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Potential of Inhaled Bacteriophage Therapy for Bacterial Lung Infection

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Abstract

Phage therapy as a promising alternative antimicrobial to treat multidrug resistant (MDR) bacteria related lung infections, has drawn significant attention in clinical trials and bench-scale study in the recent decade, and the therapeutic effect of local delivery of phage has been demonstrated by several clinical reports. This book chapter discusses the current clinical development of inhaled phage therapy followed by the advancement of phage formulation designs for respiratory delivery of phage using various inhalation devices and their *in vivo* efficacy. The development of combination therapy of phage and antibiotics to combat MDR bacteria associated lung infections is also covered to reflect the current clinical practice. Lastly, we also share our insights on the challenges of advancing inhaled phage therapy and potential directions for future research.

Keywords: pulmonary delivery, multidrug-resistant bacteria, respiratory infection, dry powder inhaler, nebulization, phage formulation, inhaled phage therapy

1. Introduction

Lung infection is a leading cause of morbidity and mortality worldwide [1]. Currently, antibiotics remain the mainstay treatment options for bacterial lung infections [2]. With the rapid emergence of multidrug-resistant (MDR) bacteria, last-line antibiotics such as colistin and carbapenem have been increasingly used for life-threatening infections. However, nosocomial outbreaks caused by pan-drug resistant (PDR) 'superbugs' have also been increasingly reported worldwide, creating significant therapeutic challenges for the treatment of lung infections [3–5].

Bacteriophage (phage) therapy has been proposed as a promising alternative to antibiotics in combating bacterial infections, including those caused by the MDR pathogens. A comprehensive review from Abedon summarized earlier clinical studies of phage application, with most reported cases from Eastern Europe as these countries more practical experience [6]. Overall, phage therapy for respiratory infections have not been extensively studied and only a handful of human studies reported [6–8].

Although recent failure of the "Phagoburn" trial against burn wound infections is discouraging, a lesson we learnt is the importance of the stability of phage preparations and the efficient delivery of sufficient amount of viable phage to the

site of infections [9]. Pulmonary delivery of phage would hold the greatest promise in achieving optimal concentration of phage in the lung for effective treatment. In this book chapter, we first introduce the clinical progress of inhaled phage therapy and highlight recent advancement made in the delivery of phage preparations using various inhalation devices. As most experimental phage therapeutic investigations were conducted with concomitant antibiotic treatment, we also discuss the development of phage-antibiotic combinations to treat lung infections. Lastly, we summarize the challenges that must be overcome in order to translate inhaled phage therapy to clinical applications.

2. Clinical development of inhaled phage therapy

In the past decade, a few success stories in experimental inhaled phage therapy were reported. Hoyle et al. reported a successful inhaled phage therapy to manage chronic lung infection caused by MDR *Achromobacter xylosoxidans* [10]. The 17-year-old female patient was unsuccessfully treated with many rounds of antibiotics before she was given a phage cocktail treatment containing two *Achromobacter* phages in the Eliava Phage Therapy Center. The phage cocktail was given by nebulization once daily and orally twice daily for 20 days. The treatment was repeated 4 times at 1, 3, 6 and 12 months after the initial treatment. The patient's subjective conditions were significantly improved and her lung function-FEV1 increased from 1.83 L to 3.33 L together with intermittent antibiotic regimen. Successful phage treatment was also reported for a 12-year-old lung-transplanted cystic fibrosis (CF) patient suffered from persistent lung infection caused by PDR *A. xylosoxidans* [11]. After two rounds of inhaled phage therapy, the patient's respiratory condition slowly improved and the bacterial load was significantly reduced. Similar favorable therapeutic efficacy was also reported in another clinical case [12], where a five-year-old cystic fibrosis patient suffering from severe lung infections was treated with a commercially available phage preparation (pyophage) by nebulization.

Aslam et al. reported the early clinical experience of phage therapy in lung transplant recipients in the USA [13]. Three patients with life-threatening MDR infections caused by *Pseudomonas aeruginosa* (n = 2) and *Burkholderia dolosa* (n = 1) received phage cocktails via both intravenous injection and nebulization with concurrent antibiotic treatments for variable duration. Two patients responded clinically with the phage treatments and were discharged from hospitals, while the third patient infected by *B. dolosa* was dead due to infection relapsed. Nonetheless, no phage therapy-related adverse events were identified. While these experimental use of inhaled phage therapy as an adjunct treatment has demonstrated the clinical benefits in treating lung infections caused by MDR superbugs, well-designed clinical trials are needed to convincingly evaluate its clinical efficacy.

To date, there have been three phage therapy clinical studies registered with the ClinicalTrials.gov to evaluate the safety and efficacy of phage therapy against lung infections (**Table 1**). "MUCOPHAGES" (NCT01818206) assessed the effect of a cocktail of 10 phages on *P. aeruginosa* from sputum samples isolated from CF patients. Although the trial was completed in 2012 according to the clinical trial registry, no information about the outcome of this trial was published. In 2020, two other trials were launched. One trial (NCT04636554) is attempting to apply personalized phage treatment in Covid-19 patients with bacterial co-infections microbial for pneumonia or bacteremia/septicemia. Another trial launched by Armata Pharmaceuticals is a Phase 1b/2a, double-blind, randomized, placebo-controlled trial (NCT04596319) aiming to study the safety, tolerability, and preliminary efficacy of inhaled AP-PA02 in subjects with CF and chronic pulmonary

ClinicalTrails. gov Identifier	Phase	Target condition/ Disease	Phage	Design	Trail status
NCT01818206	NA	Cystic Fibrosis	A cocktail of 10 bacteriophages	Single Group Assignment	Completed in 2012
NCT04596319	1b/2a	Chronic *Pseudomonas aeruginosa* Lung Infections and Cystic Fibrosis	AP-PA02 cocktail	Parallel Assignment (Randomized, double-blind, placebo-controlled)	Recruiting as of the preparation of this book chapter
NCT04636554	NA	Covid-19 patients with bacterial co-infections	Phages against *A. baumannii*, *P. aeruginosa* or *S. aureus*	Expanded Access (Intermediate-size Population, Treatment IND/ Protocol)	Recruiting as of the preparation of this book chapter

NA: not available.

Table 1.
Clinical trials of phage therapy for lung infections.

P. aeruginosa infection. This is the first randomized trial on inhaled phage therapy and the AP-PA02 cocktail is an advanced version of AP-PA01 which was used in the successful experimental study documented in Aslam et al. [13]. The findings from this trial are expected to set a landmark for the development of inhaled phage therapy.

3. Nebulization

3.1 Liquid formulation

Majority of the phage studies for lung delivery focus on liquid formulations as minimal formulation development is required to prepare phage cocktails with sufficient stability for a short storage period. The long term storage stability of phage in liquid formulations was often reported. Cooper et al. demonstrated a phage cocktail of 3 *Pseudomonas* phages (GL-1, GL-12.5 and LP-M10) suspended in phosphate buffered saline (PBS) was stable at both 4 °C and room temperature with no statistically significant titer loss (≤ 0.5 log) for 6 months [14]. As most commonly used phage stabilizers, including PBS, salt-magnesium buffer (SMB) and Tris-H buffer are not yet approved for inhalation. Dilution of phage suspension with 0.9% sodium chloride (NaCl) is usually needed for pulmonary administration [10]. Carrigy et al. showed minimal impacts on the phage stability with the NaCl dilution process, suggesting the suitability of this approach [15].

To date, nebulization has been the exclusive choice for pulmonary delivery of phage suspension in human studies due to its high delivery efficiency and capability of delivering a large volume of liquid phage formulation (> 1 mL) to patients including those cannot administer the dose voluntarily. Several types of commercial nebulizers are available to aerosolize phage into fine droplets using different aerosol generation mechanisms, including air-jet nebulization, vibrating mesh nebulization, ultrasonic nebulization, and colliding liquid jets [16, 17]. The suitability of these nebulizers in delivering phage to lungs has been previously evaluated in terms of deactivation of phage upon the nebulization process.

Jet nebulizers use compressed air to atomize the liquid phage suspension into primary droplets and their subsequent impaction onto the baffle would further breakdown into smaller droplets suitable for inhalation. LC-star nebulizer [16, 18], Collison 6-jet [19–21], LC Sprint jet nebulizer [22], AeroEclipse [23] and atomizer [24] have been used to deliver therapeutic phages. Leung et al. showed the air-jet nebulization had negligible impacts on the stability of the *Podovidae* PEV2 phage, while significant titer loss was found in *Myoviridae* PEV40 phage (~1 log loss) and *Siphoviridae* D29 phage (~3 log loss) [22]. Based on the cryo-transmission electron microscopy analysis, they found the nebulization-induced titer loss was correlated with morphological damage to phages. They further suggested that the length of phage tail may be an important consideration when delivering phages via jet nebulization, particularly for phage cocktails containing phages of different morphologies. The influence of the final formulation composition for nebulization of D29 phage was evaluated by Liu et al. using a Collison 6-jet nebulizer [19, 21]. They reported that deionized water was the optimal spray liquid for D29 aerosol generation and they postulated that the high ion strength and salt concentrations in the PBS and 0.9% NaCl were detrimental to the phage upon jet nebulization. These results were in accord with Carrigy et al. and Leung et al. nebulizing buffered D29 using other jet nebulizers [15, 22]. Liu et al. also studied the impact of relative humidity (RH) on the stability of nebulized D29 and found a low environmental humidity condition was more favorable for D29 nebulization [19]. Later, Verreault et al. reported that the stability of nebulized phage aerosols at different temperatures and humidity is phage-dependent with some being more robust and some being more vulnerable [21]. Overall, these studies highlighted the importance of controlling the temperature and RH for phage nebulization.

Vibrating mesh nebulizers produce aerosol droplets by extruding the liquid formulation through a membrane with calibrated holes based on the converse piezo-electric effects. Several studies compared the aerosol delivery of phage between jet and mesh nebulizers [15, 16, 23–25]. Golshahi et al. showed both the LCstar (air-jet) and eFlow (mesh) nebulizers were suitable for the delivery of phages active against *Burkholderia cepacia* Complex by imaging the lung deposition and mathematical model prediction [16]. In some studies, mesh nebulizers were found to be more detrimental to phage than air-jet nebulizers [23, 24], but reasons for the poorer delivery of mesh nebulizer were unclear. In contrast, better phage recovery was noted after nebulizing using a mesh nebulizer compared with the jet nebulization in some other studies [15, 25]. Visual evidence on the correlation between the titer reduction and morphological change of a *Myoviridae* PEV44 phage after nebulization was provided by Leung et al., showing more "intact" phage was detected in the mesh-nebulized phage samples under TEM image. The more destructive effect of jet nebulization is likely caused by stresses associated with the droplet production and re-nebulization processes. Based on the collected experimental data and a mathematical model, Carrigy et al. estimated phage were re-nebulized an average of 96 times before exiting the mouthpiece of the jet nebulizer [15]. A review from Prichard et al. revealed that 86% of the disclosed nebulizer technology have chosen vibration-mesh nebulizers as the delivery devices, particularly for stress-sensitive drugs [26]. The mixed findings of phage nebulization in the literature can be attributed to many factors, such as phage types, formulation composition, experimental conditions (like temperature, humidity and sample collection methods) and different models of the same nebulizer type. Therefore, the survival of individual phages within a cocktail should be tested with different delivery devices for the optimization of phage cocktail – inhalation device combinations.

Ultrasonic nebulizers use a piezoelectric transducer to generate ultrasonic wave in the liquid drug formulation and aerosolize it at the solution surface. Upon the

nebulization process, a portion of the ultrasonic energy converts to heat, which could be detrimental to heat-sensitive biologics, like phages. Only one study reported the use of an ultrasonic nebulizer to deliver phage to treat lung infections in a mink model, but little data on the nebulization process was available [27]. More recently, Marqus et al. assessed the capability of a novel low cost and portable hybrid surface and bulk acoustic wave (HYDRA) nebulizer to deliver a *Myoviridae* phage K and lysostaphin to target *Staphylococcus aureus* [28]. Negligible titer reduction was noted (0.1 log loss), possibly due to the relatively low powers and high frequencies (approximately 10 MHz) of the nebulizer. Furthermore, the size of the aerosols generated by HYDRA is smaller (DV50 1.85 μm), well within the respirable range, demonstrating its suitability for pulmonary delivery of phages.

3.2 *In vivo* efficacy of inhaled phage therapy achieved with nebulization

The *in vivo* efficacy of phage liquid formulation has been studied in rodent and mink models. Semler et al. established *B. cenocepacia* respiratory infection model in mice and then treated with liquid phage formulation delivered by a LC-star jet nebulizer or intraperitoneal injection (IP) [18]. After a 2-day treatment, the lung bacterial load was only reduced by ~0.5 log in mice received phage via IP injection, but a 2-log bacterial reduction was observed in mice treated with inhaled phage. This finding is in contradiction with a previous study showing that phage delivered by the IP route was more efficacious than intranasal instillation in treating a *B. cenocepacia* respiratory infection in mice [29]. Semler et al. accounted the discrepancy to the efficiency of phage delivery to lungs that nebulization is a more effective way in delivering phage particles to the lung than intranasal instillation. Also, the capability of IP injected phage reaching lung is significantly affected by the clearance rate of phage in blood which is phage-dependent. The *in vivo* delivery efficiency of D29 phage using a Collison 6-jet nebulizer and IP route was compared by Liu et al. [20]. Approximately 10% of D29 phage could reach to the lung of mice after nebulization and complete phage elimination was noted in 72 h, whereas only 0.1% of the phage could reach the lung by IP injection and no phage was detected after 12 h. The importance of phage dose on the pharmacokinetics/pharmacodynamics (PK/PD) of inhaled phage therapy was recently confirmed by Chow et al. using *Pseudomonas* phage PEV31 [30].

Carrigy et al. recently demonstrated the prophylactic function of nebulized D29 phage for protection against *Mycobacterium tuberculosis* infection in a mouse model [31]. Phage was delivered with a vibrating mesh nebulizer and a dose of 6.6 log phage reached the lung and remained there for 90 min post-delivery, suggesting that phage was not rapidly cleared in the mouse lung. Low doses of *M. tuberculosis* (5–100 CFU) were given to mice 30 min post phage administration. This phage pretreatment was able to significantly reduce the bacterial burden in mouse lungs at 24 h and 3 weeks post infection. The prophylactic effect of phage was also demonstrated in a rat model against methicillin-resistant *S. aureus* infection [32]. Phage was given by a vibrating mesh nebulizer 4 h before the bacterial challenge, higher survival rate (60–70% improvement) with a 2 log bacterial reduction in the rat lungs were observed. Both studies demonstrated prophylactic treatment with sufficient dose of nebulized phage may provide protection to immunocompromised individuals and health care professionals who are at risk of exposure to "superbugs".

There is accumulating evidence that bacterial clearance by phage therapy requires the synergy between phage and host immune system. Therefore, the translation of preclinical data collected from rodent to humans should be treated with care due to the significant difference in their immune systems [33]. Cao et al. explored the phage antibacterial effect of hemorrhagic pneumonia in a mink model [27]. Effective treatment

outcomes were achieved at multiplicity of infection (MOI) of 10 with an 80% survival rate at 12 days after phage administrated by means of ultrasonic nebulization.

4. Dry powder inhalers

4.1 Powder formulation

Although nebulization has been the method of choice for phage delivery in treating lung infections in clinical settings, dry powder formulations are preferred to liquid formulations in terms of storage, transportation and administration [34]. Compared to nebulizers, dry powder inhalers (DPIs) are easier to handle without the need of a power source, fewer cleaning requirements and quick delivery [35]. Current research on pharmaceutical development of inhaled phage dry powder mainly focuses on formulation optimization for sufficient powder dispersibility to deliver phage to the lung and storage stability. The choice of excipients plays a key role among all the techniques to produce phage dry powder. Zhang et al. published a comprehensive review to discuss how the choice of excipients affecting the stability of phage in the solid-state [36]. Overall, sucrose, lactose and trehalose are the most popular disaccharides in phage powder formulations. Freeze drying (FD), spray drying (SD) and spray freeze drying (SFD) have been used to generate inhalable phage dry powders with these excipients.

FD is a commonly employed technique to stabilize drugs in solid state [37]. Puapermpoonsiri et al. used FD to generate dry powder of phage-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres designed for pulmonary delivery [38]. Although phages were successfully incorporated into the PLGA microparticles, the poor shelf-life of the encapsulated phage which completely deactivated within 7 days either stored at 4 °C or 22 °C was discouraging. In their follow-up study, they investigated the feasibility of using a high concentration of sucrose (0.5 M) or PEG6000 (5%) to stabilize the FD phage cake [39]. Although rapid phage reduction was still noted over the first 7–14 days, phage remained relatively stable in the powder formulations thereafter. Since then, a number of studies have studied the impacts of various excipients on the production loss and storage stability of FD phages [40–43]. Among all excipients examined, sucrose and trehalose were identified as the most promising stabilizers to preserve phage viability upon the dehydration in the drying process and upon storage. The residual moisture content was found to play an important role in maintaining phage stability. Similar to other protein therapeutics, a 3–6% moisture content of the powder cake was found to be optimal for phage preservation [39, 41]. Although the mechanisms of phage stabilization in dry powder by these sugars are still unclear. Two most acceptable hypotheses for the stabilization of proteins in the solid state by sugars are water replacement and vitrification, which may also be applicable to phages because they are mostly composed of proteins.

In general, FD powder is not respirable, and a separate milling step is required to reduce the particle size to <5 μm, suitable for pulmonary delivery. However, the high-energy milling may cause additional phage loss due to the generation of heat and mechanical stresses. Golshahi et al. prepared FD formulations of KS4-M and ΦKZ phages with 60% lactose and 40% lactoferrin suitable for pulmonary delivery without milling [44]. The size of the phage powder was within the inhalable range (< 5 μm) and acceptable aerosol performance with a fine particle dose of >10^6 pfu using an Aerolizer was achieved. The production loss was 1–2 log which was not desirable, but the FD phage powders were stable with negligible titer reduction within 3 months storing either at 4 °C or 22 °C.

SD is a well-established single-step technique employed for the production of many inhaled pharmaceutical products [45]. Matinkhoo et al. were among the first to study the feasibility of using SD to produce inhalable phage powders comprising trehalose and leucine with or without a third excipients (a surfactant or casein sodium salt) [46]. In these formulations, trehalose was used to protect phage against dehydration; leucine forming a crystalline shell at the particle surface was used to enhance the dispersibility of powders; and a surfactant was employed to reduce aggregation of phage during the drying process. Due to the thermal sensitivity of phage, a low drying temperature was used to produce SD powders with acceptable production loss (0.4–0.8 log) and phage lung dose (7–8 log pfu). Trehalose-alone formulation was employed by Vandenheuvel et al., but the production loss was found to be phage dependent [47]. On the other hand, trehalose-leucine and lactose-leucine systems could stabilize a panel of *Pseudomonas* phage upon the SD process [48–51]. Since the SD trehalose and lactose is amorphous, Chang et al. demonstrated that the addition of a sufficient amount of leucine (at least 20%) was critical to stabilize phage by minimizing recrystallization of trehalose/lactose during powder production process [48]. Despite a low production loss was achieved, particle merging was still significant for formulation containing 80% sugar and 20% leucine due to moisture sorption upon handling. Therefore, higher leucine content and the addition of mannitol to the excipient system was attempted to improve the morphology and reduce the moisture sorption capacity of the phage powders during handling and storage (**Figure 1a-c**) [49, 50]. Although these approaches significantly reduce the problem of particle merging and make powder handling easier, they failed to stop the recrystallization of the amorphous content at high humidity conditions (RH > 50%). Therefore, storing the SD powders at low humidity conditions (RH ≤ 20%) was generally recommended [48, 49, 52, 53]. The storage temperature was also reported to be important on phage dry powder stability. It is generally recommended to store phage drug powder at a temperature at least 50 °C below the glass transition temperature (Tg) of the powders [54].
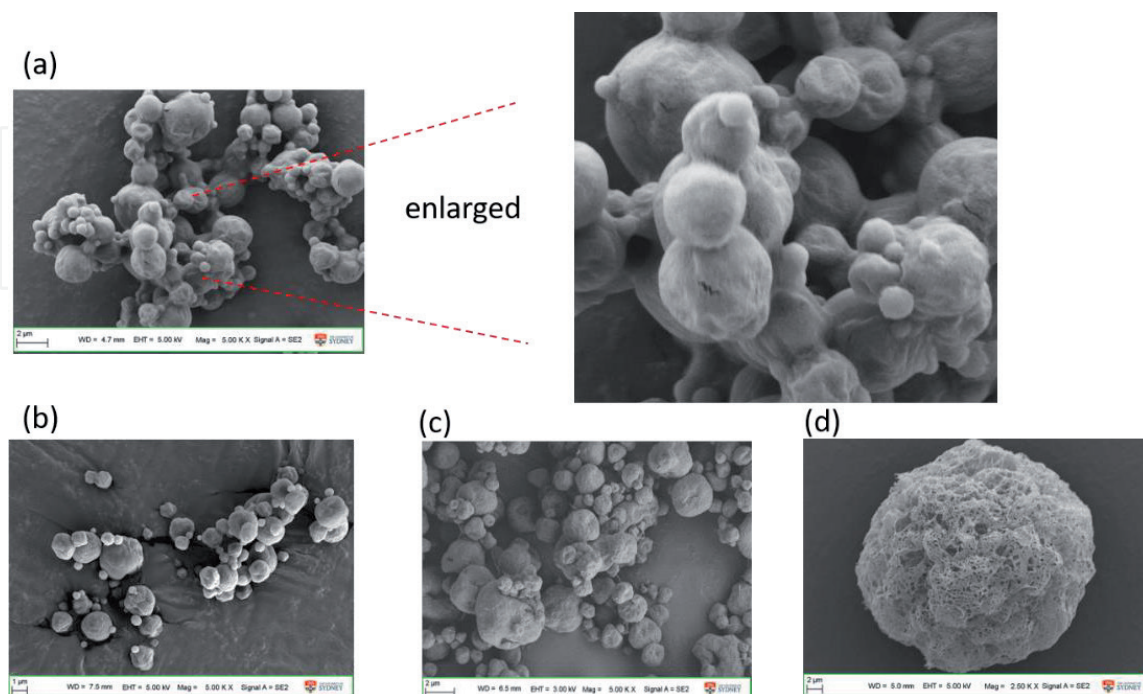

Figure 1.
Representative scanning electron microscopy images of phage powders produced by spray drying (a-c) and spray freeze drying (d). (a) 80% trehalose+20% leucine; (b) 60% trehalose +20% mannitol +20% leucine; (c) 70% trehalose +30% leucine and (d) 60% trehalose+20% mannitol and 20% leucine.

Overall, SD phage powders composed of trehalose/lactose not less than 40% of the total solid content together with leucine and mannitol was able to stabilize phage in powder form with sufficient long shelf-life (≤ 1 log titer loss in 12 months) under refrigeration or room temperature at RH < 20% and yield acceptable lung dose (10^5–10^7 pfu) [46–50, 53]. While leucine is a commonly employed surface active agent to improve the powder dispersity of inhaled pharmaceuticals, trileucine has also been increasingly used to improve aerosol performance and stability of SD powders for inhalation. Recently, Carrigy et al. demonstrated the effectiveness of a trileucine and trehalose system in preserving an anti-Campylobacter phage, CP30A, in powder form for long-distance ambient temperature transportation [55, 56].

SFD is a relatively new drying technique to produce inhalable dry powders. The produced powders are superior to those prepared by traditional FD in terms of structure, quality, and the retention of volatiles and bioactive compounds [57]. The suitability of SFD porous mannitol carriers for pulmonary delivery of drug nanoparticles and biologics have been demonstrated [58–60]. Leung et al. produced SFD phage powder and compared their differences of powder properties with the SD phage powders (**Figure 1d**). With the use of a high frequency of ultrasonic nozzle in the SFD process, a significant titer reduction (>2 log) was noted in the spraying process, making the overall production loss inferior compared with the SD process [53]. Nonetheless, the larger porous carrier provided a larger extent of protection of the embedded phage during aerosolization with a higher recovery of viable phage compared with the SD counterparts. The conventional SFD process is a two-step manufacturing process, which hinders scaling up. Ly et al. used an atmospheric spray freeze-drying (ASFD) technique, which is a single step process, to prepare D29 phage powder [61]. An acceptable titer loss (~0.6 log) was noted due to the use of a twin-fluid nozzle and improved mass and heat transfer rates.

4.2 *In vivo* efficacy of inhalable phage dry powder

Pulmonary delivery of dry powder to small animals is challenging as they cannot inhale powder actively. Intratracheal delivery using a dry powder insufflator, either the commercially available Penn-Century models or custom-made insufflators [62], are commonly employed to introduce powders directly into the lungs of the experimental animals. Chang et al. explored the *in vivo* efficacy of phage powder to treat lung infections caused by MDR *P. aeruginosa* in a mice pneumonia model [63]. After challenging the neutropenic mice with intratracheal administration of the bacterial suspension for 2 h, powder of phage PEV20 was administrated use a Penn-Century dry powder insufflator at a concentration of 2×10^7 pfu/mg. A significant bacterial reduction (5.3 log cfu) was noted after 24 h post-infection accompanies with 1 log phage propagation. The successful treatment outcomes and safety profile from this study warrant further investigation to fully evaluate the therapeutic potential of inhaled phage powder in managing lung infections.

5. Other inhalation devices

5.1 Metered dose inhaler

Pressurized metered-dose inhalers (pMDIs) are the most popular inhalers for the treatment of asthma and chronic obstructive pulmonary diseases. To date, only one study has attempted this type of device to aerosolize phage [64]. The phage cocktail suspension containing FKZ/D3 and KS4-M phages, was formulated in a reverse emulsion with Tyloxapol surfactant using hydrofluoroalkane 134a as the

propellant. A limited loss of phage activity (0.5–0.9 log) upon the actuation was observed, but the long term storage stability of the phages was not assessed. Further studies to examine the interactions between phage and liquefied propellant gas [65], and maximum loading capacity of phage/puff are required to move this inhaler choice forward.

5.2 Soft mist inhaler

Soft mist inhaler (SMI) is a relatively new generation, propellant-free inhaler that delivers drugs to the lung more efficiently than pMDIs because of the lower spray velocity and longer duration time [66]. Carrigy et al. compared the delivery efficiency of phage among vibrating mesh nebulizer, jet nebulizer and SMI [14]. SMI was showed to deliver phage D29 at high titers quickly (~5×10^8 pfu/actuation) with an acceptable titer reduction (0.6 log pfu/ml) and a higher lung delivery (3.2×10^6 pfu/actuation of inhalable active phage). This compact and light weight device may act as an attractive option for self-administration of phage aerosols.

6. Combination of phage therapy and antibiotic to treat lung infections

6.1 Mechanisms of phage-antibiotic synergy

With the emergence of phage-resistant bacteria [67], the combination therapy of antibiotics and phages has drawn increasing attention. Synergistic effect of antibiotic and phage against *S. aureus* was first reported by Himmelweit et al. back in 1945 [68]. Similar synergistic antibacterial effects have also been observed in a number of subsequent studies [69–79]. In 2007, Comeau et al., coined the term phage-antibiotic synergy (PAS) corresponding to an incident where the killing effect of bacterial strains considerably higher when phage production increases by the sublethal concentrations of particular antibiotics [80]. While many antibiotics exhibit synergistic effect in combination with phages, two specific classes of antibiotics (namely beta-lactams and fluoro-quinolones) were shown to produce a more consistent and pronounced antibacterial synergistic effect with phage therapy. The precise mechanisms contributing to phage-antibiotic synergy remain largely unknown. A few possible mechanisms have been proposed (**Figure 2**): (1) Antibiotic causes cell elongation or filamentation, thus subsequently promoting phage production; (2) Degradation of the extracellular membrane of bacteria by phage facilitates internalization of antibiotic into cells; (3) Auto-aggregation of bacterial cells leads to synergism; (4) Bacteria containing complete prophages could be induced by antibiotics which further kill bacteria [81]. The capacity of phage in resensitizing bacteria to certain antibiotics have also been reported as the host bacteria cannot develop resistance to phage and antibiotic simultaneously [82–84]. As a result, the phage-antibiotic combination can kill both phage-sensitive and antibiotic-sensitive pathogens with the phage lysing cells resistant to antibiotics and antibiotic mediated killing of phage-resistant bacterial cells and eventually inhibit the infections.

Interestingly, the sequence of phage and antibiotic administration was found to be critical in the overall antibacterial effect from the combination treatment. Chaudhry et al. showed the efficiency of removing *P. aeruginosa* PA14 biofilm was higher when the biofilm was treated with phages before antibiotics [85]. A similar observation was also reported in another study evaluating phage-antibiotics combination therapy against *S. aureus* biofilms [86]. However, the observed synergistic effects were found to be dependent on the class of antibiotics used. Pre-treatment
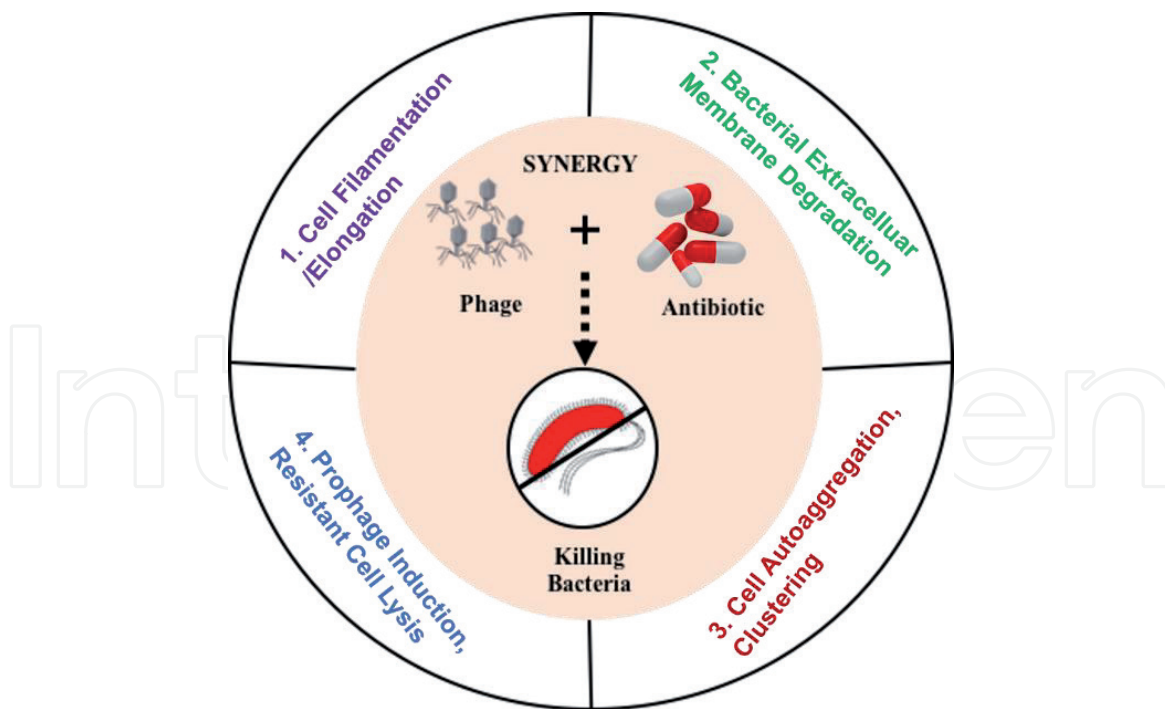
Figure 2.
Possible mechanisms responsible for phage-antibiotic synergy.

with phage led to favorable antibacterial effect when combined with linezolid or tetracycline, whereas antagonism was observed between the phage and dicloxacillin or cefazolin. Furthermore, it is noteworthy that an antagonistic effect was observed when the bacterial biofilm was treated with antibiotics preceding the phage therapy, irrespective of which class of antibiotics used [86].

6.2 Novel tools for selection of optimum phage-antibiotic combination

Since the exact mechanisms responsible for PAS are still unclear and the choice of the combinations is mostly empirical, it is not surprising that mixed results were reported in the literature [72, 82]. Also, the concentration of antibiotics used in previous studies was limited to one or two levels, which is not enough to predict the efficacious concentration when applied in clinical treatment. To solve these problems, Liu et al. developed a high-throughput platform called synogram by combining an optically based real-time microtiter plate readout with a matrix-like heat map to quickly assess the effects of various phage and antibiotic concentrations on bacterial growth [87]. They concluded that PAS is highly dependent on the antibacterial mechanism of action for antibiotic and phage pairs and their stoichiometry.

To guide the choice of phage-antibiotic combination, Rodriguez-Gonzalez et al. [88] developed an *in silico* nonlinear population dynamics model taking into account the systemic interactions between bacteria, phage and antibiotics to mimic *in vivo* application by given an immune response against bacteria. Using two *P. aeruginosa* strains, one phage-sensitive (resistant to antibiotic) and one antibiotic sensitive (resistant to phage), as the model bacteria, the phage-antibiotic combination therapy was confirmed to outperform the monotherapy. The role of the host immune response was also evaluated and the model predicted that the phage-antibiotic combination failed to eliminate the infection when innate immunity was removed or severely reduced. Their findings confirmed the clearance of infection is depending on the nonlinear synergistic interactions between phage, antibiotic, and innate immunity. The *in silico* prediction was consistent with previous experimental results obtained *in vitro* and *in vivo*. While this model is a valuable tool in

identifying potential phage-antibiotic combinations, further modification of the model to yield high-resolution temporal data in addition to the final results will be useful for quantitative comparison of the model-based predictions with experimental results.

6.3 Formulations of phage-antibiotic combination to treat lung infections

Streptococcus pneumoniae, S. aureus, B. cepacia complex, Klebsiella pneumonia and *P. aeruginosa* are the major causative pathogens for lung infections. A summary on previous work on the combination phage-antibiotic therapy against these pathogens were provided in Chang et al. [8]. Recently, Lin et al. screened a panel of antibiotics with PEV20 phage to target two *P. aeruginosa* strains and ciprofloxacin showed the highest synergistic effects. The combination was then nebulized using a jet nebulizer and a mesh nebulizer with no difference in the antibacterial effect observed between the nebulized samples and non-nebulized suspension [89]. Later, the same research team investigated the feasibility of formulating this combination into dry powder formulations [90]. PEV20 phage and ciprofloxacin were co-spray dried with leucine and with or without lactose. Both formulations maintained bactericidal synergy after dispersion using a low resistance inhaler or a high resistance inhaler, both showing acceptable FPF (60–75%). The antimicrobial efficacy of the PEV20-ciprofloxacin combination powder was also confirmed in a mice respiratory infection model with significant bacteria reduction (5.9 log) at 24 h post-treatment, while no loss of bacteria viability when mice was treated with phage or antibiotics alone [91]. The long-term storage stability of the combination powder at 4 °C and 20% R.H. was also confirmed [92].

7. Challenges for pulmonary delivery of phage and future perspective

Phage therapy is evolving as a promising alternative or an adjuvant to antibiotics for the battle against MDR bacteria. Although a few randomized, double-blind and placebo-controlled clinical trials have been conducted to assess tolerance and/ or efficacy of phage therapy in the past few years, none of the completed trials have yielded data supporting the promising observations noted in the experimental phage therapy conducted in animals and humans. Górski et al. highlighted the importance of the quality and titer of the phage preparations and their delivery efficiency to the target sites to ensure a sufficient high phage to bacteria concentration in the vicinity of infected tissues [93]. For lung infection, directly delivering phage preparation to the airways enhance the incidence of phage getting access to its host bacteria, avoiding the rapid clearance in systemic circulation. Advancements have been made in the past decade to improve the formulations for pulmonary delivery of phage. Here we highlight some hurdles remained to be tackled to bring inhaled phage therapy to clinical settings beyond compassionate use and a few prospective research directions for the commercial application of aerosol formulations.

As a sufficient amount of phage at the site of infection is the prerequisite for successful therapy, nebulizers and DPI are better choice for pulmonary delivery of phage compared with pMDI and SMI due to their capacity of high dose delivery. The detrimental effect of the various type of nebulizers to phage was found to be phage-specific, likely attributing to the tail morphology of phage [21] and compositions of the phage formulations [18]. Systematic studies to confirm their impacts on phage nebulization will provide important information in developing new phage cocktail formulations. Although liquid formulations are commonly used for phage therapy, solid phage formulations are more desirable for long-term storage and transportation.

While stable phage powder formulations have been successfully achieved with storage at ambient temperature, they are usually required to be handled and stored at low humidity conditions (RH < 20%) [48–50]. These would be easily achievable in a manufacturing setting and with pharmaceutical packaging designs. As excessive environmental moisture could also be relevant in patients' homes or in healthcare settings, the impacts of humidity on powdered phage administration should be evaluated to ensure the phage product could be used successfully in different geographic regions over the world. In preparing phage-powder formulation, trehalose, lactose, and leucine are commonly employed to stabilize phage. However, these excipients have not been approved for inhalation except lactose was approved as a carrier which is not expected to be delivered to the lower respiratory tract. Further *in vivo* studies are required to evaluate the safety profile of these excipients for both short term and long term usage.

Currently, *in vivo* data of phage therapy for lung infections mostly focused on acute infections that phage preparation was given at within a few hours post-infection. However, in clinical settings, the phages are unlikely given immediately after the onset of infection, the postponed treatment may lead to significant bacterial growth and biofilm formation, more research is needed to evaluate the therapeutic efficacy of phage therapy against chronic lung infection in animal models. Moreover, more extensive *in vivo* PKPD evaluations are needed to investigate the optimal administration dose and time for pulmonary phage therapy.

The role of the immune system on phage therapy is largely unexplored in animal studies and human trials [33, 88]. Depending on administration route, phage type and phage dose, and duration of phage therapy can lead to the generation of neutralizing antibodies [94]. Together with increasing evidences showing the interactions between phage and mammalian cells [95–97], it would be worthwhile to explore the interaction between phage formulations with lung leukocytes and epithelial cells lining the alveolar surface and the conducting airways.

Current phage formulation research is largely empirical based. To speed up the research progress for phage therapy, *in silico* models and database would be required to predict phage-excipient interaction, phage-antibiotic combination and pharmacokinetic/pharmacodynamics (PKPD) profiles.

8. Conclusion

In the past decade, highly acceptable formulations have been achieved with minimal phage loss and desirable stability for pulmonary delivery using both nebulizers and dry powder inhalers. The synergistic effect of the phage-antibiotic combination provides an efficient way to prevent the emergence of bacterial resistance and reduce the toxicity of antibiotic use. However, systematic PKPD profile of phage after administration by inhalation, and the modern tools to accurately predict the result of combination therapy are still pending. With the advent of phage research, the sound manufacturing and regulatory guidelines towards successful clinical trials to bring phage therapy to clinical settings will be beneficial to the patients suffering from bacterial infections.

Acknowledgements

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