the identification of peptides or antibodies that interact with both beta-lactamase and the bacterial cell membrane. This dual-targeting strategy has been found to improve the efficacy of beta-lactam antibiotics against resistant pathogens and to limit the chances of resistance development. It does this by attacking several bacterial defenses at once.

4.5 High-Throughput Screening for Next-Generation Beta-Lactamase Inhibitors

Combination of phage display and high throughput screening has enabled the identification of beta lactamase inhibitors from large libraries. Thus, automated systems have been employed for the screening of phage display libraries to select high affinity beta lactamase binders. For example, a high throughput phage display screening platform has been used to discover new inhibitors of carbapenem resistant beta lactamases. They are particularly difficult to target owing to their wide spectrum of resistance [51]. This approach has accelerated the discovery of putative therapeutics that may be of use in the management of carbapenem resistant hospital acquired infections.

5. Advantages of Phage Display Over Traditional Drug Discovery Approaches

5.1 High Speed and Variety: Conventional drug discovery employs small molecule libraries, which are limited in diversity. By contrast, phage display enables the analysis of more than 101 of random peptides. This enormous combinatorial resource enhances the probability of identifying effective and novel inhibitors, especially for hard-to-target proteins like beta-lactamases.

5.2 Target Specificity: Phage display allows us to identify peptide sequences that interact with beta-lactamases. This leads to the development of very selective inhibitors. Traditional small molecule screening produces compounds that interact with other proteins, which can result in adverse effects. The specificity of phage display derived peptides minimizes these problems.

5.3 Rapid Optimization: Because of the iterative nature of the phage display technique, it is suitable for rapid optimization. The identified peptides can be easily altered in order to enhance their recognition specificity and affinity. For instance, the RRGHYY peptide was optimized to enhance its activity [52]. This process is more effective and more accurate than the modification of small molecules which is often a lengthy process and may require several iterations of medicinal chemistry.

5.4 Structural Insights into Drug Design: Phage display can be coupled with other structural analysis techniques including X-ray crystallography or NMR to understand how the peptides interact with the beta-lactamases. For instance, the cocrystal structure of beta lactamase inhibitory protein (BLIP) in complex with TEM-1 beta lactamase was used to identify the binding modes. This informs the development of new inhibitors [53]. Such structural information facilitates the rapid optimization of peptide inhibitors**.**

5.5 Reduced Probability of Resistance: Because that phage display peptide inhibitor can be designed to recognize non-standard binding sites on beta-lactamases, it is not as likely to be affected by the known resistance mechanisms that are a problem for antibiotics. The beta-lactamase inhibitors identified from phage display may represent a new strategy for overcoming resistance through the enzymatic pathway that cannot be reached by small molecules.

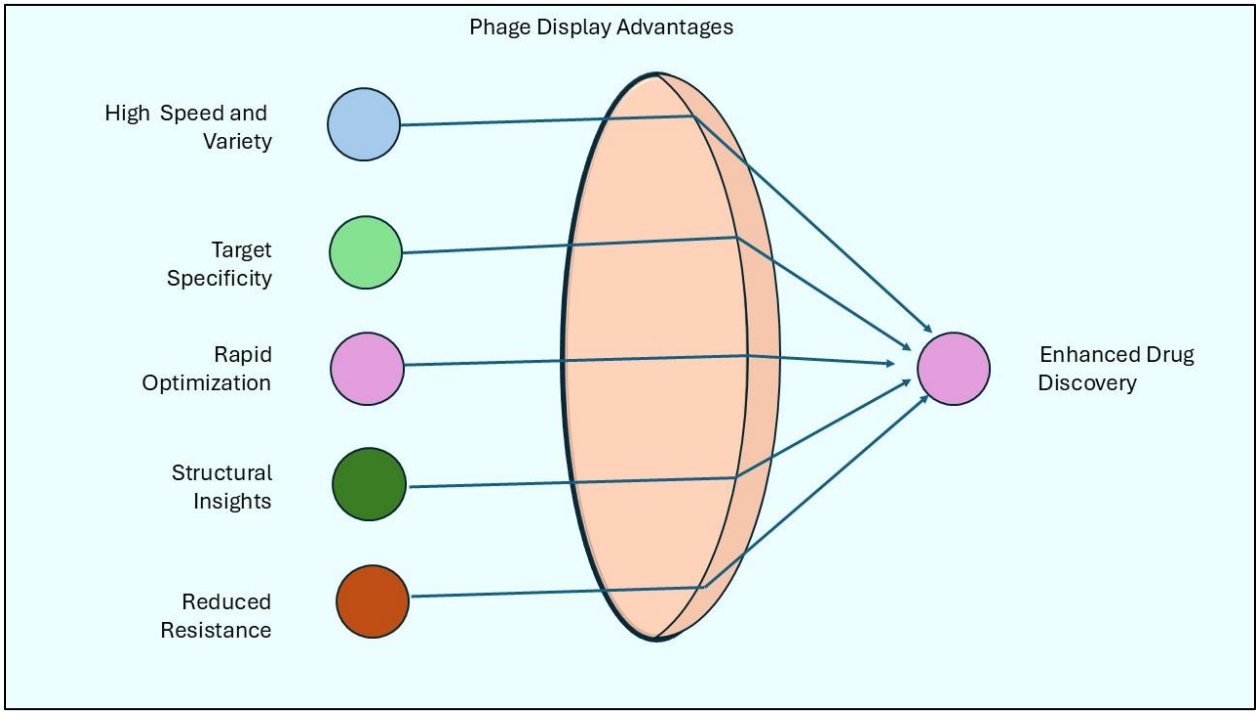

Figure 3: Schematic showing advantages of phage display in enhanced drug discovery.

As summarized in Fig. 3, the applications of phage display in the discovery of beta-lactamase inhibitors are clear. It provides a flexible and effective system for identifying and refining peptides that suppress these resistance enzymes. It enables high throughput screening, fast optimization and high specificity, and this makes it a useful tool. This opens up a new way of creating treatments that may replace current ones. Among the most notable cases is a linear 6-mer peptide RRGHYY which was identified by phage display. This peptide was found to have the capacity to inhibit class A beta-lactamase enzyme TEM-1 with a K_I of 136 μM as well as exhibit activity against class C beta-lactamases. The study showed that phage display can quickly provide candidates for potential inhibitors from a library of peptides. The peptide was then further optimized to increase its binding efficiency. This indicates that phage display can be combined with other methods in order to modify inhibitors for beta-lactamases. This peptide can be used as a reference for the design of broad-spectrum inhibitors. This may help in the fight against antibiotic resistance [52]. Another success is the identification of new peptide inhibitors of the beta lactamase inhibitory protein (BLIP). BLIP is a natural inhibitor of beta lactamases, and using phage display, peptides that are highly active against various classes of beta lactamases were identified. One study showed that these peptides were also able to interact with penicillin binding proteins (PBPs), which are involved in the bacterial cell wall synthesis. Co crystallography of these peptides with beta lactamases revealed the binding interface, which helped in the understanding of small molecule ligands [53]. These examples demonstrate the effectiveness of phage display in identifying peptide inhibitors of different beta-lactamases, which can be used to fight antibiotic resistance.

6. Challenges in Targeting Beta-Lactamases Using Phage Display

Phage display is a powerful technique for developing effective peptide inhibitors of beta-lactamases, but the structural and functional diversity of these enzymes presents a significant challenge. These enzymes, which defeat antibiotic agents through the destruction of beta-lactam rings, have a highly complex active site and catalytic mechanism with a wide range of substrate specificity. Hence, it becomes difficult to recognize broad spectrum inhibitors.

6.1 Structural and Functional Diversity of Beta-Lactamases

6.1.1 Classification and Variability: There are four major classes of beta-lactamases (A, B, C, and D), and they are classified based on sequence homology and catalytic mechanisms (Table 1). Classes A, C, and D are serine beta-lactamases, which use a serine residue in the active site to break the beta-lactam ring of antibiotics. Class B beta-lactamases are metallo-beta-lactamases, and they depend on zinc ions for catalysis (see Fig. 4).

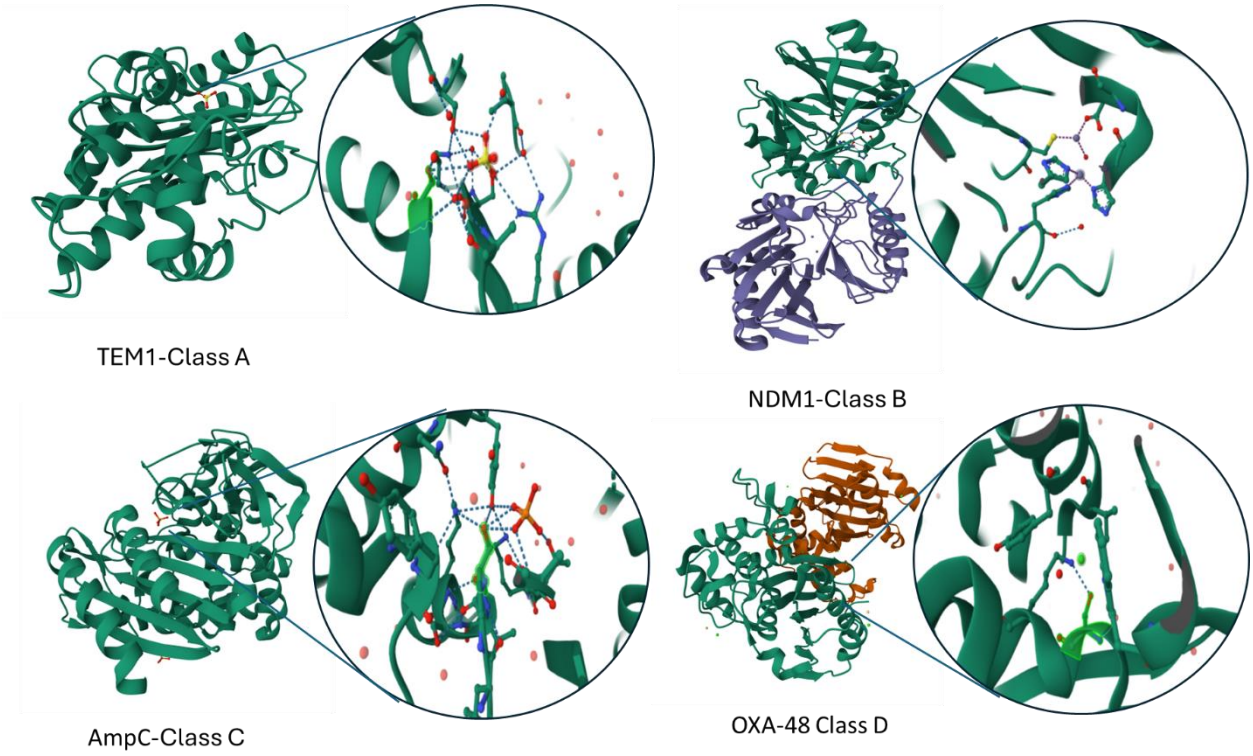

Figure 4: 3D Structure of beta-lactamases classified under Class A-D, from top left in a clock wise manner Escherichia coli TEM1 beta-lactamase, [54], Klebsiella pneumoniae apo-NDM-1 (Homo 2-mer), [50], Escherichia coli AmpC beta-lactamase, [55], Klebsiella pneumoniae OXA-48 carbapanemase, apo form, [56]. In-set the interactions of the active site amino acid highlighted in light green color which is shown for each of the classes. 3D visualisation done using Mol* available at RCSB-PDB., [57].

Class A enzymes, such as TEM-1 and SHV-1, are widespread and cause resistance to penicillins. Class C enzymes, like AmpC, are mostly found in Gram-negative bacteria and cause resistance to cephalosporins. Class B enzymes, such as NDM-1, are metallo-beta-lactamases capable of hydrolyzing a broad range of beta-lactam antibiotics, including carbapenems. The structural differences between these classes make it difficult to develop a single peptide inhibitor that can bind and inhibit all classes effectively. Phage display screens must consider the specific structural features of the target beta-lactamase class when creating peptide libraries.

Table 1: Classification of Beta-Lactamases and Their Mechanisms of Resistance

Class of Beta-Lactamase	Mechanism	Antibiotics Hydrolyzed	Example Enzymes	Resistance Mechanism
Class A	Serine-based hydrolysis	Penicillins, cephalosporins	TEM-1, SHV-1	Active site mutations
Class B	Metallo-beta-lactamase (zinc)	Broad-spectrum, including carbapenems	NDM-1, VIM-2	Zinc coordination for catalysis
Class C	Serine-dependent hydrolysis	Cephalosporins	AmpC	Active site mutations, substrate flexibility
Class D	Serine-based hydrolysis	Oxacillin, cloxacillin	OXA-23	Variable active site structure

6.1.2Active Site Diversity: Beta-lactamases' active sites vary significantly across different classes. Serine beta-lactamases (classes A, C, and D) use serine-dependent catalytic mechanisms. Metallo-beta-lactamases (class B) use zinc ions for activity. The shape, charge, and accessibility of these active sites differ between classes, and even between variants of the same class. This makes it difficult to design peptides that can inhibit all beta-lactamases.

6.1.2.1 Class A Beta-Lactamases: Serine Active Site Enzymes

Class A beta-lactamases are serine-based enzymes. They have a highly conserved active site serine residue that breaks down the beta-lactam ring of antibiotics. Structural traits include:

- **Active Site Conformation:** The active site has residues like Ser70, Lys73, Ser130, and Glu166. The catalytic serine (Ser70) is essential for nucleophilic attack on the beta-lactam ring, leading to antibiotic breakdown.
- **Extended-Spectrum Variants**: Extended-spectrum beta-lactamases (ESBLs), like TEM-1 and CTX-M, belong to this class. They have developed mutations that enable them to hydrolyze third-generation cephalosporins.

- **Structural Flexibility**: Some Class A enzymes, such as CTX-M, have mutations in the Ω-loop region, which alters the substrate profile. This allows them to accommodate broader-spectrum antibiotics [58].

6.1.2.2 Class B Beta-Lactamases: Metallo-Beta-Lactamases

Class B beta-lactamases, also known as metallo-beta-lactamases (MBLs), need metal ions, usually zinc, to break down beta-lactams. They are structurally different from serine-based beta-lactamases. Their features include:

- **Zinc-Binding Active Site:** The active site contains one or two zinc ions, which are coordinated by residues like His116, His118, Asp120, and His263. These zinc ions activate a water molecule, which attacks the beta-lactam ring rather than a serine residue.
- **Broad Substrate Range:** MBLs like NDM-1 and VIM-2 can hydrolyze nearly all beta-lactam antibiotics, including carbapenems. This is due to their spacious and flexible active site.
- **Active Site Flexibility:** The active site is more flexible and open than serine-based enzymes. This allows MBLs to bind to various beta-lactam substrates without relying on substrate-specific interactions [59].

6.1.2.3 Class C Beta-Lactamases: AmpC Enzymes

Class C beta-lactamases, also known as AmpC beta-lactamases, are serine-dependent enzymes like Class A. However, they have distinct structural features that cause resistance to many beta-lactamase inhibitors. These features include:

- **Highly Conserved Serine Active Site:** Like Class A enzymes, AmpC beta-lactamases have a serine residue (Ser64) that starts hydrolysis. The surrounding active site residues are different, making them less susceptible to classical beta-lactamase inhibitors like clavulanic acid.
- **Distinct Substrate Binding Pocket:** AmpC enzymes have a substrate-binding pocket that is more restricted than Class A. It is designed to bind larger cephalosporin molecules.
- **Resistance to Inhibitors:** The active site's structure prevents the binding of beta-lactamase inhibitors commonly used to inhibit Class A enzymes [60].

6.1.2.4 Class D Beta-Lactamases: Oxacillinases

Class D beta-lactamases, called oxacillinases or OXA-type enzymes, are also serine-based, but differ greatly in their structure and substrate profile. They are often associated with carbapenem resistance, mainly in hospital-acquired infections

Flexible Active Site: Class D beta-lactamases have a highly adaptable active site, with a flexible Ω-loop that can accommodate various beta-lactams, including oxacillin and, in some cases, carbapenems. The loop changes shape to facilitate substrate binding and catalysis.

Carbamylation of Lysine: A unique structural feature is the carbamylation of a lysine residue near the active site, which is needed for catalytic activity. This carbamylation is needed to form the active enzyme complex in the presence of CO_2.

Extended Substrate Range: OXA-type enzymes like OXA-48 can hydrolyze carbapenems unlike most Class A or Class C enzymes. This unique ability is due to the structure of their active site [61].

Table 2: Summary of Structural Differences and Implications for Inhibitor Design

Class	Catalytic Mechanism	Active Site Features	Structural Adaptations	Implications for Inhibitor Design
Class A	Serine-based	Ser70 active site	Active site optimized for penicillins and cephalosporins	Inhibitors can target conserved serine and binding residues
Class B	Zinc-dependent (metallo)	One or two zinc ions	Large, open active site; flexible for various substrates	Use of metal-chelating agents to inhibit zinc binding
Class C	Serine-based (AmpC)	Ser64 active site	Substrate-binding pocket tailored for cephalosporins	Design inhibitors that fit AmpC's specific substrate-binding pocket
Class D	Serine-based (oxacillinases)	Carbamylated lysine near active site	Flexible Ω-loop that accommodates large beta-lactams like carbapenems	Develop inhibitors targeting unique lysine carbamylation mechanism

These structural differences (Table 2) affect the beta-lactamases' substrate profiles, resistance capabilities, and susceptibility to inhibitors. Understanding these differences is essential for tailoring phage display libraries and developing inhibitors that can specifically bind and neutralize each beta-lactamase class effectively. For example, the RRGHYY peptide was effective against

class A and class C beta-lactamases, but its inhibitory activity against class B metallo-beta-lactamases was not reported. This is probably due to the differences in the active site structure and catalytic mechanism [52].

6.1.3 Substrate Specificity: Beta-lactamases also have different substrate specificities. Some enzymes, like TEM-1, are narrow-spectrum, hydrolyzing only penicillins. Others, like extended-spectrum beta-lactamases (ESBLs) and carbapenemases, can hydrolyze a broader range of antibiotics. This functional diversity means that an inhibitor effective against one enzyme may not be effective against others, even within the same class.

The challenge for phage display here is that the peptide inhibitors found through screening may have high affinity for one type of beta-lactamase, but low or no affinity for others. To address this, libraries may need to be tailored for specific subtypes of beta-lactamases or optimized through iterative rounds to find more broadly acting peptides.

6.1.4 Mutational Flexibility and Resistance: Beta-lactamases evolve quickly through point mutations. This can lead to variants that escape inhibition. Even if phage display finds a potent peptide inhibitor, a single point mutation in the active site or a nearby structural area of the enzyme can greatly reduce the binding affinity of the inhibitor. This mutational flexibility is a significant problem for developing long-lasting inhibitors, as beta-lactamases continuously adapt to selective pressures.

For example, the TEM-1 beta-lactamase, which has over 200 known variants, can develop resistance to inhibitors by mutating residues in the active site. Clavulanic acid was one of the first beta-lactamase inhibitors. It was introduced in combination with amoxicillin to combat beta-lactamase-producing *E. coli* and *Klebsiella pneumoniae*. However, in just a few years, extended-spectrum beta-lactamases (ESBLs), like the TEM and SHV types, mutated to resist clavulanic acid. This enabled them to hydrolyze extended spectrum cephalosporins although the inhibitor was present in the medium. TEM-3, one of the first ESBL variants, was detected in Europe shortly after the introduction of clavulanic acid. This mutation led to the reduction of the effectiveness of clavulanic acid and thus to treatment failures. This variability makes it harder to develop inhibitors that are broad-spectrum and of long duration. KPC (*Klebsiella pneumoniae* carbapenemase) enzymes are Class A beta-lactamases that can hydrolyze carbapenems. The inhibitor avibactam was introduced in combination with ceftazidime to target KPC enzymes. However, resistance was found to emerge quickly, with mutations in the omega-loop region of KPC enzymes. These mutations led to reduced affinity of the inhibitor to bind to the enzyme and thus lead to therapeutic failure in patients with MDR infections [62]. NDM-1 is a Class B beta-lactamase, New Delhi metallo-beta-lactamase-1, needs zinc for catalysis. NDM-1 mutants with altered zinc-binding sites have appeared in response to inhibitors that act by targeting zinc in the active site. A mutation in the zinc-binding site of NDM-1 was noted in clinical isolates that were resistant to metal-chelating agents that were being used to block metallo-beta-lactamase activity. This mutation enabled the enzyme to maintain its function with a lowered zinc affinity, such that it was resistant to chelation-based inhibition strategies [63]. OXA-48 is a Class D beta-lactamase secretion by Enterobacteriaceae and is renowned for its carbapenems hydrolyzing capacity. Vaborbactam, a new inhibitor of carbapenemases, was not very effective against OXA-48 carbapenemases. This is because the enzyme has a flexible active site which can assume structural changes without compromising on the enzyme function. After using vaborbactam with meropenem, treatment

failures were reported with OXA-48 producing strains. This was because of structural changes in the enzyme's omega-loop that reduced vaborbactam binding while retaining the ability of the enzyme to hydrolyze carbapenems [64]. *P. aeruginosa* and Enterobacter species produce AmpC beta-lactamases that have evolved to be resistant to traditional and new generation beta-lactamase inhibitors. The mutation at the active site serine residue has changed the shape of AmpC enzymes to make it resistant to both the classical inhibitors clavulanic acid and new generation inhibitors. The active site region mutation of AmpC was noted in clinical isolates from patients with cephalosporin inhibitor combination therapy. This resulted in a high rate of resistance and treatment failure in hospital acquired infections [65]. The compatibility of beta-lactamases, especially through mutations in the active sites and loop regions, limits the durability of beta-lactamase inhibitors. For instance, in KPC and OXA-48 variants, treatment failure was noted, which highlights the need for new inhibitor designs that can counter these mutations. There could be focus on inhibitors that act on many enzyme sites, drug combinations and agents that destabilize the bacterial membrane. This can prevent the bacteria from developing resistance and hence maintain the efficacy of the drugs for the long term.

7. Impact on Phage Display-Based Inhibitor Development

The structural and the functional diversity of beta-lactamases affect the phage display screening in the following manner:

7.1 Library Design Complexity: The structural diversity of active sites and the substrate specificities of the beta lactamase classes dictate the need for highly diverse phage display libraries. This makes it difficult to identify effective inhibitors since libraries must contain both conserved and variable regions of the enzymes. For instance, the studies on beta-lactamase homolog diversity in environmental samples are a clear example of the problems associated with targeting structurally diverse enzymes [66].

7.2 Specificity vs. Breadth: It is thus possible to find very selective peptide inhibitors for a given beta-lactamase, but the design of peptides with spectrum similar to that of antibiotics against different categories or types of enzymes or their variants is rather troublesome. The presence of diverse substrate specificities, for example the diverse Class D beta-lactamases like OXA enzymes also pose a problem in terms of specificity versus breadth in inhibitor development[67,68].

7.3 Iterative Optimization: For the most identified inhibitors, the binding affinity and inhibitory activity may require further optimization. This is especially important when working with enzymes that have several variants or a dynamic active site. The structural diversity of beta-lactamases is known to affect their hydrolytic activity and substrate specificity. Forloni et al. [69] however argued that this needs to be refined iteratively to be effective.

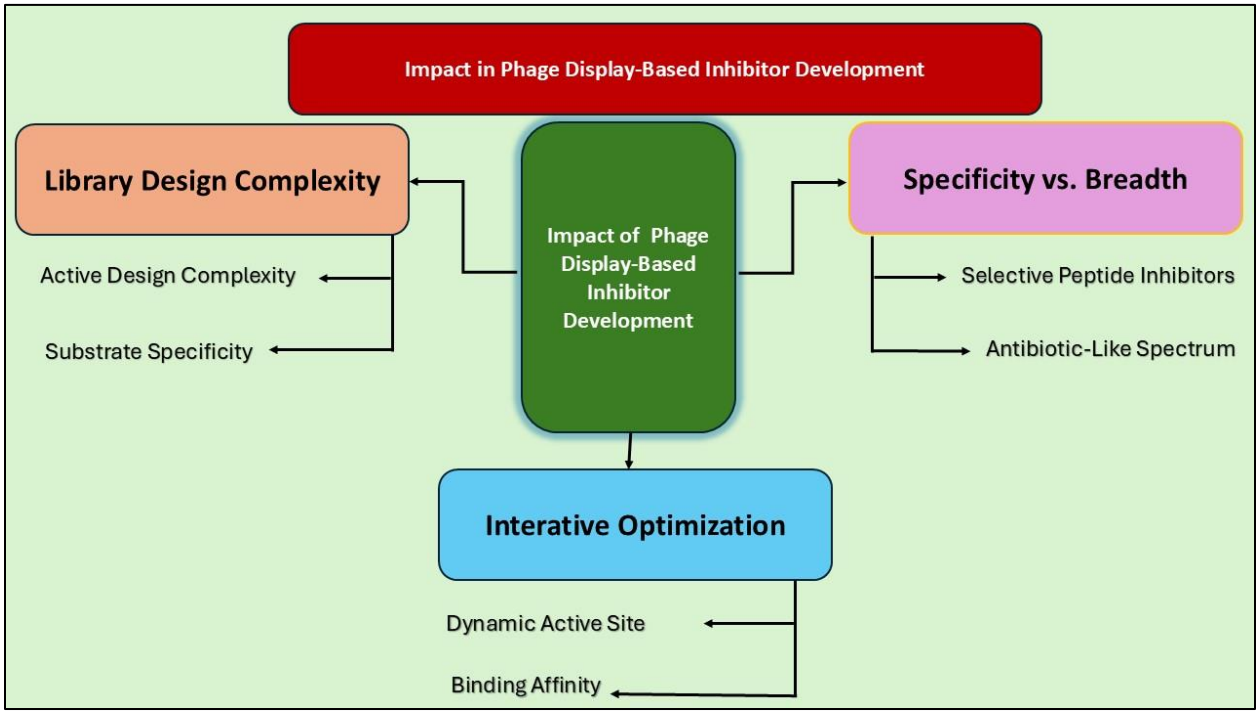

Figure 5: Impact on Phage display-based inhibitor development.

As illustrated in Fig.5, the structural and functional diversity of beta-lactamases significantly increases the difficulty of screening for the effective inhibitors via the phage display. These challenges are attributed to the complexity of designing diverse libraries, the tradeoff between specificity and spectrum in the efficacy of the libraries, and the necessity to improve the identified candidates through iterative optimization.

8. Challenges in Identifying High-Affinity Binders Using Phage Display

Developing peptide-based therapeutics starts with the identification of high-affinity binders. A number of inbuilt difficulties make this process difficult (see Fig. 6), especially when enveloping enzymes that are diverse and metabolic adaptive such as beta-lactamases.

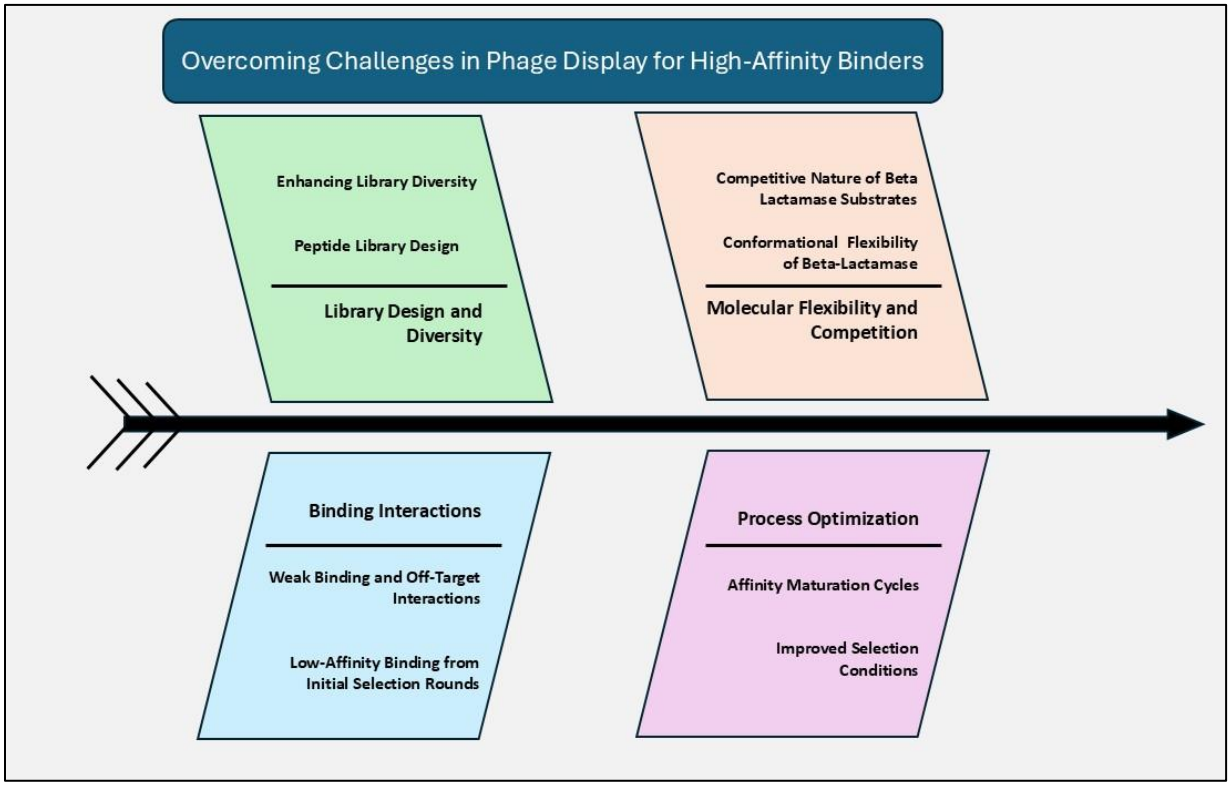

Figure 6: Summary of factors to be considered to overcome the challenges in phage display for high-affinity binders.

8.1 Peptide Library Design and Diversity: High-affinity binders are a function of the diversity and composition of the peptide library. Phage display screens contain billions of unique peptides; however, the library is not uniform, and there is library bias such that some sequences are overrepresented. This may mean that high affinity binders are not identified when they are present but in a more even distribution or in a more specialized library. The enzymes like beta-lactamases which have highly varied and evolving active sites mean that the peptide libraries must present sequences that are able to interact with different classes of these enzymes. The construction of libraries that can bind tightly throughout the different classes of beta-lactamases is a major problem. Inadequate library construction results in low-affinity or non-specific binders.

8.2 **Weak Binding and Off-Target Interactions:** Phage display tends to select weak binders in the initial cycles of biopanning. These binders may not be enough to prevent the activity of the beta-lactamase enzyme. Early binders may have micromolar affinity, which is not enough for the development of effective inhibitors. However, optimizing these binders to nanomolar or picomolar ranges is a process that can take many rounds of affinity maturation. Moreover, the following can occur as off-target binding: Peptides can stick to the enzyme or to other components of the screening system through non-specific interaction. This can result in false positive results where the peptides identified in vitro appear to be effective but do not inhibit the enzyme in more stringent biochemical or in *vivo* assays.

8.3 Conformational Flexibility of Beta-Lactamases: Beta-lactamases are modular proteins that undergo conformational changes upon substrate binding. This is because the shape of the protein

changes during the binding of the peptide, thus making it difficult to identify high affinity binders. Peptide binders that are effective in a static system may not be efficient in a system where the enzyme changes its conformation. For example, inhibitors identified by phage display may be ineffective when the enzyme changes its conformation during the catalytic cycle or when it interacts with an antibiotic. There are two aspects: Phage display can be combined with dynamic binding assays or allosteric inhibitors but identifying allosteric sites with phage display is more challenging.

8.4 Competitive Nature of Beta Lactamase Substrates: Antibiotics, which are the substrates of beta-lactamases, have a high affinity towards the active site of the enzyme. This is because phage display screens may fail to identify such peptides in a very competitive binding environment, especially for beta-lactamases with high affinity towards their substrates.

8.5 Low-Affinity Binding from Initial Selection Rounds: The first rounds of phage display panning usually result in the identification of low-affinity binders. These early binders are usually used as a starting point for optimization, but the initial binding affinities may not be sufficient to produce clinically meaningful inhibitors. The necessity of several rounds of affinity maturation to increase the binding strength. For instance, the RRGHYY peptide had moderate inhibition with a K_i value of 136 µM [52]. This is encouraging, but it is not to the level of a potent therapeutic, so it needs further optimization.

8.6 Affinity Maturation Cycles: After the potential binders have been recognized, the next step is affinity maturation to increase bond strength. It is also slow and requires at least one more round of screening to identify the improved variants. Optimizations can also increase the affinity at the expense of specificity and result in off target effects. To address these challenges, several strategies can be used:

8.7 Enhancing Library Diversity: Preparing more diverse and specific peptide libraries that are directed to certain classes of proteins or active site motifs will increase the chances of identifying high affinity ligands. Using phage libraries with non-canonical amino acids can increase peptide chemical diversity.

8.8 Improved Selection Conditions: Using dynamic selection conditions such as panning under competitive conditions with substrate analogues or inhibitors can aid in the identification of peptides that have higher affinity and specificity towards beta-lactamases. This method will replicate the conditions that are seen in vivo.

8.9 Allosteric Inhibition: Phage display can also be employed to identify peptides that interact with the allosteric sites of beta-lactamases instead of using it to recognize peptides that interact with the active site. Allosteric inhibitors can indirectly affect enzyme activity. This is especially helpful for metallo-beta-lactamases (class B) which cannot be inhibited easily through direct binding with the active site.

8.10 Combination of Phage Display and Structural Biology: Phage display can be combined with structural biology methods such as x-ray crystallography or NMR to understand the binding interactions. This knowledge can be used to direct the design of high affinity binders and help guide the construction of peptide libraries.

8.11 Multi-Round Affinity Maturation: A strategy of progressive panning can assist in the selection of peptides to become higher affinity binders through multi-round panning.

Computational methods can be used to suggest mutations that may enhance the peptide affinity before rescreening. All the challenges discussed above are mentioned in the Table 3.

Table 3: Challenges in Phage Display for Beta-Lactamase Inhibitor Discovery

Challenge	Description	Solution
Structural Diversity	Different active site structures across beta-lactamase classes	Design libraries targeting conserved or allosteric regions
Weak Affinity Binding	Low-affinity peptides are often identified in early screens	Employ iterative affinity maturation and multi-round panning
Competitive Substrate Binding	Substrates bind tightly to the active site	Target allosteric sites or use competitive selection conditions
Proteolytic Degradation of Peptides	Peptides are degraded in vivo	Use cyclic peptides or peptidomimetics for increased stability
Evolving Resistance	High mutation rates lead to inhibitor escape	Develop dual-target or multi-target inhibitors

9. Problems of the Model

When using the phage display technique to develop peptide inhibitors of beta-lactamases, stability and specificity are important. Both of these factors are important for the effectiveness of the therapy in the clinical setting. Peptide based drugs are especially efficient in terms of selectivity and affinity, but they are rather unstable.

9.1 Proteolytic Degradation: The major problem with respect to peptides is proteolytic degradation. The human body has enzymes that attack peptide bonds. This results in a short half-life of therapeutic peptides. Peptides selected by the phage display such as the RRGHYY peptide are also vulnerable to such degradation when used *in vivo*.

9.2 In Vivo Stability and Bioavailability: Peptides are generally less bioavailable when administered *in vivo*. They are poorly absorbed, rapidly metabolized and have a short half-life in the bloodstream. Even though phage display is excellent at identifying potential binders *in vitro*, they may be short lived *in vivo* and thus of limited therapeutic value.

9.3 Some Specificity Issues

Phage display can be used to identify peptides with high affinity for a particular target, but the problem of selectivity is significant. This means that the inhibitor must recognize the target enzyme and no other proteins.

9.3.1 Off-Target Effects

Beta-lactamases are numerous. There is a high probability that the peptides will recognize other proteins, especially serine hydrolases or other proteins with similar structures. For instance, the peptides that are selected for beta lactamase inhibition may interact with human proteases or other bacterial enzymes and lead to off target effects which may be toxic or lessen efficacy. Off target effects can lead to toxicity of healthy tissues or cytotoxicity or organ specific toxicity. This can lead to unwanted interactions that may be toxic or have other adverse effects. Some peptides, especially those with high affinity to the membrane, may lead to hemolysis (rupture of red blood cells), which is a safety concern. For example, peptide-based therapies that target specific membrane proteins can interact with erythrocytes and cause hemolytic toxicity. Hemolytic activity is usually regulated by the peptide structure or charge to minimize the interaction with red blood cells [64]. Immunogenic reactions, where the body perceives the therapeutic peptide as an antigen, can also take place. This risk is higher with peptides of foreign or synthetic origin. The peptide backbone can be modified, or PEG chains can be conjugated to the peptide to avoid immunogenic reaction by the hiding of epitopes that may induce the response [70].

To avoid the adverse effects of off target binding, the following have been employed:

To make the peptides less sensitive to non-specific binding, non-natural amino acids or cyclic structures can be incorporated. A study which establishes the effectiveness of using cyclic peptides to enhance peptide selectivity and reduce off target effects in vivo was reported by Lucana et al in 2021 [71]. By linking the peptides to the nanoparticles, it has been possible to guide the peptides to the target cells more specifically, without reacting with the other tissues. These findings show that nanocarrier bound peptides have improved cellular delivery, with reduced protease degradation and off target effects [72]. Bioinformatics tools are currently being employed to design peptides with high selectivity and reduced toxicity.

Computational modelling helped to identify the highly specific binding sites in order to design selective peptides with minimal toxicity [22]. Pegylation of an anti-cancer peptide was found to decrease its immunogenicity and off target cytotoxicity in a study by Evans et al in 2020[73]. Another study also established that, through careful design, dimeric peptides could reduce off target toxicity. These peptides had selective inhibition of the target proteases without affecting other proteins. This made them less cytotoxic and thus more suitable for therapy.

This work is important for the future development of peptide therapeutics as it focuses on the issues of specificity and off target effects. The safety of peptide drugs has been improved through structural modifications, conjugation techniques, and computational studies. These mitigation strategies can be used by peptide researchers to enhance the therapeutic index of peptide drugs.

9.3.2 Cross-Reactivity Between Beta-Lactamase Classes

Another issue is achieving specificity across different beta-lactamase classes. The structural diversity among class A, B, C, and D beta-lactamases means that a peptide with high affinity for class A beta-lactamases, such as TEM-1, may not effectively inhibit class B metallo-beta-lactamases or class D oxacillinases. Cross-reactivity or lack of broad-spectrum inhibition can limit the therapeutic usefulness of the identified peptides. This is particularly true when trying to combat

a wide range of resistant bacterial strains. Several strategies have been developed to improve the stability and specificity of peptides (Table 4).

Table 4: Strategies to Improve Stability and Specificity of Phage Display-Derived Inhibitors

Strategy	Improvement Area	Example Solution	Benefit
PEGylation	Stability (in vivo half-life)	Attaching PEG molecules to peptide inhibitors	Increased half-life and reduced proteolysis
Cyclization	Stability	Designing cyclic peptides	Improved resistance to proteolytic degradation
Dual-target Inhibition	Specificity (cross-class inhibition)	Designing peptides to bind two beta-lactamase sites	Increased binding efficacy and reduced resistance
Allosteric Targeting	Specificity	Identifying peptides that bind allosteric sites	Less chance of developing resistance mutations

10.1 Peptidomimetics for Enhanced Stability

Peptidomimetics are man-made molecules that resemble peptides but have some structural changes. These may be cyclic backbones or nonnatural amino acids, which make them more stable to proteolysis. The cyclic structures also enhance stability through steric hindrance of the proteases. The stapling of divinyltriazine to cyclic peptides enhanced serum stability as compared to the linear peptides. This stapling process enhanced the resistance to enzymatic degradation and thus made cyclic peptidomimetics very efficient in extending the half-life in the environments rich in proteases [74]. This paper presents nonnatural amino acids in the design of beta-lactamase inhibitors to enhance stability and reduce enzymatic cleavage. Peptidomimetic beta-lactamase inhibitors were developed by grafting peptides with enhanced stability, which retain their function under thermal and enzymatic stress, and are, therefore, suitable for high-protease environments [75]. Among them, the triazine moiety conjugation enhances plasma stability to increase the in vivo half-life of the peptide. The triazine stapled peptides were proteolytically stable and thus, it was proved that structural alterations in the peptidomimetics can lead to a significant increase in the duration of the therapeutic effect [76].

10.2 PEGylation for Enhanced Pharmacokinetic and Physiological Properties

PEGylation is the process of coupling PEG into a molecule to enhance stability, bioavailability and protease resistance. It achieves this through the formation of a protective shield. PEGylation is normally done on peptides to make them less prone to degradation. Metabolic stability, half-life and bioavailability of PEGylated peptides were improved and recommended for clinical use in long term exposure applications [73]. The immunogenicity of PEGylated anti-cancer peptides was reduced, and the stability of the peptides was enhanced for the long-term therapeutic use [70].

Both peptidomimetics and PEGylation provide vital improvements in peptide-based therapeutics. This is accomplished by enhancing the stability, bioavailability and proteolytic resistance of the

peptides. These strategies enable the development of effective long lasting peptide therapies in protease rich environments such as those that are involved in the resistance of bacteria to beta lactam antibiotics. Lipidation to Improve the Peptide Inhibitor Stability and Efficacy. Lipidation is the process of incorporating lipid groups into the peptides. Peptides are usually used in various applications including therapeutic purposes, but they have some drawbacks which include low membrane permeability, sensitivity to protease cleavage and a short plasma half-life. It has been established that lipidation, the covalent linkage of lipid groups, enhances the bioavailability, stability and cell penetration of peptide-based drugs through increasing their lipophilicity. In a study performed on β peptides, lipidation assisted in their penetration of plasma membranes in mesenchymal stem cells and the lipidated β peptides accumulated well within the mitochondrial network. Thus, lipidation can be used to enhance cell targeting and delivery efficacy in therapeutic application [77]. A lipidation pattern was proposed for cationic cell penetrating peptides (CPPs) to increase their membrane affinity and penetration. The double lipidated CPPs had the best cell permeability with good membrane stability, which is crucial for clinical practice [78]. However, lipidation does not only enhance peptide cell penetration but also protects the peptide against enzymatic degradation. It creates a hydrophobic environment around the peptide, thus making it the least prone to proteolysis. Lipidation has been used to stabilize peptide inhibitors by incorporating fatty acids that increase plasma stability. For example, packaging the peptides in lipid nanocapsules enhanced the stability of the particles, reduced the degradation and enhanced the antimicrobial activity of the particles, thus revealing the protective role of lipidation [79]. Circulation times and pharmacokinetic properties of lipidated peptides are usually better due to increased lipophilicity. A study with calcitonin gene-related peptide (CGRP) analogs revealed that lipidation supported the maintenance of receptor antagonist activity and prolongation of the terminal half-life, which contributed to improvement in vivo activity. The study concluded lipidation as a viable strategy to enhance the peptide stability for the sustained therapeutic effect [80]. Lipidated self-assembling peptides formed diverse and stable nanostructures at different temperatures and pH. This shows that lipidation is important in enhancing the stability of peptides and extending their usage in therapy [81]. Lipidation can increase the target specificity through the preference for the interaction with a given cell type or the tissue environment, thus decreasing the toxicity and the off-target effects. A new strategy of lipidation through the tryptophan site was established to enhance the peptide stability and target specificity. This targeted lipidation not only enhanced the stability of the peptide but also enhanced its efficacy in the induction of insulin secretion with low cytotoxicity [82]. Lipidation has been determined as a useful oxidative modification for the enhancement of stability, availability and the curative nature of peptide inhibitors. These examples demonstrate lipidation's growing utility in developing peptide-based therapeutics.

10.3 Increased Rounds of Selection (Biopanning): To enhance specificity, phage display can be modified to include negative selection rounds where peptides that bind to off-target proteins are removed. Developing peptide inhibitors specific to beta-lactamases is challenging because of the structural similarity between different classes of beta-lactamases and human serine proteases. Based on the reviewed references, it appears that negative selection techniques specifically designed to avoid off-target peptide binding in beta-lactamase screening have not been extensively documented in the context of phage display or similar methods. The main focus in beta-lactamase inhibitor research has often been on enhancing binding affinity and optimizing stability. Specificity improvements are generally achieved through structural changes. In summary, while

negative selection rounds are widely used in phage display for other targets, they do not appear to have been used prominently in phage display for beta-lactamase inhibitors. This is an opportunity for future research to explore and validate negative selection strategies to enhance specificity and clinical viability.

10.4 Structural Template Assisted Design to Enhance Selectivity of Inhibitors of Beta Lactamase

Structure guided design approaches use the structural information of the beta-lactamases to design inhibitors that can recognize specific protein targets with minimal recognition of other proteins. This has been useful in producing inhibitors that are more effective and have fewer side effects. This is especially important for difficult targets such as the metallo- and serine-beta-lactamases. The researchers also designed antimicrobial peptides with high binding affinity and stability. The study used molecular docking and dynamics simulations to determine the specific amino acid residues that are involved in the binding of the peptide in the active site of the beta-lactamase. This approach led to the identification of peptide candidates that display high specificity in the recognition of beta-lactamase with minimal sequence similarity to other proteins. Inhibitors of metallo-beta-lactamases such as NDM-1 pose a problem of undesirable interaction with human metalloproteins. A 2021 study on computationally designed peptide macrocycles was successful in this regard by optimizing the peptide conformations to be stable in the enzyme's active site. This design strategy enabled the authors to predict the binding affinity and select the stable peptide structure that can specifically target the bacterial protein [83]. The research was carried out on Class D beta-lactamases (OXA types) using a structure-based screening to discover non-beta-lactam compounds. This revealed that some amino acid interactions near the active site are important for determining the specificity of an inhibitor, thus enabling the design of peptides that are selective for OXA beta-lactamases. This avoids the problem of cross reactivity with other bacterial enzymes [84]. The carboxylate binding pocket of the beta-lactamase was recently targeted using a DNA encoded chemical library to design peptide inhibitors. This structure-based design led to the identification of non-beta-lactam pharmacophores that can specifically interact with the active site of the enzyme without interacting with the human proteins. Such targeted approaches can enhance the therapeutic index by reducing the chances of non-specific interactions [43]. A novel strategy was reported for the development of lysine targeted affinity labels that could inhibit both serine and metallo-beta-lactamases through the modification of active site residues such as Lys211. The structure-based design of this study helped in reducing the possibility of off target effects and increasing the efficacy of the inhibitors[85]. Structure guided design has emerged as an important strategy in the development of peptide inhibitors that act against beta-lactamases. These strategies enable the selective targeting of bacterial enzymes with minimal effects on other proteins (see Table 5).

Rational Design and Computational Modeling: After the screening, the peptides can be modeled to predict their behavior when bound to off-target proteins. Peptides can be further optimized by making changes in the side chains to reduce the off-target binding while maintaining high affinity for the beta-lactamase. Rational design and computational modeling are very important techniques in the production of beta-lactamase inhibitors with high specificity. These methods employ structural and biochemical information to predict and optimize the interaction of inhibitors with the target enzymes. CTX-M-15 beta-lactamase inhibitors were designed using a stepwise virtual screening strategy, and the inhibitors were developed through docking and molecular dynamics

simulations [86,87]. Rational design has also been applied to metallo-beta-lactamase (MBL) inhibitors to help restore the function of beta-lactam antibiotics. A comprehensive review highlighted how computational modelling and molecular dynamics simulations have enabled the development of inhibitors that interact specifically with metallo-beta-lactamases [87]. A computational assay was introduced to predict the antibiotic activity in Class A beta-lactamases. Mechanistic insights into the enzyme's breakdown pathways were gained from this assay, which was focused on the TEM-1 beta-lactamase. It suggested that certain inhibitors might be designed to specifically block these pathways [88]. In a 2020 study, the novel non-beta-lactam inhibitors of Class D beta-lactamases were designed through structure based screening and molecular dynamics simulations. This rational design process allowed the determination of the key residues in the enzyme's active site that contribute to the specificity [84]. Rational design and computational models were recently employed in the effort to discover new drugs from approved drugs as potential beta-lactamase inhibitors. The study aimed at understanding the interactions of these drugs with the conserved residues in the beta-lactamase substrate binding pocket which provides a quick and economical way of identifying inhibitors that are specific, for instance, diosmin for NDM-1 beta-lactamase [89]. Rational design and computational modeling have been found to be useful in the production of beta-lactamase inhibitors with high specificity [90].

Multi enzyme screening: In phage display, the screen can be carried out using many beta-lactamases to discover broad spectrum inhibitors. For instance, if a peptide interacts with both class A and class C beta-lactamases but not with class B, then the subsequent optimization can be directed towards enhancing the cross-class specificity. Rowland et al.[91] reported bicyclic peptide inhibitors for *E. coli* PBP3 in a 2024 study. These inhibitors had antimicrobial activity against Enterobacterales species including MDR strains [91]. In a recent study, Masadeh et al. [92] investigated a novel HLM peptide which was identified by phage display and had a broad spectrum of activity against resistant strains including MRSA and NDM *E. coli*. The peptide had synergistic effects with antibiotics [92].

Dual target Peptides: Design of dual specific peptides that can recognize and inhibit two or more classes of beta lactamases is a possible strategy. These peptides may contain several binding domains or flexible linkers. The novel chimeric peptide from the beta-lactamase inhibitory protein (BLIP) was explored by Alaybeyoglu et al.[93]. Budeyri Gokgoz et al. [94] investigated the interaction between BLIP and beta-lactamase and found that co-expression of BLIP with beta-lactamase decreased enzyme activity in resistant bacteria. This provides a model for the design of dual specific peptides that can recognize and inhibit several enzyme classes through targeted binding domains [94].

Allosteric Inhibition: Thus, through the inhibition of the conserved allosteric sites of the different beta-lactamase classes instead of the active site, the peptides can block a set of enzymes. In a study by Ahmad et al. [95], a dual-binding mechanism was observed where one beta-lactam molecule was found to bind at the catalytic site while another molecule attached non-covalently at an allosteric site [95].

These mechanisms give an understanding of the multi-binding domain designs that can give dual specificity against different beta-lactamases.

Table 5: Ways to Enhance the Robustness and Selectivity of Inhibitors Derived from Phage Display

Challenge	Description	Solution
Structural Diversity	Different active site structures across beta-lactamase classes	Design libraries targeting conserved or allosteric regions
Weak Affinity Binding	Low-affinity peptides are often identified in early screens	Employ iterative affinity maturation and multi-round panning
Competitive Substrate Binding	Substrates bind tightly to the active site	Target allosteric sites or use competitive selection conditions
Proteolytic Degradation of Peptides	Peptides are degraded in vivo	Use cyclic peptides or peptidomimetics for increased stability
Evolving Resistance	High mutation rates lead to inhibitor escape	Develop dual-target or multi-target inhibitors

The challenges of stability and specificity must be addressed for phage display-derived peptide inhibitor treatments to be clinically usable in the management of beta-lactamase-mediated antibiotic resistance. The problems of stability such as proteolytic degradation and low bioavailability can be reduced through structural changes like peptidomimetics, PEGylation or cyclization. The specificity can be enhanced by improving the selection strategies, structural knowledge and computational approaches. Thus, the peptide inhibitors obtained from the phage display can be made suitable for therapeutic application and may become important antibacterial tools in the fight against the bacterial resistance.

11. Recent Advances and Potential Solutions

With the help of sophisticated techniques such as structural biology, computational modeling and iterative optimization of phage display it is possible to design peptide inhibitors that can circumvent the evolutionary plasticity of beta-lactamases and remain effective against the resistant bacteria. Recent improvements in the strategy for identifying beta-lactamase inhibitors from library screening using multi-target and high-throughput systems have greatly improved the identification of potent and broad-spectrum inhibitors.

11.1 High Throughput Phage Display Platforms

The application of HTS in phage display has greatly accelerated the drug discovery process by enabling the automation of the screening of large peptide libraries against beta-lactamases. HTS technologies enable the researchers to test more than a billion peptide variants at a time. This has

greatly improved the speed and efficiency of identifying lead compounds. Many HTS systems allow for parallel analysis, in which many targets can be assessed at the same time. Robotics and microfluidics have been integrated into the biopanning process. These platforms enable the rapid screening of the peptide libraries against various beta-lactamases and the potential inhibitors could be identified within few weeks where it might have taken months [90]. High throughput methods are further exemplified by Jeffs et al. [96] who created a whole cell biosensor platform for beta-lactamase inhibitor discovery specifically for this purpose.

11.1.1 Miniaturized Platforms: Phage display systems that are based on microfluidics allow for the reduction of the scale which leads to the reduction in the consumption of reagents and the time of the process. These platforms can also be combined with NGS to visualise the binding events in real time and the sequences of the bound peptides can be determined readily. In a study which employed automated phage display with HTS, peptide inhibitors of class A and class C beta-lactamases were identified quickly due to the ability of the platform to analyze a large number of compounds at a go and at the same time perform testing [52].

11.2. Affinity Maturation and Directed Evolution

Phage display allows for affinity maturation, a process that consists in sequential mutation of peptides identified as potential peptides to be modified in order to increase their recognition and affinity. Some strategies of directed evolution have been incorporated into phage display workflows.

11.3 Next-Generation Sequencing (NGS) Integration

The integration of next-generation sequencing (NGS) into the phage display screening has greatly enhanced the analysis and selection. NGS allows for the tracking of peptide enrichment during the biopanning. NGS has enabled the researchers to sequence the entire population of phage displayed peptides and thus know how the peptides are modified during the process of biopanning. This assists in the rapid identification of potential binders and reduces the number of experimental cycles required. The NGS has helped the researchers to monitor the enrichment of peptide sequences towards beta-lactamases. This reduced the time for discovery of high affinity inhibitors. Phage display high-throughput platforms are crucial in drug discovery. Phage display high-throughput technology, including NGS and multi-target screening is vital in drug discovery, particularly for peptide and antibody drugs. In this manner, high-throughput technology mimicks this process in a way that is similar to using a powerful sieve to quickly pinpoint the right candidates. For instance, in identifying peptides that can block antibiotic resistant beta-lactamase enzymes, high-throughput screening enables the scientists to test thousands of peptides at a go. This makes it possible to identify specific inhibitors that would have taken many years to discover manually [22]. These techniques have enabled the researchers to determine high affinity inhibitors, the efficacy of which is determined across multiple targets and the means of selecting drug candidates. In their study, Li et al. [22] discussed the application of phage display in the identification of peptide-based drugs and monoclonal antibodies with the help of NGS. NGS is a powerful tool that allows scientists to sequence millions of DNA sequences at a time to help identify the genetic makeup of potential drug compounds. In phage display, the peptides are ‘displayed’ on the surface of viruses (phages) and the peptides that bind to the target of interest can be isolated and sequenced using NGS. This gives the precise structure of each binding peptide.

For example, if a phage library has millions of variants, then NGS can be used to rapidly determine which of them are good binders [97]. This method has been used to produce a large amount of data on peptide binding profiles so that potential drug candidates can be identified easily [22]. Li et al. [22] proposed the PHDs-seq platform for the assessment of the phenotypic responses to drugs on multiple targets at once. The platform shows how the readout of multiple targets can help in the identification of compound effects on different biological pathways and may be a useful tool for the screening of peptides with specific action on multiple targets including enzymes like beta-lactamases [22]. Flow cytometry in high-throughput mode has enabled real-time assessment of peptide binding to target proteins. Busby et al. [98] reports the progress in high-throughput flow cytometry and fragment-based ligand discovery that enables the assessment of thousands of compounds. Such techniques are employed in drug discovery for the identification of peptide fragments that are potential binders [98].

11.4 Rational Library Design: From the structural data of beta-lactamase enzymes, it is possible to construct peptide libraries that include specific and conserved regions. This decreases randomness and enhances the chances of obtaining high affinity inhibitors [99]. Dotter et al. [100] investigated chemical modifications in the peptide libraries with a focus on the post translational modifications that improve peptide stability and affinity. These modifications are particularly important in phage display applications. Maria et al. [101] described a dual-display phage system with superfolder GFP (sfGFP), which allows controlled switching of the displayed peptides and the visualization of their interactions [101]. Ochoa and Cossio [102] created PepFun, an open-source software to assist in the rational design of phage display libraries. These advancements explain how the concept of rational peptide library design in phage display can enhance target specificity, binding affinity and structural robustness and how these methods can be exploited for drug discovery and therapeutic purposes.

11.5 Structure-Based Design: The available structural information from x-ray crystallography and cryoEM enables the construction of libraries whose sequences are likely to include binding motifs or elements of the sequence that are likely to interact with the conserved regions of the beta-lactamase enzyme. This increases the chances of identifying high affinity binders while at the same time reducing the number of rounds of screening required. For example, rational design was used to select conserved regions across beta-lactamases and the identification of broad-spectrum inhibitors was more effective [90]. In a study by Boragine et al., [103], the authors designed peptides that compete with BLIP for binding to beta-lactamase enzymes with the help of structural data from BLIP.

11.6 Non-Canonical Amino Acids: Another improvement is the application of techniques that enhance the chemical composition of the phage display libraries. The incorporation of noncanonical amino acids or chemically modified residues increases the peptide sequence diversity. Phage display libraries have been created to include non-native amino acids. A study showed that the incorporation of noncanonical amino acids in phage display libraries enhanced the affinity of the peptides against metallo-beta-lactamases which are otherwise difficult to inhibit.

11.7 Phage Display on Non-Linear and Cyclic Peptides: It has been found that cyclic peptides or non-linear peptides are more stable and have higher binding affinity than their linear counterparts. This is because cyclic peptides are less conformationally flexible and thus resistant to proteolysis and have higher binding affinity owing to their preorganized structures. Cyclic

peptides are generally more stable in the biological fluids and also have higher affinity. The MOrPH-PhD platform developed by Gu et al. [104] combines phage display with post-translational cyclization. This system enables high-throughput selection of peptides with constrained conformations. This makes the method especially relevant for drug discovery. In the article, Zheng et al. [105] describes a new strategy of backbone to side chain cyclization.

11.8 Cross-Class Inhibitors: Multi-target and high-throughput strategies are used to discover cross-class inhibitors that can interact with and inhibit several classes of beta-lactamases [59]. The efficacy of ceftolozane/tazobactam in the treatment of complicated intra-abdominal infections has been documented [106]. This paper also includes a description of its use in the management of bacteria resistant to multiple antipseudomonal drugs, including beta-lactams. This combination is used in both monotherapy and empirical therapy. This highlights its role as a cross-class beta-lactamase inhibitor in the management of beta-lactam resistance in various infections [106]. In a review by Bassetti et al. [107], several novel beta-lactam/beta-lactamase inhibitor combinations are discussed that are in the late stages of clinical development. In the INCREMENT-SOT Project, a clinical study was conducted to assess the efficacy of broad-spectrum beta-lactamase inhibitors in the management of kidney transplant patients with extended-spectrum beta-lactamase (ESBL)-producing Enterobacterales ([20]). Reddy et al. [87] explained the application of metallo-beta-lactamase inhibitors (MBLIs) with beta-lactam antibiotics in the treatment of carbapenem-resistant infections. The authors also recommended the development of broad-spectrum beta-lactamase inhibitors that can interact with both serine beta-lactamases (SBLs) and metallo-beta-lactamases (MBLs). These real-time case reports demonstrate how cross-class beta-lactamase inhibitors are used in clinical practice, particularly in high-risk and multidrug-resistant infections.

11.9 Allosteric Inhibition: Allosteric inhibitor discovery has been improved such that alternative strategies to active site inhibition are possible. Allosteric sites are less likely to be mutated compared to active sites. The development of multi-target screening, high-throughput platforms, and affinity maturation techniques for the peptide inhibitors of beta-lactamases is presently underway. These strategies allow the identification of broad-spectrum inhibitors.

12. Recent Advances in Engineering and Optimization of Phage Libraries

12.1 Rational Design of Phage Display Libraries: The most important achievement in phage display technology is the rational design of peptide libraries. The typical phage display libraries are randomly selected peptides, whereas the rational designed libraries are based on the structure of beta-lactamases.

12.2 Next-Generation Sequencing (NGS) for Library Optimization: The integration of next-generation sequencing (NGS) into phage display workflows has greatly improved library optimization. NGS provides a powerful tool for the comprehensive analysis of phage library populations during the selection processes. This technology helps in understanding the dynamics of the peptide population during the selection process, which in turn, assists in the design of better libraries for the subsequent cycles. NGS is applied to identify potential peptide candidates faster by tracking the evolutionary history of the peptides that are enriched. Also, NGS can be used to remove non-functional or low affinity binders from the library. NGS coupled phage display enabled the identification of the phage particles displaying the enriched peptide sequences after each biopanning round.

12.3 Library Design and Screening: Phage display coupled with computational modeling and machine learning has become an important strategy to design effective libraries. These technologies enable the prediction of peptide sequences that may have high binding affinity and specificity to the target protein. Thus, the machine learning algorithms can predict how the mutation in the peptide sequence would affect its binding to the beta-lactamases and, therefore, help to select potential peptides for the initial screening.

13. Potential Solutions Based on Optimized Phage Libraries

13.1 Broad-Spectrum Inhibitors: Phage display can be used to design libraries that are specific to the conserved regions of the multiple beta-lactamase enzymes and produce broad-spectrum inhibitors. These inhibitors can be used against several beta-lactamase enzymes including those with high mutation rate. Broad-spectrum peptide inhibitors were identified against both class A and class B beta-lactamases [52].

13.2 Specificity: To exclude the off-target peptides and increase the specificity of the peptides selected for the interaction with beta-lactamase, phage display strategies with negative selection rounds should be gradually increased. Class A beta-lactamases including TEM-1 and SHV-1 have structural similarities with some human proteases, which increases the risk of non-specific binding. The negative selection rounds can be carried out against these human proteases to sharpen the tonicity of the peptide inhibitors. Screening for Class A beta-lactamase inhibitors, using structurally similar human serine proteases as counter-screens can help to eliminate peptides that are bound non-specifically to human proteases. Some peptide inhibitors may bind to the target beta-lactamase of interest (AmpC), but also to other beta-lactamases due to their sequence similarity. The absence of these off-target beta-lactamases in the screening will help to select for inhibitors that are specific to AmpC. A study to screen inhibitor for AmpC beta-lactamases involved a counter selection strategy that incorporated Class A and Class D beta-lactamases to push the library towards peptides with higher specificity to AmpC. In addition to the bacteria enzymes, it is important that beta-lactamase inhibitors do not interact with human immune-related proteins. Thus, using human plasma proteins, such as albumin, as the negative selection targets, the researchers can modify the peptide library to exclude the interaction with the immune system. During the development of peptide inhibitors for NDM-1, a negative selection round using human plasma proteins can be included. This reduced immunogenic binding can lead to the identification of NDM-1-specific inhibitors that may have reduced immunogenicity in preclinical models. When peptide inhibitors are being screened for beta-lactamases, the bulk amplification methods can sometimes select for parasitic or non-specific phage. Emulsion amplification coupled with negative selection rounds provides a good solution. The use of negative selection rounds in phage display screening increases the specificity of peptide inhibitors for beta-lactamases.

13.3 Allosteric Inhibitors: Phage libraries can be optimized to focus on the allosteric sites, which are less likely to mutate than the active sites, to develop inhibitors that remain effective even with the changing beta-lactamases. Peptides that recognize the conserved allosteric regions can be identified using phage display. Allosteric peptides against class A beta-lactamases were investigated as potential inhibitors.

13.4 Cyclic Peptide Optimization: The problems of the stability of linear peptides have been solved through the engineering of phage libraries to include cyclic peptides. These optimized

libraries provide peptides that are not only more stable but also more resistant to proteolysis. Cyclic peptides produced by phage display were also more resistant to enzymatic degradation and had better pharmacokinetic properties [108].

The development of phage libraries and associated technologies in combination with computational approaches has greatly enhanced the peptide inhibitor discovery for challenging proteins such as beta-lactamases. Strategies like rational library construction, diversification with non-canonical amino acids, cyclic peptide synthesis, and the application of next-generation sequencing have increased the capability of the phage display technique. These optimized libraries are now in a better position to recognize stable, high affinity, and broad-spectrum inhibitors.

14. Conclusion

Further work in high-throughput phage display platforms and computational tools, including NGS and AI-driven design, will be important. Obtaining funds specifically for improving these technologies will help researchers to create and sequence peptide libraries faster and more effectively, and to close the gap between discovery and treatment. Funding initiatives could also aim at the establishment of platforms available for smaller research labs and academic institutions. The cooperation between academic institutions, pharmaceutical companies, and governmental health organizations can be accelerated both for discovery and for commercialization. Academic labs provide novel approaches and exploratory studies, while industry partners provide funding and a way to translate research into practice. The establishment of joint centers or consortia for phage display applications may create the necessary multidisciplinary resources for the development of new therapies.

Since antibiotic resistance is becoming a worldwide problem, research on cross-class inhibitors, especially those acting against multi-resistant beta-lactamases cannot be overlooked. Cross-class inhibitors that act on various types of beta-lactamase enzymes may be useful in the management of current treatments. Future research should focus on the design of reasonable peptide libraries towards the conserved regions of the different beta-lactamase classes. The development of rational library design needs further exploration to optimize the peptide specificity and efficacy. Collaborating with structural biologists and bioinformatics experts may help to build targeted, structure-based libraries. Combining structural information, the library design can be directed towards difficult targets like metallo-beta-lactamases and other enzyme classes which are currently insensitive to antibiotics. Beyond the discovery stage, additional funding should be made available for early-stage clinical trials to establish the safety and efficacy of the peptide inhibitors developed by phage display. Working with regulatory bodies at an early stage of the development process can also facilitate the way to approval.

With the help of funding, cooperative relationships, and concentration on the next-generation approaches, phage display can become a critical strategy for the development of modern therapies. The future of phage display in the fight against antibiotic resistance looks promising owing to the developments in library construction, computational chemistry, and envelope-based screening. The development of broad-spectrum inhibitors, allosteric inhibitors, and synergistic combination therapy will be crucial in the fight against the genetic plasticity of beta-lactamases and other

mechanisms of antibiotic resistance. Continual innovations in peptide engineering and optimization will ensure that phage display remains a valuable tool in the global fight against multidrug-resistant bacteria and the restoration of antibiotic effectiveness.

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