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REVIEW

Phage therapy as a renewed therapeutic approach to mycobacterial infections: a comprehensive review

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Abstract: Mycobacterial infections are considered to a serious challenge of medicine, and the emergence of MDR and XDR tuberculosis is a serious public health problem. Tuberculosis can cause high morbidity and mortality around the world, particularly in developing countries. The emergence of drug-resistant Mycobacterium infection following limited therapeutic technologies coupled with the serious worldwide tuberculosis epidemic has adversely affected control programs, thus necessitating the study of the role bacteriophages in the treatment of mycobacterial infection. Bacteriophages are viruses that are isolated from several ecological specimens and do not exert adverse effects on patients. Phage therapy can be considered as a significant alternative to antibiotics for treating MDR and XDR mycobacterial infections. The useful ability of bacteriophages to kill *Mycobacterium* spp has been explored by numerous research studies that have attempted to investigate the phage therapy as a novel therapeutic/diagnosis approach to mycobacterial infections. However, there are restricted data about phage therapy for treating mycobacterial infections. This review presents comprehensive data about phage therapy in the treatment of mycobacterial infection, specifically tuberculosis disease.

Keywords: mycobacteriophage, tuberculosis, mycobacteria infection, phage therapy

Introduction

Mycobacterium species (spp) can create a variety of infections such as tuberculosis (TB), Searls ulcer, leprosy, and fish tank granuloma.[1] TB, caused by *Mycobacterium tuberculosis* (Mtb), is one of the most serious public health problems that can cause high morbidity and mortality worldwide.[2–4] Based on WHO TB report in 2018, TB remained to be a major global health challenge and, in 2017, TB caused an estimated number of 1.3 million deaths among HIV-negative people. In addition, there were an additional number of 300, 000 deaths from TB among HIV-positive people. Moreover, there were an estimated number of 10.0 million new TB cases equal to 133 cases per 100, 000 population, worldwide.[5] The rapid spread of infections and the alarming growth of drug resistants, especially the emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) strains, have sounded the alarm to find more new potent drugs; therefore, finding an alternative approach to the controlling and treatment of TB has become extremely vital.[6] Phage therapy can be considered as a significant alternative to antibiotics for treating MDR and XDR pathogens.[7] The natural and useful capacity of bacteriophages to infect mycobacterial hosts, as well

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as their ability to kill *Mycobacterium* spp, has involved numerous research studies for investigaing the phage therapy as a novel therapeutic/diagnosis approach to mycobacterial infections.[1,6] However, approapriately relevant data about the efficiency of phage therapy in treating various mycobacterial infections, especially TB, are largely scarce and limited. In this study, a comprehensive review of the literature has been conducted to identify in vitro and in vivo studies associated with the phage therapy in the mycobacterial infection.

Phage therapy

The increasing emergence of infection, coinfection, and drug-resistant pathogens has become a severe challenge for scientists and public health.[8,9] In recent years, several novel alternative strategies including therapeutic enzymes, pigments, phytochemicals, antimicrobial polymers, antimicrobial peptides, and silver nanoparticles have led researchers to consider the treatment of infections in the presence of MDR and XDR pathogens.[1,10,11] However, since drug-resistant bacteria have become increasingly problematic and challenging, phage therapy is considered to be an important candidate for alternative therapy.[12] Bacteriophages are viruses that are isolated from several ecological specimens including sewage, soil, and water.[13–15] The isolation of bacteriophage from an environmental sample revealed that bacteriophages did not exert adverse effects on individuals.[15–17] It is estimated that there are more than 4200 bacteriophages that exactly infect *Mycobacterium* spp; it is expected that 10^{25} phage launches a new infection cycle in every second of the day.[18] The application of bacteriophage as a candidate for alternative therapy in non-mycobacterial infection was discovered in the early 20th century, showing that bacteriophage has a high capacity to efficiently eradicate pathogenic bacteria.[12,19,20]

However, information about the use of phages in the treatment of bacterial infection in humans is little. Phages are considered to be a good candidate in treating and controlling mycobacterial infections; however, these agents have several advantages and limitations for use in humans.[21–23] The main advantages and characteristics of bacteriophages are as follows: I) phages cannot infect human cells and replicate only in the target bacterium; II) the selected phage perfectly lyses the pathogen at the site of infection; III) the administration of phages is easier and, after the initial administration, the concentration of phages increases at the site of infection; therefore, very few doses are required;[24–26] III) similar to phages TM4 and D29, the selected phage should be highly virulent against Mtb; IV) a non-pathogenic phage should be used in phage therapy; and V) the selected phage should not trigger an immune response and their effects are limited to the site of infection.[13,20,27] On the other hand, the use of bacteriophages in humans is subject to several limitations as follows: I) identifying a phage with therapeutic characteristics and demonstrating that a phage is specific to a given bacterial strain is very difficult;[21,28] II) the appearance and development of bacterial resistance against phages is theoretically possible, and the production of phage genome without antibiotic-resistant gene, genes encoded bacterial virulence factors, and integrase genes (or without genes for phage-encoded toxins) is complicated;[21,29] III) the formulation and stabilization of pharmaceutical preparations of phages is difficult and has several problems;[30] IV) it is possible that lysogenic phages integrate their DNA into the bacterial genome and horizontally transfer resistant genes to the bacteria; therefore, new resistant bacteria can develop.[31,32] It can be concluded that phages can perhaps set the ground for the emergence and development of antibiotic resistance; and V) it is possible for the immune system to lead to the reduced activity of phages under the in vivo condition.[33,34] Results of the previously published studies revealed that phage therapy could be very effective against different pathogenic bacteria such as *Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus*, and *Escherichia coli*.[35–38] However, there are limited studies and data about the role of phage therapy in the treatment of mycobacterial infections.

Is mycobacterium smegmatis the ideal delivery system in phage therapy?

Mycobacterium smegmatis (*M. smegmatis*) is a nonvirulent fast-growing mycobacterium that does not infect people with disease, even immunosuppressed peoples.[39] Since bacteriophages do not have the ability to spread throughout the membrane, several strategies are required to deliver the bacteriophage to the intracellular pathogenic bacteria.[20,40] The use of nonvirulent mycobacterium, especifically *M. smegmatis* as a delivery system, is one of the main strategies used for the treatment of mycobacterial infection (Figure 1).[41] In the phage therapy process, *M. smegmatis* plays several roles: I) *M. smegmatis* acts as a carrier to deliver phage to the intracellular pathogen; II) *M. smegmatis* can act as a host and lead to the high proliferation of bacteriophage; III) this organism

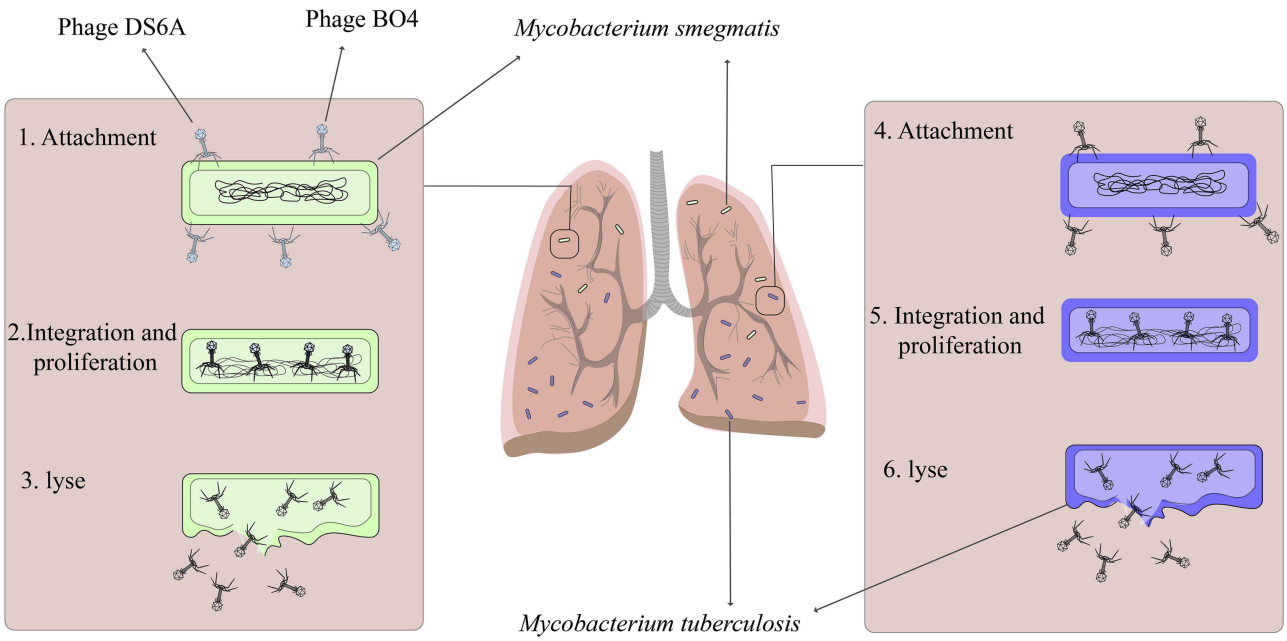

Figure 1 Steps involved in phage mediated *Mycobacterium tuberculosis* lysis using *Mycobacterium smegmatis*.

increases bacteriophage infection rates; and IV) *M. smegmatis* is an appropriate environment for bacteriophage activity within mononuclear cells such as macrophages and monocytes.[13,42] Based on the above, it can be concluded that, in phage therapy, the use of nonvirulent *M. smegmatis* in intracellular infection treatment is a better choice, and this result can be seen as "proof of concept" that opens up new opportunities for further research.

Phage therapy in tuberculosis

The emergence of MDR and XDR *M. tuberculosis* and the limitation of finding more potent drugs and alternate therapeutics for the treatment of TB have attracted different research groups to investigate the bacteriophage roles as a suitable alternative to antibiotics in the treatment of TB.[6,43] According to the natural capability to infect and kill mycobacteria, bacteriophages can be considered as an appropriate alternative to antibiotics.[42] Different bacteriophages were investigated against TB as therapeutic options as follows: 1) Phage DS-6A; 2) Phage TM4; 3) Phage D29; 4) Phage T7; 5) Phage P4; 6) Phage PDRPv; 7) Phage BTCU-1; 8) Phage Bo4; 9) Phage SWU1; 10) Phage GR-21/T; 11) Phage My-327; 12) Phage Ms6; and 13) Phage Bxz2. In this review article, the role of important mycobacteriophages in the treatment of mycobacterial infection, especifically tuberculosis disease, is explained (Table 1).

Phage DS-6A

Mycobacteriophage DS6A has a high specificity to members of the *Mycobacterium tuberculosis* complex (MTBC), and this unique feature makes this bacteriophage an important and interesting candidate for anti-TB therapy.[44] This mycobacteriophage can form plaque only on mycobacteria belonging to the MTBC.[45] The results of the previously published studies have shown that the treatment of Mtb infection with mycobacteriophage DS6A has led to a reduction in infection in liver, spleen, and lung lesions; it has shown a high ability to competently eliminate Mtb from infectious sites.[44,46] In this scenario, it is presumed that phage-infected Mtb cells may briefly transport mycobacteriophage DS6A to Mtb bacilli within macrophages (Figure 1).[13,46] However, more in-vitro and in-vivo studies are required to shed light on the mechanisms that phage uses for Mtb eradication.

Phage TM4

The length of the mycobacteriophage TM4 genome is nearly 52,797 bp, which encodes several proteins with different functions.[42] The proteins encoded by TM4 are similar to transcriptional regulators or bear a high similarity to haloperoxidases and glutaredoxins.[47] TM4 is a lytic bacteriophage with double-stranded DNA and an extensive host range that could infect both slow-growing and fast-

Table 1 The list of mycobacteriophages used against Mycobacteria infections: targets, mechanisms, and results of phage therapy

Phages	Targets	Mechanisms	Results of phages therapy
DS-6A	*Mycobacterium tuberculosis*, *Mycobacterium ulcerans*	• Plaque formation on mycobacteria belonging to the MTBC	• Reduction of infection in liver, spleen and lung lesions. • Complete Elimination of Mtb from infections sites. • Improvement of lesions (infection reduction) in lungs, spleen and livers in BU.
TM4	*Mycobacterium tuberculosis*, *Mycobacterium avium*	• Unknown • Lysing and killing *M. avium* inside mononuclear cells such as macrophages and monocytes.	• Liposomal-delivered mycobacteriophage TM4 could have direct access to intracellular Mtb, finally destroying the intracellular pathogen. • Treatment of *M. avium* infected macrophages with TM4 contributed to a considerable decrease in the number of *M. avium* bacilli.
D29	*Mycobacterium tuberculosis*, *Mycobacterium ulcerans*	• Lysin A hydrolyzing peptidoglycan of Mtb. • Lysin B disseminating mycolic acid from the peptidoglycans in Mtb cell wall. • Inducing a cellular infiltrate of a macrophagic/lymphocytic profile in BU. • D29 having an extensive lytic activity against mycolactone-producing *M. ulcerans*. • D29 increasing the levels of TNF, IFN-γ, and IL-10. • D29 resulting in the increase and maintenance of a local mononuclear inflammatory response to *M. ulcerans*.	• Complete Elimination of Mtb from infections sites. • *Mycobacterium ulcerans* lysed and eradicated due to the lytic activity of phage. • Reduction of the proliferation of the mycolactone-producing *M. ulcerans*.
T7	*Mycobacterium tuberculosis*	• Gp2 binding to the β subunit of RNA polymerase and preventing transcription by inhibition initialization of the nascent RNA transcript. • Gp2 prevents the enzymatic activity of Mtb RNA polymerase.	• T7 effectively kills the Mtb.
P4	*Mycobacterium tuberculosis*	• Bacteriophage P4 capsid protein Psu inhibiting ATPase and translocase activities of the Rho proteins in two ways: • I) The binding of Psu to Rho proteins causing Rho proteins to be unable to terminate with a Rho-dependent terminator. • II) Psu via direct interaction with Rho preteens preventing the release of RNA from a stalled elongation complex.	• P4 can effectively kill the Mtb.
PDRPv	*Mycobacterium tuberculosis*	• Unknown	• Phage PDRPv showed a lytic activity against Mtb.

(*Continued*)

Table 1 (Continued).

Phages	Targets	Mechanisms	Results of phages therapy
BTCU-1	*Mycobacterium tuberculosis*	• BTCU-1 encoding two endolysins as follows: • I) The cleavage of the peptidoglycan in the cell wall of mycobacteria is done by *lysA*. • II) The release of free mycolic acid from the mycolylarabinogalactan bond is carried out by *lysB*.	• *LysA* and *lysB* have antimicrobial activity and make significant changes in the cell shape of Mtb. • These endolysins eradicate most of Mtb during the course of their life cycle.
Bo4	*Mycobacterium tuberculosis*, NTM, *Mycobacterium bovis*	• Bo4 has antimicrobial activity and could lyse and effectively halts the growth of Mtb	• A lytic phage possibly eradicates Mtb in infectious sites.
SWU1	*Mycobacterium tuberculosis*	• Inhibiting the lipid metabolism and preventing the usual production of long-chain fatty acids. • Increasing the cell wall permeability and potentiating the efficiency of multiple antibiotics. • Increasing the susceptibility of Mtb against heat shock, H2O2, SDS, and low PH. • Changing colony formation and biofilm morphology.	• SWU1gp39 and gp67 might be included as a broad-spectrum antibiotic adjuvant or potentiator.
GR-21/T	*Mycobacterium tuberculosis*	• Unknown	• Reduction of infection in liver, spleen and lung lesions.
My-327	*Mycobacterium tuberculosis*	• Unknown	• Reduction of infection in liver, spleen and lung lesions.
Ms6	*Mycobacterium smegmatis*, *Mycobacterium tuberculosis* *Mycobacterium bovis*, BCG	• Similar to the phage D29, Lysin A hydrolyzing peptidoglycan of Mtb cell wall. • LysB encoding a protein with lipolytic activity that hydrolyzes a wide spectrum of fatty acid esters in Mtb. • Cleavage of ester bond among arabinogalactan and *mAGP*. • Cleavage of the ester bond between trehalose and mycolic acids in TDM.	• *LysA* and *lysB* have antimicrobial activity, and these endolysins eradicate most of Mtb during the course of their life cycle.
Bxz2	*Mycobacterium ulcerans*	• Unknown	• Bxz2 has the highest lytic activity against *Mycobacterium ulcerans*.

Abbreviations: BCG, Bacille Calmette Guérin; MTBC, *Mycobacterium tuberculosis* complex; Mtb, *Mycobacterium tuberculosis*; BU, Buruli Ulcer; TNF-α, Tumor necrosis factor alpha; IFN-γ, Interferon gamma; NTM, Non-tuberculosis mycobacterium; *mAGP*, Mycolyl-arabinogalactan-peptidoglycan; TDM, *Trehalose* 6,6′-dimycolate.

growing strains of mycobacteria.[48] Based on the results of studies that evaluated the gene expression and codon usage in different mycobacteriophages, it is revealed that mycobacteriophage TM4 extremely expresses genomes and has probably the strongest capability to eradicate mycobacteria strains in infectious sites.[49] In addition to the problem of MDR and XDR Mtb strains, these bacteria infect macrophages and monocytes and grow within them. Finally, in macrophages and monocytes, Mtb begins a dormant or latent phase of infection and produces the restriction effects of used antibiotics against Mtb infections.[48] Overall, all mycobacteriophages are not able to penetrate eukaryotic cells, and this limitation against intracellular bacterial infection, such as TB, leads to the introduction and development of a novel vector for phage delivery into intracellular infections.[42] Recently, researchers have used the liposomes as a delivery system to transport bacteriophage into pathogen-infected cells.[50] Liposomes have significant cell penetration features and, therefore, are quite possible to be an appropriate envelope for phages used against intracellular bacteria, especially Mtb.[42,50] Results of a previously published study revealed that liposomes penetrated into infected cells by endocytosis and were found within early endosomes after penetration.[51] Mycobacteriophage TM4 has the ability to destroy the intracellular pathogen.[52] Since liposomal-delivered mycobacteriophage TM4 could have direct access to intracellular Mtb,[42] it is concluded that liposome is a proper vector for mycobacteriophage TM4 therapy of intracellular Mtb infection (Figure 2).

Phage D29

The ability of mycobacteriophage D29 to quickly penetrate and eradicate pathogens is the reason why researchers have considered it as a new therapeutic option against MDR and XDR bacteria, especially Mtb.[53] This mycobacteriophage is used for several targets, particularly in TB diagnosis and

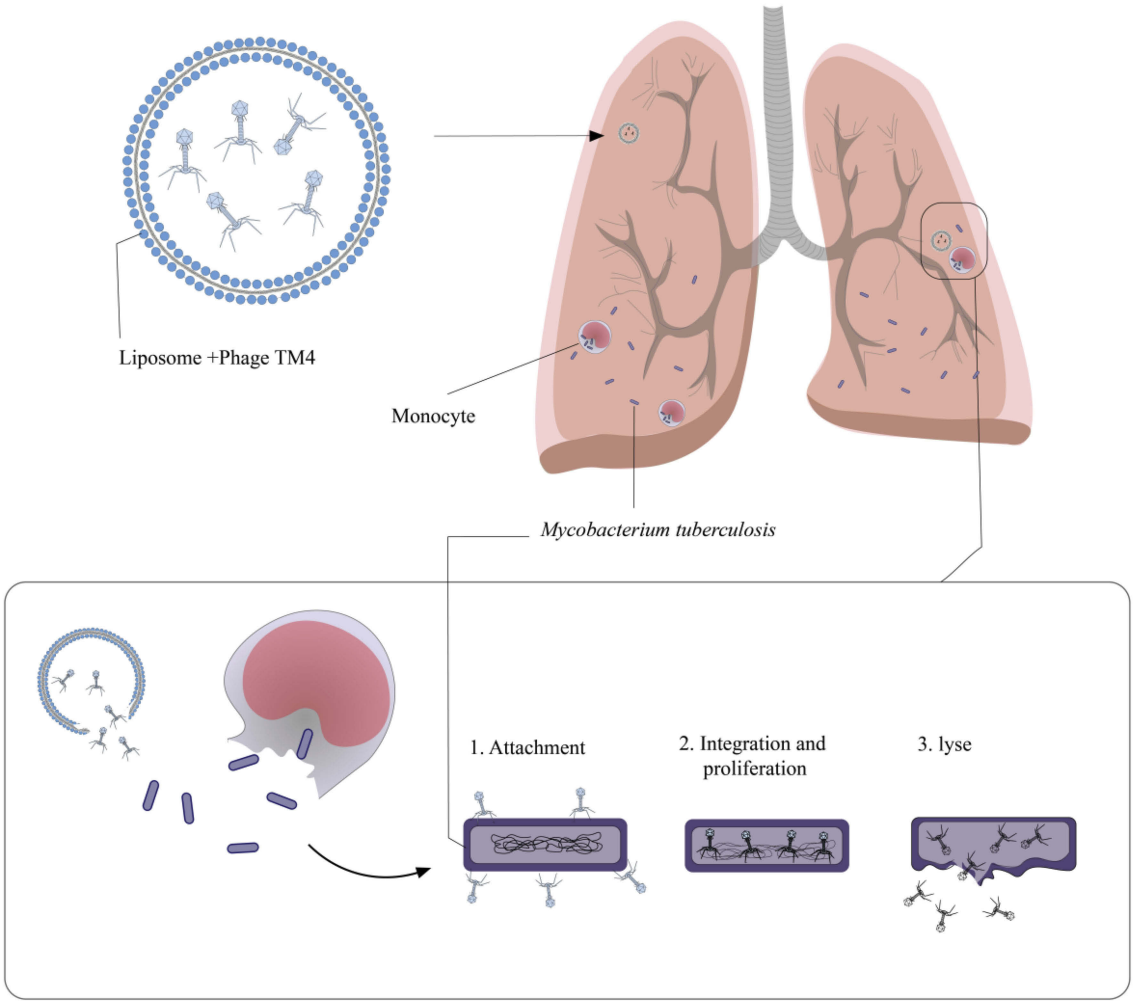

Figure 2 Steps involved in phage TM4 mediated *Mycobacterium tuberculosis* lysis using liposome.

probably in TB treatment.[54] In the mycobacteriophage D29 genome, there are three genes that make the lytic cassette. These three genes encode Lysin A, Lysin B, and holin protein.[55,56] Lysin A coded by gp10 hydrolyzes peptidoglycan of bacterial cell walls.[57] Gp12 encodes Lysin B that leads to the dissemination of mycolic acid from the peptidoglycans in mycobacterial cell wall.[53,58] Holin as a membrane pore-forming protein participates in the transfer of lysins from the cytosol to the periplasm.[59] Lysin A of mycobacteriophage D29 has three main domains including NTD (lethal to *M. smegmatis*), LD with catalytic activity in N terminus, and one domain present at the C terminus. Specifically, the C-terminal domain of Lysin A binds to Mtb peptidoglycan and hydrolyzes it.[55,57] Therefore, these molecules (LysinA, LysinB, and holin) are potential candidates that develop phage-based therapeutics against Mtb infections (Figure 3).

Phage T7

Bacteriophage T7, an obligate lytic phage, was defined in 1945 as one of the several different bacteriophages that infects and replicates in *Escherichia coli*.[60] Bacteriophage T7 enjoys several advantages that make it a suitable model system for genome strategy and engineering. These advantages are as follows: 1) T7 is relatively independent of complex host physiology, and 2) most of the T7 genomes are transported into a newly infected cell through RNA polymerase.[61,62] Six important proteins formed the main T7 phage particle including I) gp10A as a primary capsid protein; II) gp10B as a secondary capsid protein; III) gp8 as a connector; IV) gp17 as a tail fiber; and V) gp11 and gp12 as tail proteins.[60,63] A new method for treating infectious diseases, such as TB, is used to define how bacteriophages can be effective in killing bacterial cells. Bacteriophages could produce small encoded proteins that bind to RNA polymerase.[64] RNA polymerase is responsible for transcription in microorganism, and the binding of the small phage-encoded proteins to RNA polymerase leads to the suppression of bacterial gene transcription. T7 phage protein, Gp2, encoded by the *rpoC* gene, in *Escherichia coli*, binds to the section including amino acids 1145–1198 in the beta-prime subunit of RNAP, thus inhibiting the productive engagement of RNA polymerase with the promoter.[65–68] Gp2 prevents the enzymatic activity of bacterial RNA polymerase by several mechanisms in the following fashion: I) Gp2 inhibits functionally necessary alterations in RNA polymerase; II) Gp2 inhibits the interaction between catalytic site of RNA polymerase and DNA; III) in the DNA binding channel, Gp2 inhibits the binding of DNA.[64] One of the main antibiotics used in TB treatment is rifampicin. Results of an in silico study revealed that, in the case of Mtb infection, Gp2, similar to rifampicin, binds to the β subunit of RNA polymerase, encoded by the rpoB gene; in addition,

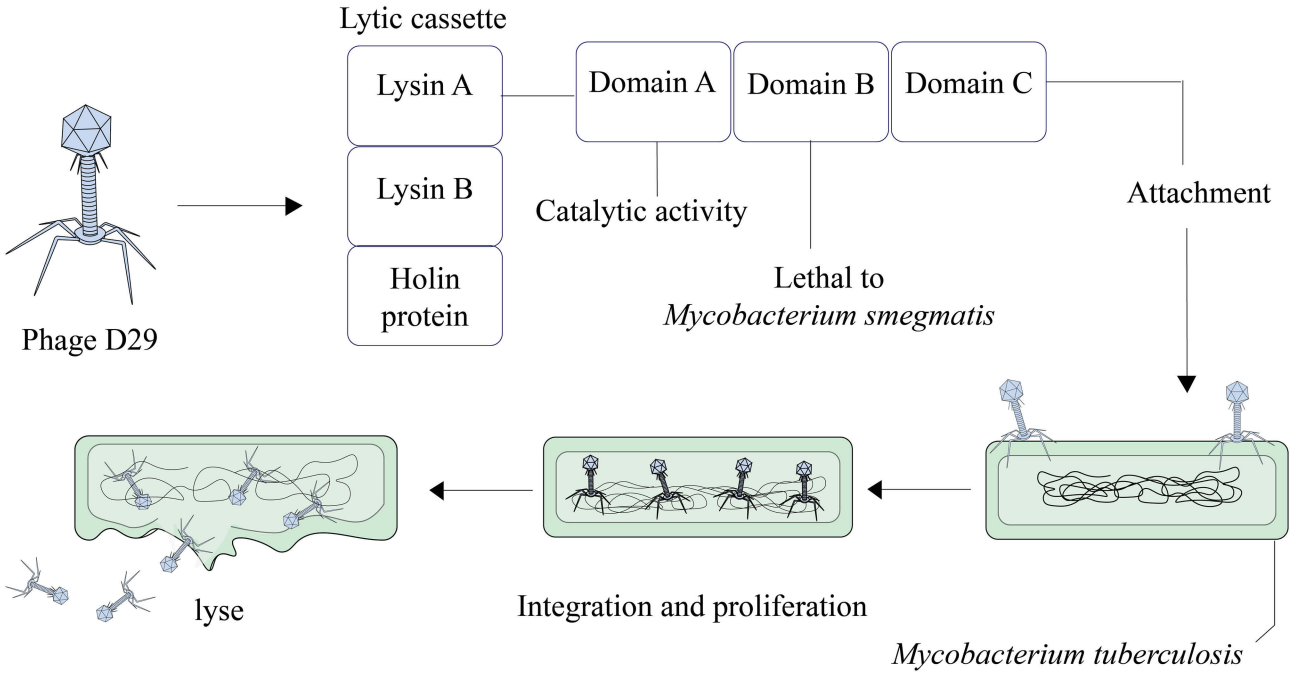

Figure 3 Overview of phage D29 mediated *Mycobacterium tuberculosis* lysis.

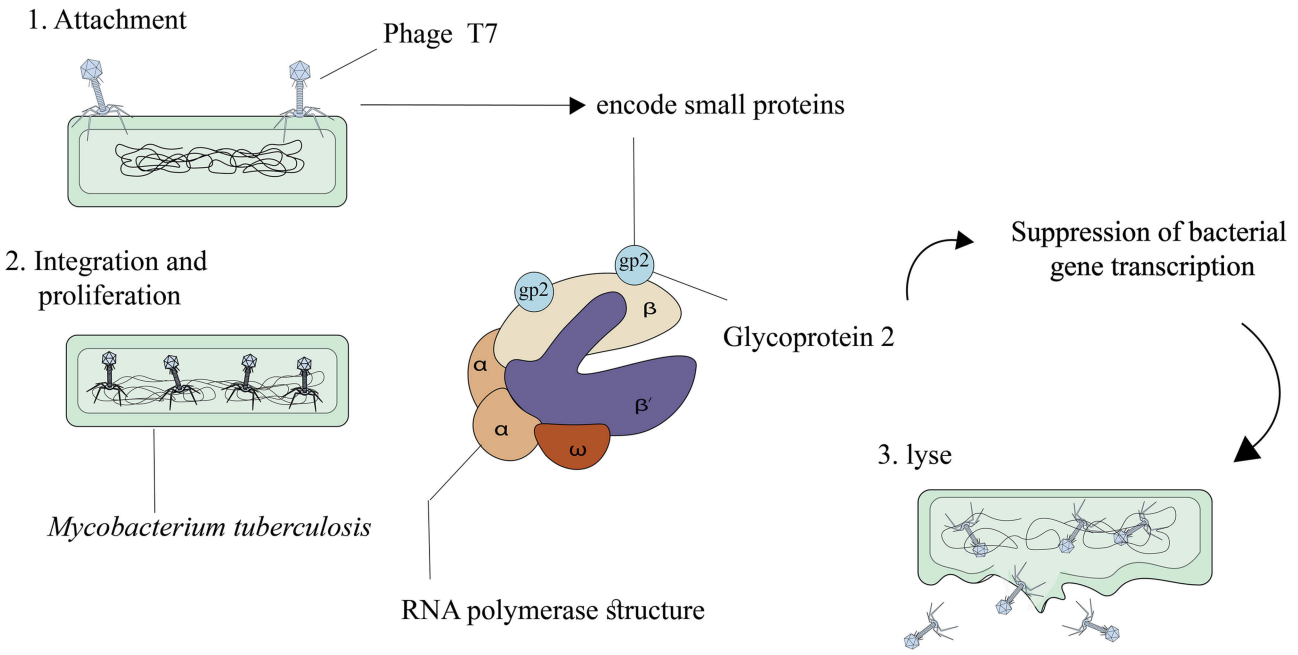

Figure 4 Steps involved in phage T7 mediated *Mycobacterium tuberculosis* lysis.

it prevents transcription by inhibiting the nascent RNA transcript, yet to a lesser degree than that in *Escherichia coli* (Figure 4).[64,69]

Phage P4

Rho is a homohexameric transcription terminator in various pathogens including Gram-negative and Gram-positive bacteria that regulates gene expression and many physiological processes and is a potential antibiotic target.[70–74] Accordingly, Rho is involved in many physiological processes; therefore, the inhibition of this transcription terminator could be beneficial as a synergistic antimicrobial treatment strategy.[75] In different bacteria such as *Escherichia coli*, Psu is a bacteriophage P4 capsid protein that acts as an unconventional capsid organizing protein and inhibits ATPase and translocases activities of these Rho proteins.[76] In recent years, the results of a study revealed that the Rho-dependent termination had the main role in the pathogenicity of Mtb.[77] Similar to the same bacteria, in Mtb, Psu binds to Rho protein and antagonizes Rho in *trans* by forming a mechanical interference to Rho translocation (Figure 5).[78] In total, the expression of Psu can kill various bacteria such as Mtb in two ways: I) the binding of Psu to Rho proteins makes Rho proteins unable to terminate with a Rho-dependent terminator; II) Psu through direct interaction with Rho proteins prevents the release of RNA from a stalled elongation complex.[76] Hence, Psu could be useful as a synergistic antibiotic treatment against Mycobacterium.

Phage PDRPv

So far, twenty-seven clusters of mycobacteriophages have been identified in the Actinobacteriophage database (Phagesdb.org). Mycobacteriophages (PDRPv) belong to *Siphoviridae* family and B1 sub-cluster. The length of the mycobacteriophage PDRPv genome is approximately 69,110 bp with a G+C content of ~66%, containing 106 open reading frames (ORFs).[12,79] The results of another study revealed that phage PDRPv had a lytic activity against Mtb. However, the exact anti-tuberculosis mechanism of this phage was not determined.[12]

Phage BTCU-1

Mycobacteriophage BTCU-1 belongs to *Siphoviridae* family and has been isolated from soil specimen, obtained from eastern Taiwan.[80] The length of the mycobacteriophage BTCU-1 genome is approximately 46 kb, and this bacteriophage has a linear double-stranded DNA with an icosahedral head and a very long tail.[81] The genome of BTCU-1 encodes several proteins with predefined functions. One of these proteins is identified as a putative phosphoribosyl transferase (PRT) and is particularly found in mycobacteriophages that infect Mycobacterium.[80,82] These

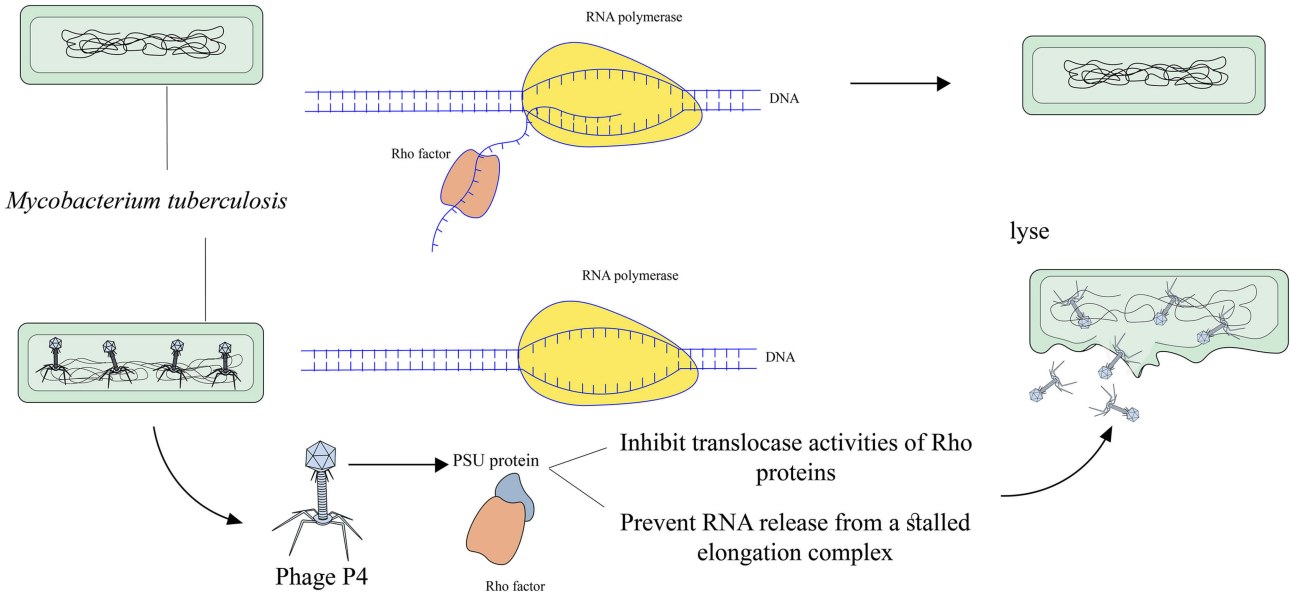

Figure 5 Overview of phage P4 mediated *Mycobacterium tuberculosis* lysis.

proteins eradicate most bacteria during the course of their life cycle.[80] Moreover, different mycobacteriophages encode lytic endolysins that have antimicrobial activity and can be effective against MDR and XDR MTB strains.[83–85] BTCU-1_ORF7 (*lysA*) and BTCU-1_ORF8 (*lysB*) are two presumed lytic genes in the genome of mycobacteriophage BTCU-1 that encode two endolysins with antimycobacterial activities.[81] These endolysins possess two separate basic functions. The cleavage of the peptidoglycan in the cell wall of mycobacteria is done by *lysA*. On the other hand, it is presumed that the release of free mycolic acid from the mycolylarabinogalactan bond is carried out by *lysB*.[81,86] Finally, it can be concluded that *lysA* and *lysB* can make significant changes in the cell shape of mycobacterium, and these findings recommend that these endolysins are good candidates for treating and controlling mycobacterial infections (Figure 6).

Phage Bo4

The length of the mycobacteriophage Bo4 genome is approximately 39,318 bp, and this bacteriophage has a dsDNA genome with a G+C content of 66.76%. It is identified that genome of mycobacteriophage Bo4 contains 58 ORFs.[87] This mycobacteriophage has a long noncontractile tail with isometric and icosahedral heads.[87,88] These features revealed that mycobacteriophage Bo4 could be considered as a lytic phage that infected and possibly eradicated pathogenic mycobacteria in the infected sites. Moreover, this phage can be a valuable tool for phage typing of Mtb.[89] Different mycobacterial species including non-tuberculosis mycobacterium (NTM), mycobacterium *bovis*, and Mtb can be infected by this mycobacteriophages.[87] However, mycobacteriophage Bo4 has the capability to infect MDR and XDR Mtb. In vivo conditions, in blood, and in lysosomal macrophages, Bo4 could lyse and effectively halt the growth of Mtb, showing that mycobacteriophage Bo4 has antimicrobial activity.[87] Finally, these features make it an ideal candidate and a potentially useful tool for diagnosing and developing phage-based anti-TB therapies (Figure 1).

Phage SWU1

In recent years, a novel mycobacteriophage SWU1 has been isolated from environmental samples, especially from a soil sample in China using *M. smegmatis* mc^{2}155 as the host microorganism.[90] The length of the mycobacteriophage SWU1 genome is approximately 52,474 bp with a G+C content of 62.4%, containing 94 and 3 candidate protein-coding and tRNA genes, respectively.[91] SWU1gp39 is a new gene from mycobacteriophage SWU1, which is absent in other mycobacteriophages and the exact function of this gene has not yet been determined.[41] Overall, the degree of antibiotic resistance in Mtb is closely related to cell wall permeability. SWU1gp39 can inhibit the lipid metabolism of Mycobacterium and prevent the usual production of long-chain fatty acids.[41,92] Therefore, SWU1gp39 could increase the cell wall permeability in Mtb and potentiate the efficiency of multiple antibiotics such as rifampicin,

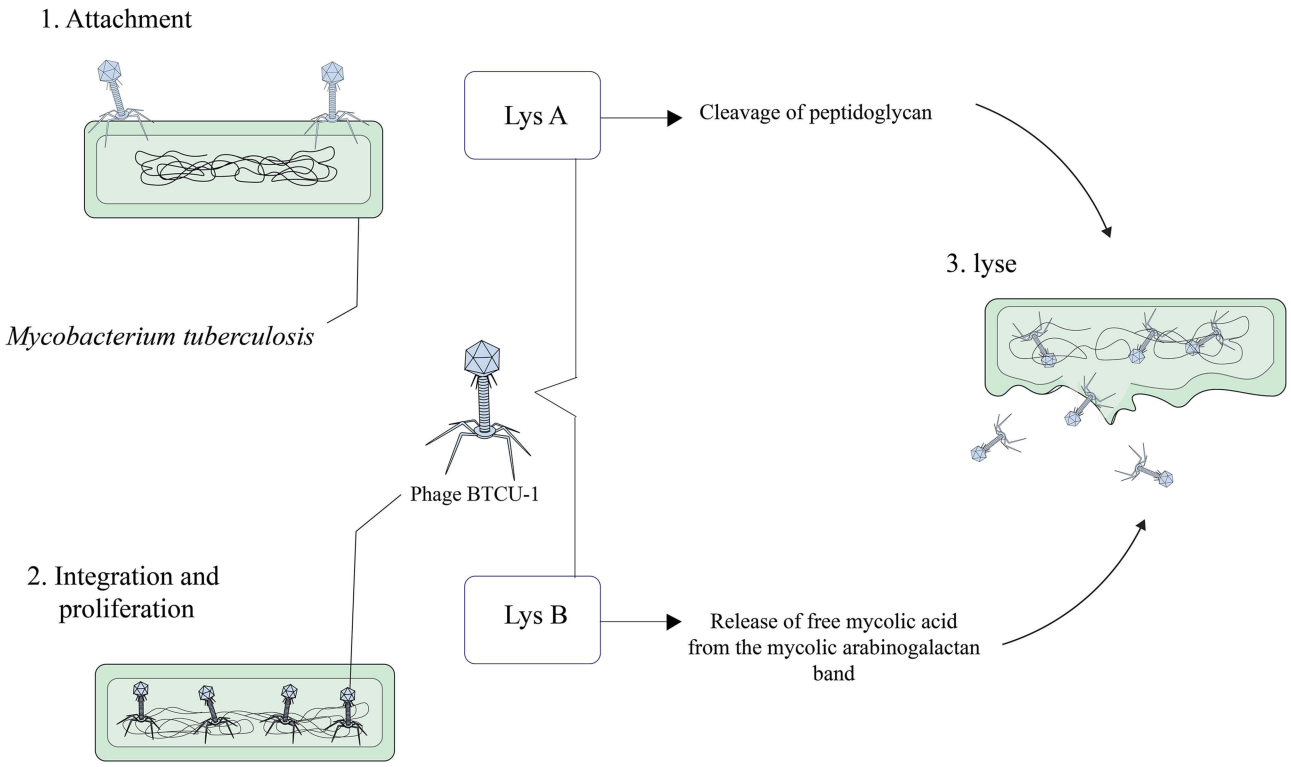

Figure 6 Overview of phage BTCU-1 mediated *Mycobacterium tuberculosis* lysis.

isoniazid, vancomycin, ofloxacin, ciprofloxacin, norfloxacin, ampicillin, and erythromycin.[41] Moreover, SWU1gp39 increases the susceptibility of Mtb to various stresses including heat shock, H2O2, SDS, and low PH.[41,93] On the other hand, a putative GTPase-activating protein (GAP) is encoded with mycobacteriophage SWU1 A321_gp67.[94] The GAP superfamily comprises 6 subfamilies including Ras, Rho, Ran, Rab, Rheb, and ARF.[95] These proteins can be involved in several processes including signal transduction, cell differentiation, cell cycle, and protein synthesis through regulating the activity of GTPase.[96] Gp67 is a mycobacteriophage SWU1 late-stage gene that could change colony formation and biofilm morphology and may play a role in the reproduction and release of the phage progeny.[97] Gp67 can downregulate the transcription of various genes such as MSMEG_0235, MSMEG_6092, MSMEG_1876, and mmpL4b.[94] These genes have multiple roles in biofilm formation, cell wall integrity, and development of colony morphology.[98] Moreover, gp67 can increase the susceptibility of Mtb against different antibiotics such as streptomycin and capreomycin via several procedures including (a) making changes in cell wall integrity and cell wall structure and (b) preventing and disrupting biofilm formation.[94] In total, it can be concluded that SWU1gp39 and gp67 might be utilized as a broad-spectrum antibiotic adjuvant or potentiator and be included into the existing antibiotic regimen for better control and greater efficiency of anti-tuberculosis drugs (including isoniazid and rifampicin) in bacterial killing (Figure 7).

Phage Ms6

Mycobacteriophage Ms6 is a temperate phage with a linear double-stranded DNA that has a lytic cassette composed of five genes.[99,100] Similar to the phage D29, in Ms6, Lysin A (a 384 amino acid polypeptide) has a central peptidoglycan recognition protein (PGRP) in a super-family conserved domain and hydrolyzes peptidoglycan of bacterial cell walls.[101] Ms6 Gp1 is highly similar to a chaperone-like protein and participates in the transport of LysA to the extracytoplasmic setting.[43] In the lysis cassette, Ms6 LysB is localized between lysA and hol genes. The length of Ms6 LysB is 996 bp that encodes a protein with lipolytic activity and possesses a capability to hydrolyze a wide spectrum of fatty acid esters.[99–102] Results of several studies revealed that, in *M. smegmatis*, Ms6 LysB targets the outer membrane, leading to the cleavage of ester bond among arabinogalactan and mycolic acids in the mycolyl-arabinogalactan-

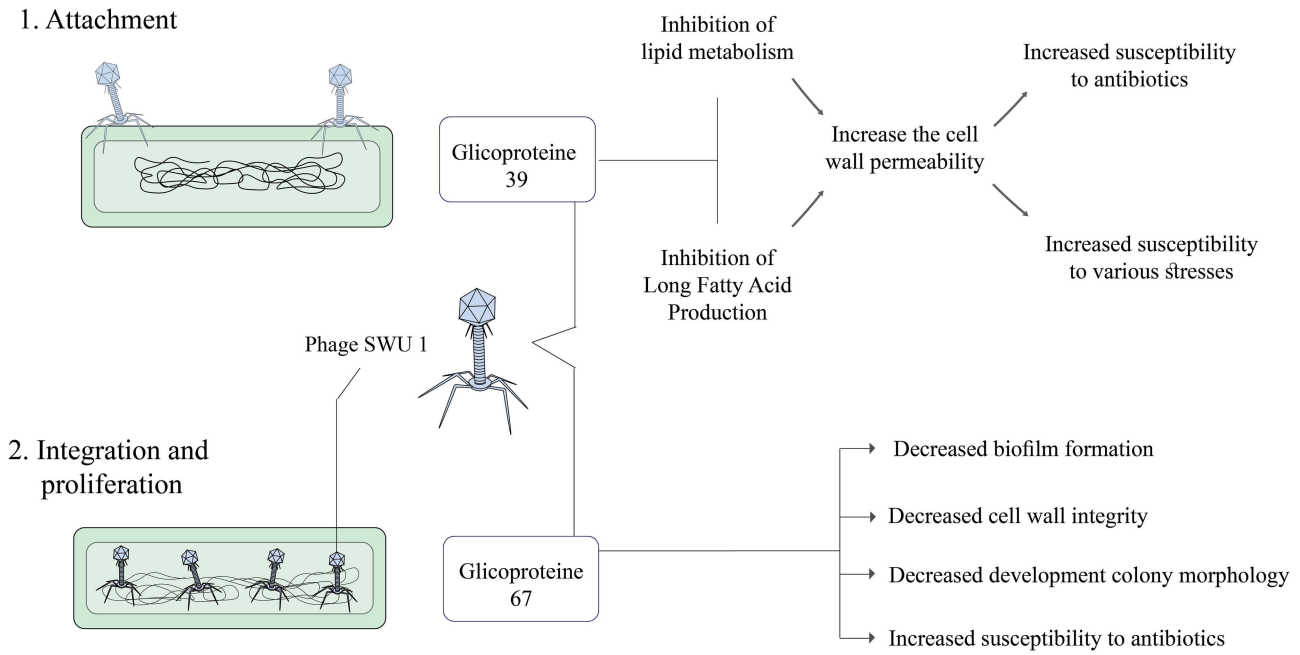

Figure 7 Overview of phage SWU1 mediated *Mycobacterium tuberculosis* lysis.

peptidoglycan (mAGP) complex.[58,101,102] Moreover, in *Mycobacterium tuberculosis* H37Ra and *Mycobacterium bovis* BCG, Ms6 LysB leads to the cleavage of the ester bond between trehalose and mycolic acids in trehalose 6,6′-dimycolate (TDM).[101] In addition to LysA (*gp2*) and LysB (*gp1* and *gp3*), in Ms6 lytic cassette, holin is encoded by *gp4* (*hol*).[99] In Ms6, holin protein has several roles including controlling the activation of the endolysin and controlling the access of endolysin to murein.[43,103] Finally, according to the above-mentioned statements, it can be concluded that mycobacteriophage Ms6 with different endolysins is a worthy candidate in Mtb infection therapy (Figure 8).

Phage therapy in mycobacterium avium infections

Mycobacterium avium (*M. avium*) is the slowest growing intracellular pathogen that replicates and persists within the mononuclear phagocytes.[104,105] This pathogen causes the dissemination of infection in immunocompromised patients. The patients with acquired immune deficiency syndrome (AIDS), especially patients with <50 $CD4^+$ T cells/mm^3, are susceptible to disseminated infection caused by this organism.[106] However, disseminated infections caused by *M. avium* have been reported to be highly frequency in non-AIDS individuals.[48] The application of protease inhibitors in the treatment of human immunodeficiency virus (HIV-1) infection has a substantial effect and contributes to a significant reduction in the occurrence of *M. avium* bacteremia.[107] Nevertheless, when the anti-HIV treatment stops, the incidence of *M. avium* bacteremia increases.[108] *M. avium* shows resistance to a wide range of antituberculosis antibiotics, and merely a few antibiotics including clarithromycin, azithromycin, and roxithromycin (Macrolides) have shown activities against *M. avium* in vitro and in vivo.[48] On the other hand, the other problem is that *M. avium* is able to infect and replicate within mononuclear cells including macrophages and monocytes.[109] The intracellular growth of organism within macrophages and monocytes justifies the latent phase of infection in the host.[48] Therefore, the antimicrobial agents that require a microbial target in active replication are not able to eradicate these infections. Of note, it is important for the new alternative therapies to be evaluated from the viewpoint of the following two facts: 1) clarithromycin, azithromycin, and roxithromycin are used in the prophylactic form for the *M. avium* infection, and the emergence of resistance in one antibiotic will be equal to resistance to all macrolides and 2) intracellular growth of organism.[110] The use of bacteriophage against *M. avium* infection is a significant alternative and is useful as an antimycobacterial regimen for treating drug-resistant bacteria.[109] TM4 is a lytic mycobacteriophage that infects *M. avium* and does not form persistent lysogens.[42,111] TM4 can be delivered by a nontuberculous mycobacterium (*Mycobacterium*

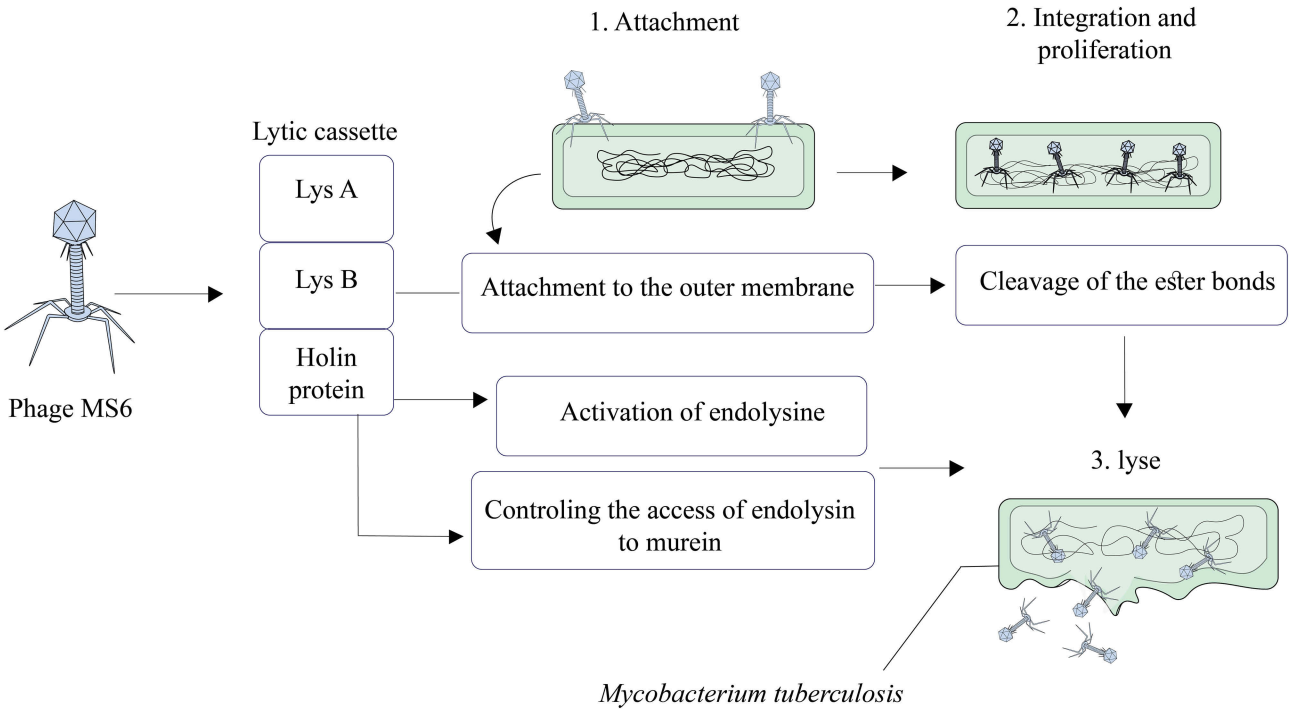

Figure 8 Overview of phage MS6 mediated *Mycobacterium tuberculosis* lysis.

smegmatis) and has the ability to lyse and kill *M. avium* inside mononuclear cells such as macrophages and monocytes.[48] It has been described previously that the treatment of *M. avium* infected macrophages with TM4 infected *M. smegmatis* and contributed to a considerable decrease in the number of *M. avium* bacilli.[109] Moreover, the treatment of *M. avium* infected macrophages with TM4 infected *M. smegmatis* led to the fusion of vacuole concealing *M. smegmatis* infected by TM4 with the *M. avium* vacuole in macrophages.[48] However, accordingly, *M. avium* naturally inhibits phagosome-lysosome fusion in macrophages, and it is predictable that the mycobacterial vacuole still has the capacity to be fused with endosomal.[112] Results of another study revealed that the coinfection of macrophage with *M. avium* and *Coxiella burnetti* led to the fusion of the two vacuoles and, finally, created new vacuoles that are acidic and contain *Coxiella* and *M. avium*.[113] Moreover, it is possible for TM4 to reach the *M. avium* vacuole in different ways, and that when vacuoles are lysed, the bacteria-containing vacuole becomes acidic.[48,109] It can be concluded that these findings proposed a new concept to kill intracellular mycobacteria and warrant the upcoming progression. However, it appears that in order to understand the exact role of mycobacteriophage in *M. avium* infection treatment, further studies are required.

Phage therapy in mycobacterium ulcerans infection

Buruli Ulcer (BU) is the third most common mycobacterial infection in immunocompromised individuals, especially in HIV positive patients.[52] This infectious disease is caused by *Mycobacterium ulcerans* (*M. ulcerans*) and is a serious and chronic necrotizing skin-infection disease, which is reported to be active in more than 30 countries worldwide with high frequency in West Africa.[114,115] Although *M. ulcerans* is proposed as an extracellular pathogen, evidence shows that this microorganism can be found in macrophages throughout the initial phase of infection.[116] *M. ulcerans* secretes a lipidic exotoxin diagnosed as mycolactone. This lipid toxin was shown to be cytopathic to cultured L929 murine fibroblasts and induced apoptosis in mammalian cells.[117,118] Moreover, this exotoxin was characterized by immunosuppressive properties and, finally, led to the typically clinical sign of ulcerative BU skin lesions.[115,119] The clinical manifestation of BU is characterized by various forms including preulcerative nodule, papules, plaque, and oedematous lesions; these lesions can tend towards characteristic necrotic ulcerative forms with undermined edges.[114,116] According to the data on BU disease, the design and preparation of controlling programs for prevention

purposes can be very difficult. Moreover, no vaccine against BU is available so far; however, evidence has shown that vaccination with Bacille Calmette-Guérin (BCG) could provide temporary protection against BU.[120] To date, BU is conventionally treated by surgical resection of affected skin followed by grafting, if required.[121] However, as of 2004, the World Health Organization (WHO) recommended the combination of antituberculosis drugs rifampicin and streptomycin as standard therapy for the treatment of BU patients.[122] Although the application of antituberculotic drugs decreases the relapse rates, this treatment is susceptible to numerous drawbacks: 1) this treatment does not resolve widespread lesions and patients are often left with scars and lifelong disabilities;[123] 2) muscular injection of streptomycin for a long time requires skilled personnel; 3) the mutations related to rifampicin resistance have already identified an in-vivo experimental condition after monotherapy; 4) the application of these antituberculosis drugs is related to several adverse side effects; 5) the consumption of these drugs may contribute to the deterioriation of the lesion with paradoxical reactions or the emergence of new lesions.[124–128] The use of bacteriophages as a diagnostic and treatment method for BU provides several benefits for patients: 1) extracellular microorganisms that prevail in progressive lesions are lysed and eradicated due to the lytic activity of phages; 2) phages can be administered topically in necrotic infection sites for the treatment of ulcerative lesions; and 3) *M. ulcerans* is naturally found as an extracellular pathogen and, finally, this pathogen might be nearly available by lytic phages.[114,129] Among different bacteriophages used for the treatment of bacterial infections, plaques of phage D29 are comparatively large and adsorption of phage particles seems to be efficient, which might be the best choice for the treatment of BU.[52] Moreover, the use of phage DS-6A in animal models with disseminated tuberculosis leads to the reduction of lesions in lungs, spleen, and livers.[114] Mycobacteriophage D29 is a lytic phage, and the results of experimentally infected animal models demonstrated that a single subcutaneous inoculation of this phage reduced the proliferation of the mycolactone-producing *M. ulcerans* 1615.[52,114] Notably, it is revealed that lytic activity of mycobacteriophage D29 may not be restricted to *M. ulcerans* 1615, and this phage also shows the lytic activity against numerous other *M. ulcerans* isolates in vitro.[130] One of the main characteristics of mycobacteriophage D29 is that this phage can be detected in several organs including blood and spleen (in post-injection 2 h) after subcutaneous injection.[114] Moreover, mycobacteriophage D29 could be found in the draining lymph nodes for longer periods of time (at least 15 days).[114,130] Results of a previously published study revealed that the application of mycobacteriophage D29 to the treatment of BU in vivo led to the pathologic reduction and the prevention of ulceration.[114] In total, it can be concluded that mycobacteriophage D29 reduces the number of *M. ulcerans* through several mechanisms: 1) mycobacteriophage D29 induces a cellular infiltrate of a macrophagic/lymphocytic profile; 2) mycobacteriophage D29 has an extensive lytic activity against mycolactone-producing *M. ulcerans* isolates, especially *M. ulcerans* 1615; 3) mycobacteriophage D29 increases the levels of TNF, IFN-γ, and IL-10 in vivo; and 4) treatment with mycobacteriophage D29 leads to the increase and maintenance of a local mononuclear inflammatory response to *M. ulcerans*.[114–116,131]

Discussion and conclusion

The rapid prevalence of mycobacterial infections and drug-resistance bacteria, especially the emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), has prompted researchers to find a novel alternative approach to controlling and treating TB. Mycobacteriophages are considered as natural antibacterial agents and are parasites on bacteria. Moreover, mycobacteriophages are extremely specific to their host. Therefore, phage therapy can be considered as a novel candidate for treating and controlling mycobacterial infections. Although phage therapy is a novel therapeutic approach against bacterial infection, especially MDR and XDR bacteria, the clinical use of this approach is susceptible to several limitations as follows: I) the administration of a large dose of phages in patients probably leads to the onset of the immunological response; therefore, this limitation restricts the use of specific phage more than once; II) the rate of clearance of phages in the body is very high; III) typically, the intracellular pathogens do not have access to the phage, and the transport of phages inside intracellular pathogens requires a delivery system such as non-virulent mycobacteria (*M. smegmatis*) or liposomes. Finally, it is suggested dedicating a greater body of in vivo and in vitro research to demonstrate the exact role and efficiency of phage therapy in the treatment of mycobacterial infection, particularly TB.

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Author contributions

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data. They played an active role in drafting the article or revising it critically to achieve important intellectual content, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Disclosure

All of the authors declare that there are no commercial, personal, political, and any other potential conflicting interests related to the published manuscript.

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