Identification and Characterization of Novel Anti-phage Compounds using a High Throughput Approach

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Biochemistry
University of Toronto

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2016

Abstract

Bacteriophages play important roles in human health. They encode and spread diverse virulence factors, and have been implicated in regulating microbiome symbiosis. In this thesis, I performed a growth curve-based high throughput screen and identified eleven novel anti-phage compounds that prevent λ phage-mediated bacterial cell lysis. Six lead compounds were further investigated to elucidate their inhibitory activities against a diverse collection of phages. I found that these compounds showed different phage inhibition profiles, suggesting diverse inhibitory mechanisms. Furthermore, I examined compound effects on individual steps of the λ infection cycle. I found that Ro 90-7501 inactivates phage particles, dequalinium chloride prevents genome injection, and mitoxantrone blocks an early step of the infection cycle. The phage inhibitors identified here represent powerful new tools for fundamental phage research. Additionally, they can be further developed and optimized for use as therapeutics against infections by pathogenic bacteria expressing phage-encoded virulence factors.
Acknowledgments

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B. anthracis</td>
<td>Bacillus anthracis</td>
</tr>
<tr>
<td>BHI</td>
<td>brain heart infusion</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>cI</td>
<td>lambda phage repressor protein</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded deoxyribonucleic acid</td>
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<tr>
<td>E. coli</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>gp</td>
<td>gene product</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium ions</td>
</tr>
<tr>
<td>λ</td>
<td>Enterobacteriophage lambda</td>
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<tr>
<td>λcI&lt;sup&gt;857&lt;/sup&gt;</td>
<td>mutant lambda phage with a temperature sensitive repressor protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>LOPAC</td>
<td>Library Of Pharmacologically Active Compounds</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharides</td>
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<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>nanometre</td>
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<tr>
<td>OD$_{595}$</td>
<td>optical density measured at 595 nm</td>
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<tr>
<td>P</td>
<td>phage titre with the specified compound</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>------</td>
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<tr>
<td>P. aeruginosa</td>
<td>Pseudomonas aeruginosa</td>
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<td>phage titre without compound</td>
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<td>RNA polymerase</td>
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<td>Streptococcus mitis</td>
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<td>Streptomyces peucetius</td>
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<td>S. typhimurium</td>
<td>Salmonella typhimurium</td>
</tr>
<tr>
<td>SM</td>
<td>suspension media</td>
</tr>
<tr>
<td>t</td>
<td>time</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
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<td>UV</td>
<td>ultraviolet</td>
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Chapter 1
Introduction

1.1 Overview

Bacteriophages, or phages, are viruses that exclusively infect bacteria. They are the most abundant biological entities in the biosphere, outnumbering their bacterial hosts by a factor of ten. Phages have well-established ecological roles. It is estimated that phage infections occur at a rate of $10^{23}$ per second, and that these infections are responsible for removing 20%-40% of the prokaryotic biomass each day. In addition to their great impact on natural ecosystems, it has come to recent attention that phages also play key roles in human health. With many bacterial virulence factors encoded on the phage genome and transmitted between different bacteria by phage-mediated horizontal transfer, it is clear that phages are not just “eaters” of bacteria but are also carriers of disease-causing genes. Additionally, phages are a natural component of the human microbiome. With the help of recent advancements in sequencing technology, the “dark matter” of the phageome (an ensemble of phages in the human body) is just starting to be investigated. Emerging evidence has suggested that the phageome has a considerable impact on microbiome symbiosis, which ultimately exerts great influence on human health and disease. In this thesis, I will describe a method of identifying and characterizing compounds that specifically inhibit phage activities.

1.2 Bacteriophage life cycle

Up to 95% of all known phages are tailed phages with a dsDNA genome. Tailed phages consist of a protein shell (capsid) that encapsulates DNA attached to a tail, which is responsible for host receptor binding and for providing a channel for phage DNA delivery from the capsid to the host bacteria. Tailed phages can be classified into three groups based on tail morphology: Myoviridae with long contractile tails, Siphoviridae with long noncontractile tails, and Podoviridae with short tails. For the majority of the work presented in this thesis, the well-characterized long noncontractile-tailed λ phage is used as the model phage.

Like other viruses, phages need to be inside a host cell to reproduce (Figure 1). Some phages are lytic; they kill the host bacteria to complete the replication cycle. Other phages are temperate; they can either kill the host bacteria, or establish lysogeny. Infection by phage λ, a temperate
phage, is initiated at the outer cell membrane when the phage tail fiber protein binds to the bacterial membrane receptor, LamB. Through a mechanism that is not completely understood, the phage DNA is injected from the phage head into the cytoplasm of the bacteria. This requires the phage to traverse the bacterial cell envelope, which consists of inner and outer membranes as well as a peptidoglycan layer between these two membranes. Once inside the bacteria, the linear phage DNA is joined at the cohesive ends by the host DNA ligase to form a double-stranded circular molecule. From here, the lytic/lysogenic decision has to be made. When bacteria are starved in poor growth media or infected with a high multiplicity of infection, lysogenic growth is favored. Lysogeny of phage λ is established by the integration of the phage DNA into the bacterial chromosome. The resulting phage, now called a prophage, can be passed down to daughter cells at each cell division. During lysogeny, most of the phage genes are silenced by the phage repressor protein, cl. However, exposure to DNA damaging agents such as UV radiation can activate the host SOS response. This results in the cleavage and inactivation of the cl repressor and irreversible transition to lytic growth. In the lytic cycle, the phage genome is replicated and phage particles are produced and assembled. The host cell is eventually lysed by phage-encoded proteins to release progeny phages, which are capable of initiating another round of infection. Interestingly, the lytic cycle of phage λ can be experimentally controlled by using a mutant strain, λcI857, which carries a temperature sensitive repressor protein that permits lytic growth at high temperatures.
Figure 1. The phage replication cycle. Lytic phages pursue a lytic cycle (outer circle) while temperate phages can pursue either a lytic or lysogenic life cycle (inner circle). In the lytic cycle, phages bind to the bacterial surface receptor (1), inject their DNA (2), replicate it (3), produce new virion particles (4), assemble new phages (5), and eventually lyse their host cell (6). In the lysogenic cycle, instead of actively replicating their DNA, phages integrate their genome into the bacterial chromosome to form a prophage, where they enter a quiescent stage and replicate with the bacterial chromosome. The resulting bacteria, now called a lysogen, can be induced by certain signals such as UV radiation, chemical exposure or oxidative stress. This causes the excision of the phage genome from the bacterial chromosome and entry to the lytic cycle.

1.3 The importance of bacteriophages to human health

1.3.1 Phage-encoded virulence factors

Phage lysogens are commonly found in bacteria; sequencing data reveals that an average bacterial genome contains three prophages, with some extreme cases where 20% of the bacterial genome is prophage-derived\(^1\). Prophages carry genes that can enhance the virulence of the host bacteria, and these prophage-encoded virulence factors are involved in all stages of bacterial pathogenesis\(^4\). Given that integrated phages can excise from one strain of bacteria and infect another strain, phages facilitate the horizontal transfer of these virulence factors and contribute to the emergence of novel pathogens\(^5\).

Historically, the most widely recognized phage-encoded virulence factors are exotoxins\(^1\). Some of these exotoxins are the major causes of life-threatening human diseases such as cholera\(^1\), diphtheria\(^1\), and botulism\(^2\). Phage-encoded exotoxins work by a variety of mechanisms,
including inhibiting protein synthesis (i.e. Diphtheria toxin and Shiga toxin)\textsuperscript{21,22}, blocking neurotransmitter release (i.e. Botulinum toxin)\textsuperscript{23}, and stimulating constitutive ion efflux (i.e. Cholera toxin)\textsuperscript{24}. The expression of some toxins relies on host cell-encoded transcription factors. However, others solely depend on the regulation of the phage life cycle. For example, The Shiga toxin genes are located within the late operons of prophage 933W in \textit{E. coli} O157:H7 and are transcribed from the late phage promoter along with the lysis genes following prophage induction\textsuperscript{25,26}. Recent studies have shown that inhibiting prophage induction is sufficient to reduce Shiga toxin production and to prevent disease progression in mice\textsuperscript{27}. Since traditional antibiotic treatment triggers prophage induction and augments Shiga toxin production, it cannot be used to combat this bacterial infection\textsuperscript{28}. Therefore, the phage life cycle may represent a promising target to fight \textit{E. coli} O157:H7 virulence.

Exotoxins only represent a subset of phage-encoded virulence factors. Phages are also known to encode other virulence factors which contribute to bacterial pathogenicity through other mechanisms. Some phages, for instance, influence early stages of bacterial infection including adhesion and invasion. Examples of phage encoded factors that facilitate bacteria adhesion to eukaryotic cells are the Pb1A and Pb1B proteins of \textit{S. mitis}\textsuperscript{29}. Pb1A and Pb1B are encoded on prophage SM1 and promote binding of the bacterium to human platelets\textsuperscript{30}. Other phages encode genes that promote tissue invasion by the bacteria. To achieve tissue invasion, many bacteria adopt a type III secretion system (T3SS), which injects effector proteins from the bacteria directly into the cytoplasm of the host eukaryotic cells\textsuperscript{31}. Many of these effector proteins are phage encoded. For example, phage SopE\_\phi of \textit{S. enterica} encodes a T3SS effector, SopE, which promotes entry of the bacteria into epithelial cells\textsuperscript{32}.

Additionally, phages encode genes that help the bacteria to survive in the human body. Some phages, for instance, help bacteria evade the host immune responses. For example, phage \phi\textsubscript{PVL} of \textit{S. aureus} encodes a cytotoxin, Panton-Valentine leukocidin, which targets and lyses leukocytes\textsuperscript{33}. Other phages encode genes that alleviate oxidative stress encountered by the bacteria. For example, phage Gifsy-2 found in \textit{S. enterica} encodes a superoxide dismutase, which converts superoxide radicals into hydrogen peroxide and molecular oxygen\textsuperscript{34}.

It is clear that phages are intimately associated with bacterial pathogenesis. Given that phages not only encode virulence factors but also disseminate these virulence factors among other
bacteria in proximity, it may be possible to attenuate or diminish the virulence of bacteria by inhibiting specific phage activities such as virulence gene expression and horizontal gene transfer.

**1.3.2 Phage impact on the human microbiome**

In addition to providing a diverse source of virulence factors that directly cause illnesses, phages have also been implicated in influencing the human microbiome. A number of comparative metagenomic studies have shown correlations between changes in the phageome and human diseases such as inflammatory bowel disease\textsuperscript{11}, cystic fibrosis\textsuperscript{9}, and schizophrenia\textsuperscript{35}. These studies suggested that phages may contribute to bacterial dysbiosis. However, the mechanisms by which phages are associated with disease conditions still remain to be further elucidated. Since current studies rely on metagenomic sequencing data after the disease state has been achieved, it is insufficient to define the causal relationship between phage activity and disease. An alternative approach would be to specifically inhibit the phage activity and observe the outcome of such inhibition on disease progression.

In addition to being associated with diseases, phages have also been shown to contribute to the dissemination of antibiotic-resistance genes in the microbiome. Upon antibiotic administration, the human microbiome faces selective pressure where resistant bacteria gain growth advantages. Phages are excellent vectors for antibiotic resistance gene exchanges through generalized or specialized transduction. Sequencing data have revealed diverse antibiotic resistance genes in phage genomes in both environmental and clinical settings\textsuperscript{36,37}. Importantly, a recent metagenomic study showed that antibiotic resistance is enriched in the phageome following antibiotic treatment in mice\textsuperscript{38}. Surprisingly, resistance was found against the challenging antibiotics as well as those unrelated to the administered drug\textsuperscript{38}. In the same study, it was also demonstrated that treating a cultured naïve microbiome ex vivo with phages from the antibiotic-treated mice resulted in increased antibiotic resistance. This study suggested that antibiotic perturbation enhances phage activity, which promotes the emergence and spread of antibiotic resistance genes in the microbiome\textsuperscript{38}. Given the implications of phages in antibiotic resistance, the phage life cycle may be targeted to minimize the transmission of antibiotic resistance genes as well as for preserving the effectiveness of existing antibiotics.
1.4 Using small molecules to inhibit phage activity

Despite the importance of bacteriophages to human health, there has been very little effort to develop interventions to prevent phage-mediated pathogenesis. Given that phages encode and spread diverse virulence factors and potentially regulate the human microbiome, the phage life cycle is an attractive target for intervention.

The most straightforward way to inhibit the phage life cycle is through the use of small molecule inhibitors. Small molecules have been used as valuable tools to probe specific biological functions and as leads to develop new therapeutic drugs\textsuperscript{39}. For example, the human genome encodes 20,000 to 25,000 genes, which can be translated and processed into approximately a million protein products\textsuperscript{40}. Great efforts have been dedicated to use small-molecule chemical probes to study the function and druggability of these protein targets\textsuperscript{41}. Unlike gene knockouts and siRNAs, small molecules target the protein rather than the gene locus or mRNA\textsuperscript{41}. Furthermore, they have defined half-lives and distribution patterns which allows spatial temporal regulation of their actions\textsuperscript{42,43}. Their selectivity, cell permeability, and virtually unlimited structural diversity make them great research tools to study the functions of novel genes and pathways\textsuperscript{41}.

With increasing accessibility of drug-like compound libraries and the development of high-throughput screening strategies, small molecules have been widely used in industry and academia for drug discovery to address key biological questions. Here, I aimed to identify phage specific small molecule inhibitors and to characterize their mechanisms of action.

1.5 Steps of the phage lytic cycle

Several key steps of the phage life cycle can be inhibited to prevent the lytic progression of phage infection. These steps include adsorption of the phage onto the host, phage genome injection, genome replication, RNA/protein synthesis, virion assembly and host cell lysis. Since phages have evolved an intimate relationship with their bacterial hosts, they require both host-encoded and phage-encoded factors to achieve these steps to ensure successful infection. These factors are excellent targets for drug inhibition. In this thesis, I employed a high throughput approach using bacterial growth curves to identify small molecules that efficiently stall phage lytic propagation without compromising the overall growth of the host bacteria.
1.5.1 Host cell adsorption and genome injection

Phage adsorption to the host bacteria is the first step of the phage life cycle. This is accomplished by binding of the phage tail fiber to specific bacterial surface receptors. This interaction defines the specificity of phage infection and primes the phage for genome injection.

Bacteriophages have adapted to use unique features of the bacterial surface for adsorption. Phages that infect Gram-negative bacteria can target proteins embedded in the bacterial outer membrane. For example, LamB, a selective porin of *E. coli* used for maltose transport, serves as the receptor for phage λ. Similarly, OmpF, another porin of *E. coli* that is involved in quinolone antibiotic resistance, is the receptor for phage T2. In addition to surface proteins, phages can also adsorb to various lipopolysaccharides (LPS), which are major components of the outer membrane of Gram-negative bacteria. LPS is a complex polymer made up of Lipid A, core oligosaccharide and O-antigens, and mainly functions to enhance bacterial structural integrity. Some phages adsorb to the O-antigen (i.e. phage P22 and phage 2 of *S. typhimurium* and *P. aeruginosa*, respectively), while others recognize the core oligosaccharide (i.e. phage T3 and phage F 0-1 of *E. coli* and *S. minnesota*, respectively). Interestingly, phage T4 of *E. coli* requires both LPS and an outer membrane protein, OmpC, for adsorption. It was shown that loss of either LPS or OmpC resulted in reduced efficiency of infection whereas loss of both receptors led to T4 resistance.

In contrast to Gram-negative bacteria, Gram-positive bacteria do not have an outer membrane. They have a thick cell wall that is composed of peptidoglycan, which provides the cell wall with structural strength. In addition to peptidoglycan, teichoic acids are also vital components of the cell wall of Gram-positive bacteria, serving to provide extra rigidity. Both peptidoglycan and teichoic acids provide receptor binding sites for phages that infect Gram-positive bacteria. For example, selective degradation of the teichoic acid or the peptidoglycan of the *S. aureus* cell wall significantly impaired adsorption of phage 3C, 71, and 77. In addition to peptidoglycan and teichoic acids, many surface proteins of Gram-positive bacteria have been found to be involved in phage adsorption. For example, GamR of *B. anthracis* which is the receptor for phage γ. For both Gram-negative and Gram-positive bacteria, phages have been reported to adsorb to bacterial pili, flagella, and capsule. Almost every structure exposed on the surface of bacteria is targeted for phage adsorption.
Mechanisms of phage adsorption and genome injection vary significantly depending on the specific phage-host pairs. For example, phage T4 initially undergoes reversible binding of its long tail fibers to OmpC or LPS, which triggers a conformational change to the base plate resulting in the release of the short tail fibers which bind irreversibly to LPS\textsuperscript{57,58}. Conformational changes of the base plate also initiate contraction of the tail sheaths which drives the inner tail tube through the bacterial outer membrane\textsuperscript{59}. To penetrate the peptidoglycan layer, lysozyme domains of gp5 located at the end of the tube degrade peptidoglycan and allow the tail tube to reach the inner membrane\textsuperscript{60}. Contact of the tail tube with the inner membrane triggers DNA release from the phage capsid through the tail tube into the host bacteria\textsuperscript{61}.

Like T4, phage T7 adsorbs to LPS through interactions with its tail fibers\textsuperscript{62}. Consequently, several internal core proteins are ejected from the capsid into the host membrane to facilitate phage DNA delivery. For example, gp14, gp15 and gp16 form a channel that spans the bacterial membrane\textsuperscript{63}. To overcome the barrier of the cell wall, gp16 possesses hydrolase activity which degrades peptidoglycan\textsuperscript{64}. After the channel is formed, phage DNA is transferred into the host cell. Interestingly, T7 phage DNA translocation is coupled with transcription\textsuperscript{65}. Early gene products inhibit the host type I restriction systems which protects later DNA sites sensitive to restriction\textsuperscript{66}.

T5 and similar phages irreversibly adsorb to the cell surface receptor, FhuA, which directly leads to DNA transfer irrespective of other factors\textsuperscript{67,68}. The exact mechanism of T5 phage DNA transfer is not fully understood; however, it has been shown that DNA injection occurs in two steps\textsuperscript{69}. The first 8\% of the phage DNA is introduced into the cytoplasm after which a pause of 4 minutes is encountered\textsuperscript{69}. Subsequently, the rest of the DNA is transferred\textsuperscript{69}.

1.5.2 DNA replication, transcription and protein synthesis

Immediately after phage genome injection, the lytic life cycle proceeds with phage DNA replication, transcription and protein synthesis. Most phages hijack the host RNA polymerase (RNAP) for transcription\textsuperscript{13}. However, some phages such as T7 encode their own RNAPs\textsuperscript{70}. To ensure efficient phage DNA transcription, some phages shut off host transcription. For example, phage T4 encodes several proteins that modify the host RNAP and transcription factors to selectively transcribe phage DNA\textsuperscript{71}. T7, on the other hand, completely inactivates host RNAP activity to promote transcription from the T7-encoded RNAP\textsuperscript{72}. 
All phages rely on the translation machinery of their host bacteria for protein synthesis. Early genes from early promoters are transcribed and translated first. These early gene products play important regulatory roles, such as lytic/lysogeny decisions for temperate phages, as well as initiating phage DNA replication. Many phage replication strategies have been reported. For example, replication of the circular \( \lambda \) DNA starts at the replication origin with the closed circle mechanism but later switches to the rolling circle mechanism, producing linear concatemeric genomes\(^1\). Phage T4, by contrast, has a more complex replication mechanism. The linear T4 DNA contains several replication origins which are capable of initiating multiple leading strand DNA synthesis\(^3\). When an origin-initiated replication fork reaches an end, the 3’ end invades another strand that is complementary in sequence and results in the formation of branched concatamers\(^4\). Furthermore, phage T7 DNA contains terminal repeats but replicates as a linear molecule\(^5\). Replication occurs bi-directionally producing linear DNA molecules with 3’ single-stranded extensions containing part of the terminal repeats which anneal to form linear concatamers\(^6\).

Later in the infection process, genes from the late operons are transcribed and translated. For example, for phage \( \lambda \), gene Q, the last gene of the early operon, is responsible for turning on late gene expression\(^7\). Gene Q encodes an anti-terminator which allows the RNAP to read through termination signals and continue to transcribe through the late gene operon\(^7\). The late genes encode structural proteins involved in assembling progeny phage particles and in cell lysis.

### 1.5.3 Virion assembly

All tailed phages contain a head and a tail which are assembled separately\(^8\). Head assembly requires the formation of a procapsid, which is an empty protein shell icosahedral in shape\(^9\). There are three crucial components of the procapsid: capsid protein, scaffold protein, and portal protein\(^9\). The capsid protein is the major building block of the procapsid\(^9\). Due to the limited size of the phage genome, most phages use multiple copies of one capsid protein to assemble the head\(^9\). To ensure the assembly of the procapsid into the right geometry, the scaffold proteins form a core inside the procapsid\(^9\). The portal protein sits on one of the vertexes of the capsid and forms a hole that is crucial for DNA passage\(^9\). It also provides a bridge that links the phage head to the tail proteins\(^9\).
Once the procapsid is formed, the phage-encoded terminase enzyme carries the replicated phage DNA to the portal protein and translocates the phage genome into the head using energy derived from ATP hydrolysis\textsuperscript{80}. The terminase enzyme is usually composed of a small subunit, which binds to phage DNA, and a large subunit, which binds to the procapsid and is responsible for DNA translocation and cleavage\textsuperscript{79}. The packaging of concatemeric DNA initiates by recognizing a specific sequence called cos or pac\textsuperscript{81}. Following initiation cleavage, the DNA is translocated unidirectionally into the empty head. A second cleavage is consequently made to generate the mature form of phage DNA\textsuperscript{81}. For some phages such as $\lambda$ and T7, the DNA is cleaved at specific sites\textsuperscript{82,83}. However, for other phages such as P1 and T1, cleavage is non-specific\textsuperscript{84,85}. Following the second cleavage, the terminase dissociates from the head and is capable of initiating another round of DNA packaging\textsuperscript{86}. Following the dissociation of the terminase, the head is capped with neck proteins, which together with the portal protein prevent the exit of packaged DNA from the head\textsuperscript{87}.

During head maturation, the procapsid undergoes structural transition to form a bigger, thinner, angular and more stable head\textsuperscript{79}. For example, the scaffold proteins of P22 and phi29 exit the procapsid during maturation while the scaffold proteins of HK97 and T4 are proteolytically cleaved\textsuperscript{88-91}. In addition to scaffold proteins, the capsid protein and the portal protein can also be subject to cleavage. Such structural changes result in head expansion to accommodate incoming phage DNA, and allow capsid protein to refold to a lower energy state\textsuperscript{79}. Several auxiliary proteins are subsequently incorporated into the head where they modulate the infectivity of the newly assembled phage\textsuperscript{79}.

The mature head joins the tail proteins in Podoviridae or a preassembled tail in Siphoviridae and Myoviridae to form a mature phage\textsuperscript{79}. Tail assembly in Siphoviridae and Myoviridae initiates from the distal end of the tail at the initiator complex, where multiple copies of the tail protein polymerize to form the cylindrical part of the tail\textsuperscript{79}. This process forms the tail tube, which forms the majority of the tail in Siphoviridae\textsuperscript{92}. In Myoviridae, once the tail tube is formed, it is wrapped by an outer contractile tail sheath\textsuperscript{93}. The length of the tail is determined by a tape-measure protein which spans the entire length of the tail and act as a scaffold for tail tube formation\textsuperscript{94}. Once the tail reaches its requisite length, it is capped by terminator proteins which subsequently interact with the neck proteins on the head to complete the assembly process\textsuperscript{95}. 
1.5.4 Host cell lysis

Once assembled, newly synthesized phages must escape from their host cell in order to find new prey for the next round of infection. For dsDNA phages of Gram-negative bacteria, this is achieved through phage-encoded lysis proteins, which disrupt the three layers of the cell envelope: inner membrane, peptidoglycan, and outer membrane\textsuperscript{96}. Endolysin is a soluble muralytic enzyme that is capable of degrading the peptidoglycan layer of the cell wall. However, it is cytosolic and requires the action of the holin, which permeabilizes the inner membrane\textsuperscript{97}. The holin function is tightly regulated to fine tune the timing of host cell lysis. This ensures optimal production of progeny virions while not compromising the chances for these virions to find new hosts\textsuperscript{97}. In addition to endolysins and holins, many phages require a third system to disrupt the outer membrane of Gram-negative bacteria. This is achieved by a spanin complex consisting of a small outer membrane lipoprotein and an integral cytoplasmic membrane protein\textsuperscript{96}. Spanin function is essential for lysis because phage mutants lacking spanins were trapped in dead spherical bacterial cells\textsuperscript{98}. It was proposed that spanins promote inner and outer membrane fusion which consequently disrupt the integrity of the outer membrane, leading to cell lysis\textsuperscript{99}.

1.6 Thesis objectives

Despite the contributions of phages to human health, little has been done to explore the use of anti-phage agents for the treatment of phage-mediated bacterial pathogenesis. The goal of my thesis project was to identify and characterize small molecules that block lytic progression of bacteriophages. Specifically, I performed a growth curve-based high-throughput assay that screened a collection of small molecules for anti-phage activity. After hit identification, I characterized the inhibitory mechanisms of action by examining their effects on individual steps of the infection cycle, including DNA injection, integration, phage particle assembly, and cell lysis.
Chapter 2
Materials and Methods

2.1 Growth-curved based high throughput screen for anti-phage compounds

*E. coli* strain K-12 BW25113 was grown overnight with shaking in LB at 37 °C. On the following day, a 2% subculture of the overnight culture was prepared in LB. 50 µl of the subculture was aliquoted to 384-well flat-bottom plates containing indicated compounds (see below). Each plate was incubated with orbital shaking at an amplitude of 3 mm in a Tecan Infinite 200 plate reader at 37 °C, with OD₅₉₅ readings taken every 15 min. At 1 hour, plates were taken out from the plate reader and 5 µl of phage λ*cI₈₅₇* at 10¹⁰ pfu/ml was added. The multiplicity of infection (the ratio of phage to bacteria) was ~10. High multiplicity of infection was chosen to screen for potent inhibitors that block phage replication during the first round of infection. The plates were then incubated in the plate reader and grown and recorded for OD₅₉₅ readings for an additional 5 hours. λ*cI₈₅₇* carries a temperature sensitive repressor gene, *cI₈₅₇*, which is unstable at temperatures above 32 °C. The screen was carried out at 37 °C, which only permits lytic growth of the phage and prevents lysogen formation which might interfere with the growth curve readings.

Using this method, I screened a collection of Approved/Bioactive/Natural Products compounds purchased from the SPARC BioCentre (http://www.sickkids.ca/Research/SPARC/Drug-Discovery/Chemical-Small-Molecule-Libraries/index.html). This class of compounds is composed of: the LOPAC Library, Prestwick Library, and MicroSource Spectrum Library. The LOPAC Library contains 1280 compounds with well documented pharmacological activities, including marketed drugs, failed development candidates and “gold standards” that have well-characterized activities. This collection of compounds impacts most cellular processes and covers all major drug target classes. The Prestwick Library contains 1280 compounds that are 100% approved by FDA, EMA and other agencies and exhibit high degree of “drug-likeness”. This collection of compounds shows high chemical and pharmacological diversity and has known safety and bioavailability in humans. MicroSource Spectrum Library contains 2400 compounds that encompass a wide range of biological activities and structural diversity. It contains drugs with known biological activities as well as natural products and derivatives with
potential novel structural diversity but undetermined activities. All compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mM. 0.2 µl of the stock solutions was added to 384-well plates to achieve a final testing concentration of 40 µM. 320 compounds were screened in one plate (in addition to 36 positive controls and 36 negative controls).

A hit was defined as a compound that prevent λcI857-mediated bacterial lysis indicated by a drop in OD595 reading post infection. This screen was of high stringency and it only allowed the identification of compounds that fully rescued bacterial growth post phage infection at high multiplicity of infection. Hits were individually purchased from SPARC BioCentre and verified by repeating the growth curve assay.

2.2 Compounds used in follow-up experiments

Mitoxantrone, dequalinium chloride, acriflavine, propidium iodide, and Ro 90-7501 were purchased from Sigma-Aldrich. Ellipticine was purchased from EMD Millipore. Stock solutions of mitoxantrone, dequalinium chloride, acriflavine and propidium iodide were made in double distilled water (ddH2O). Stock solutions of Ro 90-7501 and ellipticine were made in DMSO.

2.3 Phage panel testing

Having established initial hits, the specificity of each hit was determined by examining its inhibitory effect on a panel of phages that infect E. coli, P. aeruginosa and S. aureus. The phages include λ, HK75, T5, T6, T7, P2, JBD44, DMS3, P68, AHJD44 and φ80. Phage λ, HK75, T5, T6 and T7 were propagated and tested on E. coli strain BW25113. Phage P2 was propagated and tested on E. coli strain C1a. Phage JBD44 and DMS3 were propagated and tested on P. aeruginosa strain PA14. Phage P68, AHJD44 and φ80 were propagated and tested on S. aureus strain 8325-4. E. coli and P. aeruginosa were grown in LB and S. aureus were grown in BHI media.

Instead of infecting bacteria at a high multiplicity of infection and looking at the growth curves in a qualitative manner, a more quantitative approach was used to examine the inhibitory effects of the hit compounds on phage replication. Indicator bacteria were grown overnight in corresponding media (see above). 1% subculture was prepared with indicated compounds at a final concentration of 40 µM. Phages were added to the subculture at a multiplicity of infection of 10⁻⁴. A low multiplicity of infection was used to minimize background phage level and to
allow quantification of phages produced after multiple rounds of infection. The bacteria-
compound-phage mixture was grown at 37 °C with shaking for 6 hours. To assess phage
production, bacteria were spun down and the supernatants containing phages were collected.
Indicator bacteria were suspended in molten 0.7% top agar and overlayed on LB agar. Serial
dilutions of the phages were spotted on the lawn of bacteria and incubated overnight at 37 °C.
The number of phages produced was quantified by counting plaque formation.

2.4 Compound inactivation assay

Phage λ (~10^{10} pfu/ml) was incubated with each lead compound at a final concentration of 40
µM and incubated at 37 °C with shaking for 2 hours. Serial dilutions of the phage/compound
mixtures were made in LB and spotted on a lawn of indicator bacteria on an agar plate. The plate
was incubated overnight at 37 °C. Active phages were enumerated by counting plaques.

2.5 Electron microscopy

Cesium chloride banded phages with and without Ro 90-7501 treatment were applied to carbon
coated grids, washed with ddH₂O, and stained with 2% uranyl acetate. EM pictures were taken
with a transmission electron microscope at 50,000x to 80,000x magnification.

2.6 Potassium efflux assays – phage DNA injection

Bacterial cultures were grown in LB overnight at 37 °C. On the following morning, 1%
subculture was made in LB in the presence of each compound (final concentration of 40 µM)
and grown at 37 °C in LB to an OD₆₀₀ of 0.5. 5 ml aliquots of the culture were collected by
centrifugation, washed in suspension medium (SM; 100 mM NaCl, 10 mM MgSO₄, and 50 mM
Tris at pH 7.5), and resuspended in 5 ml SM. Washing in SM removed extracellular potassium
ions present in the medium and minimized background readings. Compounds were added to the
suspension to a final concentration of 40 µM. The cells were allowed to equilibrate in a 37 °C
water bath for 5 min before 150 µl of PEG precipitated λ phage (~10^{11} pfu/ml) was added.
Potassium levels were monitored for 15min using an Orion ionplus potassium electrode (Thermo
Scientific).
2.7 Lysogen formation assay

Bacterial cultures with compounds were grown at 30 °C in LB supplemented with 0.2% maltose and 10 mM MgSO₄ to an OD₆₀₀ of 0.5. Phages were added to cells at a multiplicity of infection of 10, and incubated at 30 °C for 15 min to engage lysogen formation. The cell-phage mixture was diluted 10 times in LB and recovered for 1 hour at 30 °C. Dilutions of the cell culture were plated on LB plates to individual colonies and incubated at 30 °C overnight. 52 colonies were collected on the following day and replica plated at 30 °C and 42 °C. Lysogens were counted as those that can grow at 30 °C but not at 42 °C.

2.8 Heat induction of a temperature sensitive lysogen

Cells that harbor a λcI₈₅₇ prophage were grown at 30 °C for 3 hours and induced by heating in a water-bath set at 50 °C for 2 min. The cultures were then shifted to 42 °C for 15 min before incubated at 37 °C for 1 hour. Cell lysis was monitored by measuring OD₅₉₅ every 10 min in a TECAN plate reader. To assess phage particle formation, samples were taken at 1 hour post induction, and phage titers were calculated by spotting dilutions on a lawn of indicator bacteria.
Chapter 3
Results

3.1 High-throughput screening identified 11 compounds that inhibited the bacterial killing activity of phage $\lambda cI_{857}$

The growth-curve profile of the host bacteria was used as the determining factor for phage inhibitor screening. Using a Tecan plate reader, which can simultaneously monitor growth curves of 384 bacterial cultures, I performed a high-throughput screen to identify compounds that inhibit $\lambda cI_{857}$-mediated bacterial lysis. I screened a total of 4960 compounds from three small molecule libraries: LOPAC, Prestwick, and Microsource Spectrum.

The screen was carried out in a 384-well plate format. Bacterial cultures were added to plates pre-aliquoted with compounds and grown for 1 hour before infection with $\lambda cI_{857}$. A multiplicity of infection of ~10 was used in the screen, which ensured that every cell was infected and would die after one round of infection. This made the screen highly stringent and only allowed the identification of compounds that fully prevented phage-mediated bacterial lysis during the first round of infection.

Growth was monitored at OD$_{595}$, with measurements taken every 15 min. As a positive control, bacterial growth was monitored without phage infection in the absence of compounds. As a negative control, cells were infected with $\lambda cI_{857}$ in the absence of compounds. As expected, robust growth was observed in the positive control cultures (Figure 2, blue), and bacterial cell lysis was observed in the negative control cultures (Figure 2, green). Hits were identified by visually inspecting growth curves with each compound post phage infection. They resulted in continued bacterial growth post infection (Figure 2, red).

The growth-curve based screen identified eleven compounds that inhibited the killing activity of $\lambda cI_{857}$ (Figure 2). These included five anthracyclines/anthracycline derivatives (daunorubicin, doxorubicin, epirubicin, idarubicin, and mitoxantrone), two acridines (acriflavine and ethacridine lactate), propidium iodide, ellipticine, Ro 90-7501 and dequalinium chloride.

Anthracyclines are one of the most effective classes of anti-neoplastic drugs originally isolated from $S. peucetius$ and have been used to treat many cancers, including leukemia, breast cancer, prostate cancer, and lung cancer$^{100}$. Anthracyclines have been identified as inhibitors of
topoisomerase II\textsuperscript{101}. They intercalate into DNA and form an anthracycline-DNA-topoisomerase II complex, which prevents topoisomerase II from resealing DNA, causing dsDNA breaks\textsuperscript{102}.

Like anthracyclines, the two acridines (acriflavine and ethacridine lactate), propidium iodide, and ellipticine all have DNA-intercalating properties. Ellipticine shows anti-neoplastic activities by forming ellipticine-DNA adducts\textsuperscript{103}. Acriflavine and propidium iodide have been used as fluorescent DNA dyes\textsuperscript{104,105}, and ethacridine lactate has been used as an antiseptic\textsuperscript{106} and abortifacient\textsuperscript{107}. The remaining two hits identified from the screen are Ro 90-7501, which is a synthetic compound that has anti-amyloid properties\textsuperscript{108}, and dequalinium chloride, which has been used as a topical bacteriostat with antibacterial and antifungal properties\textsuperscript{109}.

For further characterization, I focused on one anthracycline (mitoxantrone) and one acridine (acriflavine) because of their lower toxicity to bacteria compared to other compounds in the same group. I also characterized dequalinium chloride, propidium iodide, ellipticine, and Ro 90-7501. First, I determined the minimal inhibitory concentration (MIC) of these six lead compounds by testing serial dilutions in bacterial cultures against \textit{\textlambda}cI\textsubscript{857} infection (Table 1). The MIC represents the lowest concentration where \textit{\textlambda}cI\textsubscript{857}-mediated cell lysis is inhibited based on bacterial growth-curve profiles. I found that mitoxantrone is the most potent inhibitor with an MIC of 5 \textmu M while acriflavine is the least potent compound with an MIC of 30 \textmu M.

It is worth noting that many of the lead compounds have bactericidal activities. For example, dequalinium chloride is an antiseptic drug that is active against \textit{E. coli} with an MIC of 120 \textmu M\textsuperscript{109}. Ellipticine, on the other hand, possesses antimicrobial activity against \textit{E. coli} with an MIC of 65 \textmu M\textsuperscript{110}. Importantly, these compounds inhibited phage activity at much lower concentrations with little or no effects on bacterial growth.
Figure 2. Eleven compounds inhibit the killing activity of λcI857. Overnight BW25113 cultures were diluted 50 times in LB and incubated in the presence (red) and absence (green) of each compound in a 384-well format. The cultures were grown at 37 °C with shaking for 1 hour. The cultures were then infected with λcI857 at a multiplicity of infection of 10 and grown for an additional 5 hours. Cell density (OD₅₉₅) was monitored every 15 min using a TECAN plate reader. As a positive control, the growth curve of an untreated and uninfected culture (blue) was also monitored.
Table 1. Summary of hit compounds identified in this study.

<table>
<thead>
<tr>
<th>Chemical classification</th>
<th>Compound name</th>
<th>Library originated</th>
<th>MIC (μM)</th>
<th>Structure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthracyclines / anthracycline derivatives</td>
<td>Mitoxantrone</td>
<td>LOPAC/Microsource/Prestwick</td>
<td>5</td>
<td><img src="" alt="Structure of Mitoxantrone" /></td>
<td>Anti-tumor, DNA intercalating agent</td>
</tr>
<tr>
<td></td>
<td>Daunorubicin</td>
<td>Prestwick</td>
<td>-</td>
<td><img src="" alt="Structure of Daunorubicin" /></td>
<td>Antineoplastic; DNA topoisomerase II inhibitor; inhibit DNA synthesis</td>
</tr>
<tr>
<td></td>
<td>Epirubicin</td>
<td>Microsource/Prestwick</td>
<td>-</td>
<td><img src="" alt="Structure of Epirubicin" /></td>
<td>Anti-tumor, DNA intercalating agent</td>
</tr>
<tr>
<td></td>
<td>Idarubicin</td>
<td>LOPAC</td>
<td>-</td>
<td><img src="" alt="Structure of Idarubicin" /></td>
<td>Anti-tumor, DNA intercalating agent</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>Microsource/Prestwick</td>
<td>-</td>
<td><img src="" alt="Structure of Doxorubicin" /></td>
<td>Anti-tumor, DNA intercalating agent</td>
</tr>
<tr>
<td>Acridines</td>
<td>Acriflavine</td>
<td>Microsource</td>
<td>30</td>
<td><img src="" alt="Structure of Acriflavine" /></td>
<td>Anti-infective, DNA intercalating agent</td>
</tr>
<tr>
<td></td>
<td>Ethacridine lactate</td>
<td>Microsource</td>
<td>-</td>
<td><img src="" alt="Structure of Ethacridine lactate" /></td>
<td>Anti-septic, abortifacient</td>
</tr>
<tr>
<td></td>
<td>Propidium iodide</td>
<td>Prestwick</td>
<td>10</td>
<td><img src="" alt="Structure of Propidium iodide" /></td>
<td>DNA intercalating agent</td>
</tr>
<tr>
<td></td>
<td>Ellipticine</td>
<td>LOPAC</td>
<td>20</td>
<td><img src="" alt="Structure of Ellipticine" /></td>
<td>DNA topoisomerase II inhibitor</td>
</tr>
<tr>
<td></td>
<td>Ro 90-7501</td>
<td>LOPAC</td>
<td>20</td>
<td><img src="" alt="Structure of Ro 90-7501" /></td>
<td>Inhibits amyloid beta42 fibril formation.</td>
</tr>
<tr>
<td></td>
<td>Doqualinium chloride</td>
<td>LOPAC</td>
<td>20</td>
<td><img src="" alt="Structure of Doqualinium chloride" /></td>
<td>Antibacterial, antifungal</td>
</tr>
</tbody>
</table>
3.2 Lead compounds show unique phage inhibition profiles

To further characterize the six lead compounds, their specificity and potency were tested against a panel of phages that infect Gram-negative bacteria (*E. coli* and *P. aeruginosa*), as well as Gram-positive bacteria (*S. aureus*). I quantified the magnitude of phage inhibition by calculating phage titers at a defined end-point post infection (Figure 3). Indicator bacterial strains were grown in the presence and absence of each compound and infected with respective phages at a multiplicity of infection of \( \sim 10^{-4} \). A low multiplicity of infection was used to minimize background phage titers and to allow multiple rounds of infection. The cultures were then grown for 6 hours and phage titers were calculated by spotting serial dilutions of the lysate on a lawn of indicator bacteria. A compound that resulted in at least a \( 10^5 \)-fold reduction in phage titer, or an inhibition index of 5, compared to the untreated control was defined as positive in inhibiting phage replication. As expected, all compounds that inhibited \( \lambda cI_{857} \)-mediated cell lysis in the growth-curve based screen displayed inhibition of \( \lambda cI_{857} \) replication when assessed by phage titer calculations (Figure 3).

The compounds showed a wide range of specificities, with some blocking a variety of phages and some inhibiting only phage \( \lambda cI_{857} \). For example, mitoxantrone showed the broadest inhibitory specificity, blocking the replication of *E. coli* phages \( \lambda cI_{857} \), HK75, T5, P2 and *P. aeruginosa* phages JBD 44 and DMS3. Ro 90-7501 showed the same inhibition pattern as mitoxantrone, except it did not inhibit DMS3. Ellipticine and propidium iodide only inhibited \( \lambda cI_{857} \) and T5, and acriflavine only inhibited \( \lambda cI_{857} \) and T6. Dequalinium chloride showed the most specific inhibitory activity, blocking only \( \lambda cI_{857} \).

To shed light on the inhibitory mechanisms of these compounds, a comparison of the *E. coli* phages used in this study is shown in Table 2. These phages exhibit different morphologies, use a variety of cell surface receptors, and employ different DNA replication mechanisms. Mitoxantrone and Ro 90-7501 targeted phages from different morphological families that use different cell surface receptors and different modes of DNA replication regimes. Therefore, these two compounds likely targeted a more general aspect of the phage infection process. By contrast, dequalinium chloride only inhibited \( \lambda cI_{857} \), suggesting the compound targets a cellular process needed only for the \( \lambda cI_{857} \) infection cycle.
Figure 3. Heat map representing reductions in phage titers in the presence of each compound. Indicator bacteria were incubated in the presence and absence of each compound and infected with respective phages at an multiplicity of infection if $10^{-4}$ at $t = 0$. Phages were allowed to replicate and propagate at 37 ºC with shaking. Phage titers with compound ($P$) and without compound ($P_0$) were calculated at 6 hours post infection by plating serial dilutions of cell lysates on lawns of indicator bacteria. Reductions in phage titers were calculated by $P_0 / P$.

*Dequalinium chloride inhibits the growth of $S. aureus$ at the concentration tested.
### Table 2. A comparison of *E. coli* phages used in this study.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Family</th>
<th>Cell surface receptor</th>
<th>Incoming phage DNA</th>
<th>DNA replication</th>
<th>Transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ</td>
<td>Siphoviridae</td>
<td>LamB</td>
<td>Linear with cohesive ends</td>
<td>Closed circle &amp; rolling circle</td>
<td>Host RNAP</td>
</tr>
<tr>
<td>HK75</td>
<td>Siphoviridae</td>
<td>FhuA</td>
<td>Linear with cohesive ends</td>
<td>Closed circle &amp; rolling circle</td>
<td>Host RNAP</td>
</tr>
<tr>
<td>T5</td>
<td>Siphoviridae</td>
<td>FhuA</td>
<td>Linear with terminal repeats</td>
<td>May be bidirectional or rolling circle (?)</td>
<td>Modified host RNAP</td>
</tr>
<tr>
<td>T6</td>
<td>Myoviridae</td>
<td>Tsx</td>
<td>Linear with terminal redundancy (Modified nucleotides)</td>
<td>Single strand invasion</td>
<td>Host RNAP</td>
</tr>
<tr>
<td>T7</td>
<td>Podoviridae</td>
<td>LPS</td>
<td>Linear with terminal repeats</td>
<td>Bidirectional with recombination with extension</td>
<td>T7 RNAP</td>
</tr>
<tr>
<td>P2</td>
<td>Myoviridae</td>
<td>LPS</td>
<td>Linear with cohesive ends</td>
<td>Closed circle</td>
<td>Host RNAP</td>
</tr>
</tbody>
</table>
For the remaining work of this thesis, I characterized the inhibitory mechanisms of the six lead compounds using assays that examine different steps of the $\lambda$ life cycle (Figure 4). Compounds were tested at the initial screening concentration (40 µM) in each assay, unless otherwise stated.

Figure 4. The life cycle of phage can be blocked at many different steps. A variety of assays, noted in bold, can be used to determine which step of the phage life cycle is inhibited.
3.3 Ro 90-7501 inactivates phage particles by forcing genome ejection

It is possible that the lead compounds inhibit phage activity by inactivating phage particles before infection occurs. To test this hypothesis, I incubated λcI857 with each lead compound for 2 hours and measured phage titers by spotting serial dilutions on a lawn of indicator bacteria. I found that incubation with Ro 90-7501 reduced phage titers from ~10^{10} pfu/ml to below the detection limit of 500 pfu/ml (Figure 5). The inactivation was fast because reducing the incubation time from 2 hours to 15 min resulted in the same inactivation effect. Importantly, the inactivation is irreversible because even though the effective concentration of the compound was significantly reduced in subsequent serial dilutions and plating procedures, no phage activity was restored.

It is shown in Figure 5 that incubation of λcI857 with mitoxantrone, dequalinium chloride, acriflavine, propidium iodide, and ellipticine resulted in no change in phage titer compared to the untreated control. This indicates that these compounds targeted the phage life cycle in association with the host bacteria. However, it is also possible that these compounds inactivate phages in a reversible manner. The inactivating effect may have been alleviated by making serial dilutions of the compound-phage mixture in fresh media and plating on agar plates in the absence of compounds.

Figure 5. Ro 90-7501 irreversibly inactivates phage particles. λcI857 were incubated with each compound for 2 hours at 37 °C with shaking and phage titers were determined by plating serial dilutions on a lawn of indicator bacteria. Error bars represent standard deviations of two experimental replicates.
To further assess the effect of Ro 90-7501 on phage particle inactivation, I incubated λcI857 with Ro 90-7501 and examined morphological changes to the phage particle using EM. DMSO was also tested because Ro 90-7501 was dissolved in DMSO. Analysis of the DMSO-treated phages revealed normal looking phage particles, with full icosahedral heads attached to tails (Figure 6A). The dark uranyl acetate stain was retained on the outside of the phage head, indicating that the phage heads were filled with DNA, which prevented the stain from penetrating into the head. However, treating λcI857 with Ro 90-7501 resulted in the formation of phage particles with heads that were devoid of DNA (Figure 6B). This was shown by the dark internal staining of the phage head and a halo-like structure of the protein shells that were unable to effectively retain the uranyl acetate stain. Similar EM structures of empty phage heads were observed in a previous study of a mutant HK97 phage lacking the gp6 connecting protein111.

**Figure 6. Ro 90-7501 inactivates phage particles by forcing genome ejection.** Transmission electron microscopy images showing λcI857 treated with DMSO (A) and Ro 90-7501(B) at 50,000x and 62,000x magnification, respectively.
The formation of DNA-less phage particles, or phage ghosts, has also been observed in cases where phages were treated with 10 mM EDTA\textsuperscript{112}. EDTA inactivates phages by sequestering positively charged ions such as Mg\textsuperscript{2+}\textsuperscript{113}. It is proposed that Mg\textsuperscript{2+} ions play an important role in stabilizing the densely packed phage DNA by counteracting the repulsive negative charges. Therefore, removal of Mg\textsuperscript{2+} ions destabilizes the phage DNA, leading to DNA ejection and the formation of phage ghosts. It is possible that Ro 90-7501 forces phage DNA ejection through a similar mechanism. To test this hypothesis, I incubated $\lambda cI\textsubscript{857}$ with Ro 90-7501 in the presence of 10 mM MgSO\textsubscript{4} and determined changes to the phage-inactivation effect by spotting serial dilutions of the mixture on a lawn of indicator bacteria. Strikingly, the addition of MgSO\textsubscript{4} abolished the phage-inactivation effect of Ro 90-7501 (Figure 7). It is possible that the additional MgSO\textsubscript{4} competes with the endogenous DNA-bound Mg\textsuperscript{2+} ions for Ro 90-7501 binding, thereby saturating the Mg\textsuperscript{2+} chelating sites responsible for phage inactivation.

Alternatively, it is possible that Ro 90-7501 causes phage genome ejection indirectly by interacting with structural components of the phage head. It has been previously shown that destabilization of the portal vertex on the $\lambda$ head triggers $\lambda$ DNA ejection\textsuperscript{114}. Furthermore, $\lambda$ mutants lacking the head stabilization protein, gpD, are extremely sensitive to inactivation by chelating agents such as EDTA\textsuperscript{115}. Therefore, Ro 90-7501 might disrupt structural proteins of the phage head which might indirectly lead to genome ejection. Addition of MgSO\textsubscript{4} may stabilize the phage DNA, thereby preventing genome ejection from the phage particle.

![Figure 7. The addition of MgSO\textsubscript{4} abolished the phage-inactivation effect of Ro 90-7501. Ro 90-7501 was incubated with phage $\lambda cI\textsubscript{857}$ in the absence (top) and presence (bottom) of 10 mM MgSO\textsubscript{4} for 1 hour. Ten-fold serial dilutions of the mixtures were spotted on indicator bacterial lawns.](image-url)
3.4 Dequalinium chloride inhibits phage activity by preventing genome injection

Genome injection is a crucial step in the phage infection cycle, where the infecting phage must transfer its genome from the phage head into the host cell cytoplasm. Previous studies have shown that blocking phage genome injection by superinfection exclusion proteins can prevent phage killing of the host bacteria\textsuperscript{116}. Therefore, genome injection is a possible target for the lead compounds to block phage activity.

In order to determine the effect of each compound on phage genome injection, I measured the passage of $\lambda$ genome into the host cell using $K^+$ efflux assays. Genome injection of phages into susceptible bacteria leads to a transient flow of $K^+$ ions from inside to outside of the cell, and this process can be monitored using a $K^+$-sensitive electrode. Cells grown in the presence and absence of each compound were infected with phage $\lambda cI\textsubscript{857}$ and $K^+$ levels were measured post infection. I observed robust levels of $K^+$ efflux that were comparable to the untreated control from cells treated with mitoxantrone, acriflavine, Ro 90-7501, ellipticine, and propidium iodide (Figure 8A-E). However, the addition of dequalinium chloride resulted in significantly lower levels of $K^+$ efflux, suggesting that dequalinium chloride interferes with phage genome injection (Figure 8F). It is worth noting that cells treated with dequalinium chloride released more $K^+$ than a mutant strain, $\textit{lamB}$, which lacks the outer membrane receptor for $\lambda$ (Figure 8F). This indicates that genome entry was partially inhibited. This agrees with the previous observation that dequalinium chloride partially inhibited multiple rounds of $\lambda$ replication (Figure 3). Even though the inhibition is incomplete, dequalinium chloride was able to prevent decreases in OD\textsubscript{595} readings in the growth curve assay (Figure 2).
Figure 8. Dequalinium chloride inhibits λ genome injection. *E. coli* wild-type strain BW25113 and a mutant strain, *lamB*, that lacks the λ cell surface receptor were grown to mid-log phase in the presence and absence of mitoxantrone (A), acriflavine (B), Ro 90-7501 (C), ellipticine (D), propidium iodide (E), and dequalinium chloride (F). Cells were washed twice in suspension medium (SM: 100 mM NaCl, 10 mM MgSO₄, and 50 mM Tris at pH 7.5), reconstituted with each compound and infected with *λ*cI₈₅₇ at a multiplicity of infection of 10. K⁺ ions released was monitored by a K⁺-selective electrode showing wild-type strain (▲) and *lamB* mutant (×) in the absence of each compound, and wild-type strain (●) and *lamB* mutant (♦) in the presence of each compound.
3.5 Mitoxantrone inhibits an early step of the infection cycle

The $\lambda$ genome enters the host bacteria in a linear form. Immediately upon entry, the phage DNA is joined at the cohesive ends by the host DNA ligase to form a continuous double-stranded circular molecule. DNA circularization within the host cell protects the phage genome from host exonuclease degradation, and is a common mechanism found in bacteriophages. Subsequently, transcription and translation of the early genes play an important role in the decision between lytic and lysogenic growth. Therefore, the early steps of the infection cycle can be investigated by examining the ability of the infecting phage to form lysogens. I grew cells in the presence and absence of each compound to mid-log phase. Cells were then concentrated and infected with $\lambda cI857$. The cell-phage mixture was incubated at 30 ºC to initiate lysogen formation and dilutions were plated at 30 ºC to obtain individual colonies. I picked 52 colonies and replica plated them at the non-permissive (42 ºC) and permissive (30 ºC) temperatures. Lysogens were identified as those that grew only at the permissive temperature.

As expected, infection of cells in the absence of compounds resulted in the formation of ~100% lysogens, while infection of the $\text{lamB}$ mutant strain resulted no lysogens (Figure 9). The addition of dequalinium chloride reduced the rate of lysogen formation to 80% (Figure 9). This is in agreement with its partial inhibitory effect on genome entry shown in the previous section. Interestingly, the addition of mitoxantrone decreased the rate of lysogen formation to 60%, suggesting that this compound blocks phage activity by inhibiting an early step of the infection cycle (Figure 9). The addition of the other lead compounds did not significantly affect the yield of lysogen formation.

The incomplete inhibition of lysogen formation by mitoxantrone and dequalinium chloride and the absence of inhibition by the rest of the compounds may be due to compound dilution. It is possible that compound inhibition is reversible. The dilution of cells in fresh LB and the plating of cells on plates without compounds may have reversed the inhibitory effect, resulting in reduced inhibition or absence of inhibition.

To test the concentration-dependent effect of mitoxantrone, I increased the concentration of the compound and repeated the lysogen formation assay. Increasing compound concentration did not slow down bacterial growth as cells took the same amount of time to reach mid-log phase. Importantly, I found that the increase in compound concentration from 40 µM to 200 µM
resulted in a significant decrease in lysogen formation from 61% to 7% (Figure 10). Therefore, the inhibitory effect of mitoxantrone on lysogen formation is concentration dependent. Further experiments are required to investigate the concentration-dependent effect of the other compounds on lysogen formation.

**Figure 9. Mitoxantrone and dequalinium chloride reduced the rate of lysogen formation.**
Cells were grown in the presence or absence of each compound to mid-log phase and infected with \( \lambda cI^{857} \) at a multiplicity of infection of \(~10\). The mixtures were incubated at 30 \(^\circ\)C for 15 min to engage lysogen formation. They were then diluted in 10 times LB and recovered at 30 \(^\circ\)C with shaking for 1 hour. Dilutions of the mixtures were plated on LB plates and incubated at 30 \(^\circ\)C overnight to get individual colonies. On the following day, 52 colonies were picked from each plate and the replica-plated at permissive (30 \(^\circ\)C) and non-permissive (42 \(^\circ\)C) temperatures. Lysogens were determined as cells that grew at the permissive temperature but not at the non-permissive temperature. LamB\(^-\) is a lamB mutant strain that lacks the \( \lambda \) surface receptor and was used as a negative control. Error bars represent standard deviations of three experimental replicates.
Figure 10. Mitoxantrone inhibits lysogen formation in a concentration-dependent manner. Lysogen formation assays were conducted as described in Figure 9. The concentrations of mitoxantrone tested were 0, 40 µM, and 200 µM. Error bars represent standard deviations of 3 experimental replicates.
3.6 Lead compounds do not inhibit heat induction of a $\lambda cI_{857}$ lysogen

Next, I wanted to examine the effect of the lead compounds on downstream stages of the phage life cycle, including DNA replication, transcription, translation, particle formation and host cell lysis. These downstream stages can be investigated by heat inducing a $\lambda cI_{857}$ lysogen, which inactivates the phage repressor responsible for maintaining lysogeny. Accordingly, I induced a $\lambda cI_{857}$ lysogen in the presence and absence of each compound by heating the liquid cultures to a temperature that inactivates the mutant repressor. I then monitored bacterial density post-induction. I observed complete cell lysis after heat induction in the presence of each compound, suggesting that these compounds do not inhibit the production or activity of the phage-encoded lysis proteins (Figure 11).
Figure 11. Lead compounds do not inhibit heat induction of a λcI857 lysogen. *E. coli* BW25113 cells harboring a λcI857 prophage were grown in the presence (▲) and absence (×) of (A) mitoxantrone, (B) dequalinium chloride, (C) acriflavine, (D) Ro 90-7501, (E) propidium iodide, and (F) ellipticine at 30 ºC for 3 hours and heat induced for 2 min in a water-bath set at 50 ºC. The cultures were then incubated at 42 ºC for 15 min before shifted to 37 ºC to grow for 1 hour. OD_{595} readings were recorded every 10 min in a TECAN plate reader.
At the same time, I examined the effect of each compound on the formation of infectious phage particles by plating serial dilutions of the cell lysate on a lawn of indicator bacteria. I observed high titers of phage particles produced in the presence of each compound that were comparable to the untreated control (Figure 12). These data indicate that the compounds do not block downstream steps of the phage infection cycle including DNA replication, transcription, translation, phage particle assembly, and host cell lysis.

It is noted that the addition of Ro 90-7501 did not inactivate phages produced post-induction despite its inactivation activity observed in Section 3.4. It is possible that Ro 90-7501 cannot pass through the cell membrane and could only inactivate extracellular phage particles when the bacterial cells are intact. During cell lysis, Ro 90-7501 may be sequestered by excess amount of cellular debris such as proteins and lipids and therefore cannot inactivate phage post-induction.

![Graph showing phage titer post induction for different compounds](image)

**Figure 12. Lead compounds do not inhibit the production of phage particles post induction.** Serial dilutions of the cell lysate from Figure 11 were spotted on a lawn of indicator bacteria and phage titers were calculated by plaque counting.
Chapter 4
Discussions and future directions

In addition to their impact on bacterial abundance and diversity, it is now well appreciated that phages play important roles in human health. While it is known that phages contribute to virulence factor production and may contribute to microbiome symbiosis, little work has been done to inhibit phage activity through the use of small molecules. In this thesis, I performed a high-throughput screen that identified eleven compounds that inhibited \( \lambda \)-mediated bacterial killing activity from three small molecule libraries. Subsequently, I tested six lead compounds against a panel of phages specific for \( E. coli \), \( P. aeruginosa \), and \( S. aureus \), and found that these compounds exhibit a wide range of phage inhibition profiles. For example, mitoxantrone inhibited most \( E. coli \) and \( P. aeruginosa \) phages tested, while dequalinium chloride only inhibited \( \lambda \). Furthermore, using a number of assays that examined individual steps of the \( \lambda \) phage infection cycle, I characterized the mechanisms of inhibition for three of the six lead compounds. I found that Ro 90-7501, dequalinium chloride, and mitoxantrone inhibit phage activity by inactivating phage particles, inhibiting genome injection, and blocking an early step of the infection cycle, respectively.

The screen appeared to be a robust method for identifying potent phage inhibitors because all eleven inhibitors identified from the initial screen were validated as true hits. The hit rate is 0.2%, which falls within the acceptable range of 0.1-1.0%\textsuperscript{117}. In this study, bacterial growth curves were used as the assay readouts for hit selection. Unlike reporter gene-based assays, this method greatly reduced the occurrence of false positives due to assay interference. Additionally, the growth profile of the bacteria was monitored throughout the infection process. This is advantageous compared to assays that rely on one endpoint measurement because it provided more information on the growth of the bacteria and the activity of the phage during infection.

In this study, a high multiplicity of infection was used, which led to bacterial killing after one round of infection. This may have overlooked compounds with inhibitory effects only noticeable after multiple rounds of infection. For example, high multiplicities of infection results in “lysis-from-without”, where the infection exhausts macromolecular synthesis of the host bacteria and cells simply die without producing virion particles\textsuperscript{118}. As a result, compounds inhibiting virion
production and assembly cannot be identified. The hit rate may be improved by using a lower multiplication of infection for screening.

Furthermore, a significant portion (3%) of the compounds screened were bactericidal or bacteriostatic. They inhibited bacterial growth and were eliminated during the hit identification process. Given that many of the hit compounds are antibiotics, it is possible that these antibacterial compounds exhibit anti-phage activity when tested at sub-MIC concentrations. Further assays may be performed to test serial dilutions of these bactericidal/bacteriostatic compounds for anti-phage activity.

Anthracyclines and anthracycline derivatives make up 5 out of the 11 compounds identified in the screen. They are daunorubicin, doxorubicin, epirubicin, idarubicin, and mitoxantrone. Interestingly, daunorubicin was first isolated from *S. peucetius* in the 1950s in an effort to find anti-cancer compounds from soil-dwelling bacteria\(^{100}\). Later it was found that minor changes to the structure of daunorubicin expanded its tumor-targeting specificity and/or lowered its cytotoxicity\(^{119}\). This led to the development of doxorubicin, epirubicin, idarubicin, and mitoxantrone.

In spite of the intensive research in medicinal chemistry carried out on this family of compounds, little is known about the potential biological role of anthracyclines in the organisms that produce them. The production of anthracyclines is complex and imposes a large energy cost to the producing bacteria. This energy cost must be compensated by a selective advantage for enhancing bacterial survival in a highly competitive environment. It has been shown that daunorubicin, doxorubicin and their derivatives exhibit modest antimicrobial activity against several *Streptomyces* strains, as well as other Gram-positive bacteria and fungi\(^{120,121}\). In addition to contributing to inter-bacterial warfare, the data generated in this thesis suggests that the production of anthracyclines may also help bacteria defend against phage infection. However, the anti-phage activity of the anthracycline examined in this study (mitoxantrone) was only observed in phages that infect Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Further studies are required to assess the inhibitory effect of anthracyclines on *Streptomyces* phages to assess their biological significance against phage infection.

Through phage panel testing, I observed that mitoxantrone exhibited the broadest anti-phage activity. It inhibited infection by *E. coli* phages \(\lambda\), HK75, T5, and P2, as well as *P. aeruginosa*
phages DMS3 and JBD44. However, it did not inhibit phages T6 or T7. K+ efflux and lysogen formation results suggest that mitoxantrone does not block genome injection but instead blocks an early step of the infection cycle prior to DNA integration. Furthermore, mitoxantrone did not block induction of a λ lysogen and subsequent production of phage particles, indicating that it does not inhibit downstream steps, including DNA replication, transcription, translation, virion assembly, and host cell lysis. These findings are in agreement with a previous study on the anti-phage activity of daunorubicin (an analogue of mitoxantrone)\textsuperscript{122}. Pariso and Soller showed that daunorubicin inhibits various dsDNA phages that infect \textit{E. coli}\textsuperscript{122}. They also showed that the inhibitory effect is not due to defects in adsorption, genome injection or host cell lysis. It was hypothesized that the main site of action of daunorubicin is at the phage genome\textsuperscript{122}.

The λ genome enters the host cell as a linear molecule and must be circularized at the terminal 12 base-pair cohesive ends (cos sites) by the host DNA ligase to prevent exonuclease degradation\textsuperscript{13}. DNA circularization is not only important for DNA stability but is also required for genome integration during lysogen formation\textsuperscript{13}. Since DNA is circularized in all phages that were blocked by mitoxantrone, it is possible that mitoxantrone inhibits phage activity by preventing DNA circularization (Table 2). Phages T6 and T7 were not inhibited by mitoxantrone, possibly because they do not circularize and replicate as a linear molecule.

The effect of mitoxantrone on phage DNA circularization can be examined by a fluorescence polarization anisotropy assay \textit{in vitro}. Fluorescence polarization anisotropy has been widely used to study nucleic acid hybridization, where a single stranded DNA molecule is fluorescently labeled and hybridization with the complimentary strand will result in an increase in the fluorescence polarization value\textsuperscript{123}. Complementary base pairing at the cos sites of the λ DNA can be investigated by fluorescently labeling one of the cohesive ends. The cos ends can be first dissociated by heating at 85 °C before mitoxantrone is added. Subsequently, the solution can be cooled slowly to allow complementary base-pairing with polarization values recorded over time. If mitoxantrone prevents DNA circularization, the kinetic profile of fluorescence polarization will be lower than that of the untreated control.

Ro 90-7501 was identified as a potent phage inhibitor that irreversibly inactivates λ by destabilizing the phage head, causing loss of the genome. Given that the addition of MgSO\textsubscript{4} removed the inhibitory effect of the compound, it is possible that Ro 90-7501 triggers genome
ejection by sequestering endogenous Mg$^{2+}$ ions. The metal-chelating property of Ro 90-7501 can be examined by a Mg$^{2+}$ dependent enzymatic assay. For example, the ATP-dependent enzymatic reaction catalyzed by glucokinase requires Mg$^{2+}$ as essential counter ions$^{124}$. Glucokinase facilitates the phosphorylation of glucose to glucose-6-phosphate, which is coupled to an NADP$^{+}$-dependent glucose-6-phosphate dehydrogenase reaction$^{124}$. This reaction converts NADP$^{+}$ to NADPH, which can be quantified by measuring absorbance at 340 nm$^{124}$. In principle, the more free Mg$^{2+}$ ions present in the sample, the higher the absorbance will be obtained. To test whether Ro 90-7501 sequesters Mg$^{2+}$ ions, increasing concentrations of Ro 90-7501 can be added to this enzymatic reaction. If Ro 90-7501 chelates Mg$^{2+}$, the absorbance will decrease with increasing Ro 90-7501 concentration.

It is also possible that Ro 90-7501 interacts with structural components of the phage head, which weakens the protein capsid and indirectly causes genome ejection. Methods to characterize protein-small molecule interactions have been reviewed previously$^{125}$. For example, these interactions may result in changes in protein stability in vitro. Such changes can be measured by differential scanning techniques such as differential static light scattering and differential scanning fluorimetry$^{125}$.

Dequalinium chloride is the most specific phage inhibitor, inhibiting only λ. I was able to show that dequalinium chloride significantly reduced the release of K$^{+}$ ions upon infection, indicating that it inhibits genome injection. Inhibition was partial but was sufficient to prevent phage-mediated bacterial lysis based on OD$_{595}$ readings. It has been previously shown that dequalinium chloride has a high affinity for membranes$^{126}$. Therefore, it is likely that it binds to and modifies the bacterial cell membrane and thus interferes with the interaction between phage λ and its cell surface receptor. It is possible that dequalinium chloride directly inhibits the phage genome injection. However, it is also possible that dequalinium chloride indirectly inhibits genome injection by preventing initial phage adsorption. This hypothesis can be tested by an adsorption assay. In this assay, bacterial cells treated with and without dequalinium chloride are infected with phage λ and incubated without shaking to allow phage adsorption. Aliquots are taken out at different time intervals and the cells are collected by centrifugation. This step removes adsorbed phages that pellet together with the bacteria. The unadsorbed phages will be quantified by spotting serial dilutions of the supernatant on a lawn of susceptible bacteria. If dequalinium
chloride inhibits phage adsorption, more phages will be left in the supernatant and a higher titer will be observed.

It was found that the six lead compounds all inhibit phage infection at an early stage. This was shown by the observation that none of the compounds inhibit heat induction of a λcI₈₅₇ lysogen, which eliminates the possibility of inhibition on any downstream steps post-induction. This was expected because the limited size of the phage genome usually requires the phage to hijack the host DNA replication, transcription and translation machineries for its own replication. Targeting these steps may also compromise the growth of the host bacteria, thereby resulting in a negative phenotype during the initial screening.

I was unable to determine the inhibitory mechanisms of action for propidium iodide, ellipticine, and acriflavine. Based on K⁺ efflux results, these three compounds are unlikely to inhibit DNA injection. Additionally, they do not inhibit induction of the λ lysogen, suggesting they do not inhibit downstream steps post-induction. Propidium iodide, ellipticine and acriflavine all possess DNA-binding properties and it is likely that they inhibit phage activity by targeting the phage genome. Even though the phage inactivation and lysogen formation experiments showed that these three compounds do not inactivate free phage particles or prevent DNA integration into the host chromosome, it is possible that the inhibitory effects of these compounds were reversed by dilution. For example, when testing the formation of lysogens and the reduction in phage titer after compound treatments, cells and phages were diluted in fresh media and plated on agar plates in the absence of the compound. These procedures exponentially decreased the compound concentration, which may have reversed the inhibitory effect. Therefore, the next step will be to conduct experiments with a consistent phage-to-compound ratio. Specifically, compounds will be applied to all the dilution solutions and agar plates to prevent compound dilution effects.

One of the limitations of the high-throughput screening assay is that only phage λ was tested for compound inhibition. However, bacteriophages are extremely diverse and employ different mechanisms to ensure successful infection. For example, λ uses LamB as the outer membrane surface receptor, but T5 and T7 use FhuA and LPS, respectively, for entry. The replication of other phages may require additional phage or host-encoded proteins, which can be potentially targeted by compounds. For example, phage T7 encodes a T7 RNA polymerase for phage gene transcription. Furthermore, phage Mu encodes a Mu-specific transposase for genome
integration and replication\textsuperscript{13}. The $\lambda$-based screen may have overlooked many of these potential compound targets. Therefore, it may be possible to obtain different sets of “hits” by performing the screen using other well-characterized phages.

Another limitation of the study is that the screen only identified compounds that exclusively inhibit steps within the lytic cycle. An important step overlooked by the screen is lysogen induction. Lysogen induction occurs when DNA damaging agents or oxidative stress trigger the bacterial SOS response, which leads to the cleavage and inactivation of the phage repressor protein\textsuperscript{15}. This causes the excision of the phage genome and activation of phage genes required to enter the lytic cycle. Previous studies have shown that lysogen induction is not only important for the production of phage-encoded toxins, but also may contribute to microbiome dysbiosis\textsuperscript{11,26}. Therefore, it will be of great interest to design future screens that specifically look for compounds that either inhibit the SOS response or repressor cleavage to block lysogen induction.

This thesis represents the first comprehensive attempt to identify novel phage inhibitors through a high throughput approach. Eleven compounds were identified that block phage activity through diverse mechanisms. These compounds may be potentially used as a starting point for the development of more effective therapeutics to prevent the production of phage-encoded virulence factors, stop the horizontal transfer of virulence genes, and protect the microbiome from dysbiosis. In addition to their fascinating clinical applications, these anti-phage compounds can be used to fuel and advance fundamental research on phage biology and be used as valuable tools for next-generation phage-based innovations and discoveries.
References


