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A commentary on the development of engineered phage as therapeutics

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The use of engineered phages offers a unique opportunity to improve on wild-type (WT) phages to generate ever more successful therapeutics to combat bacterial infections. Here, we discuss how phage engineering could be used to overcome some of the technical challenges of phage therapy, and suggest some areas in which more research will be crucial to the development of further novel phage therapeutics.

Keywords: Wild type bacteriophages; Engineered bacteriophages; Bacteriophage therapy; Phage cocktails; Regulatory approval; Genetically modified organisms; Spectrum of activity; PK/PD; Stability

Introduction

Bacteriophages (phages) have a varied history of use as therapeutic agents to treat bacterial infections. In recent years, substantial progress has been made in the evolution and refinement of phage therapy, with the development of engineered phages as therapeutics themselves and/or as delivery vehicles for therapeutic agents. Here, we explore and opine on some of the drivers for this increased interest in WT phage therapy and discuss some of the associated technical therapeutic challenges and how these could be overcome by phage engineering, particularly of tailed, double-stranded DNA phages. A comprehensive review of the current state of the art is provided elsewhere [1].

Antibiotic resistance: an emerging issue

According to the WHO, antibiotic resistance is one of the biggest threats to global health. The phenomenon of antibiotic resistance is not new, and many bacteria have developed resistance mechanisms to combat naturally produced antibiotics from other organisms occupying the same environmental niche. For example, serine beta-lactamases are estimated to have originated more than 2 billion years ago [2]. The constant environmental battle among microbes, and between microbes, and the human use of antibiotics, drives the real-time evolution of antibiotic defence mechanisms, leading to the clinically important antibiotic resistance seen today [3].

Commercial antibiotic development has sought to keep pace with the spread of antibiotic resistance [3]. Next-generation versions of long-used antibiotics, designed to overcome and/or evade existing resistance mechanisms, have been life saving. Unfortunately, a growing number of infections, such as pneumonia and food poisoning, and those caused by specific organisms, including *Clostridium difficile*, *Neisseria gonorrhoeae*, *Mycobacterium tuberculosis*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, are becoming harder to treat because of the increasing prevalence of resistance to available antibiotics.

Use of phages to combat antibiotic-resistant bacteria

Antibiotics are not the only means of treating infections; viruses that target bacteria,

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called bacteriophages (phages), have been used therapeutically for this purpose for > 100 years [4]. The advent of the antibiotic era during the 1930s led to a decline in the use of such phage therapy in many areas of the world; however, given the current backdrop of increasing antibiotic resistance, interest in phages as therapeutic agents is being revived.

It is estimated that there is a total of ~ 10^{31} phages on the planet, and phages are widespread in the environment [1,5,6]. For example, 10^4–10^8 virions/ml can often be found in aquatic systems, and ~ 10^7–10^8 virions/g in soil. The ubiquity of phages illustrates their success, as obligate parasites, at infecting their target bacterial host, replicating, and, ultimately, killing the host to liberate progeny phage. Importantly, these abilities of phage are largely unaffected by the antibiotic resistance profile of the target bacteria, making phage therapy attractive for the treatment of bacterial infections [7]. Phage therapy using naturally occurring or WT phages is expanding, and several institutions and companies are pursuing therapeutic indications [7,8]. Although no drug is without its flaws, the use of phage engineering offers a unique opportunity to improve on WT phage therapy.

Phage engineering methods

Phage engineering is being driven by increased understanding of phage biology, genomics, interaction with host bacteria, and pharmacokinetic/pharmacodynamic (PK/PD) properties, as well as commercial considerations, such as stability, manufacturing, and general 'drugability', to improve phages as therapeutics. It has been enabled by the development of phage engineering methods [1].

Classical phage and microbiological techniques have long been used to generate both lytic and temperate phage mutants, such as through chemical mutagenesis [9]. Phenotypic screening of the resultant mutant libraries, possibly combined with 'phage training', could result in the identification of mutants with beneficial properties [10]. Temperate phages can also be easily modified during the lysogenic phase of their life cycle, using standard selection-based techniques for generating bacterial mutants, then recovering the engineered phage by induction. By contrast, similar methods are difficult to apply to lytic phages, which kill their bacterial host as an obligate part of their life cycle, making the selection (and, therefore, isolation) of modified lytic phages complex. However, recent advances have led to the development of more sophisticated methods of precision phage genome engineering by rational design, particularly of lytic phage; many of these methods are equally applicable to temperate phage.

The Bacteriophage Recombineering with Electroporated DNA (BRED) method is one means of precision phage engineering [11]. BRED relies on the addition of a recombineering system to a suitable phage host, which is then co-electroporated with phage genomic DNA and DNA sequences carrying regions of homology flanking the mutation(s) of interest. Further screening identifies recombinant phage carrying the desired engineered genome. Another example is the Host Range Determinant Selection (HORDS) method, which uses phage host range determinants (HRD), such as tail fibres, as selectable markers for phage engineering [12]. HORDS is particularly useful for engineering obligately lytic phage, because it uses positive selection for a characteristic inherited by the phage in its lytic state, eliminating much of the laborious screening that can be required when using methods that do not involve selection.

Other approaches to phage modification include the assembly of an engineered phage genome from DNA fragments *in vitro*, followed by recovery of the engineered phage via transformation of a suitable bacterial host [1]. Cell-free transcription/translation (TXTL) systems can also be used to assemble phage particles from phage genomes engineered *in vitro* [1]. Alternatively, phage genomes can be captured and engineered on yeast expression vectors, with the engineered phage particles recovered following transformation of a suitable bacterial host [1]. CRISPR/Cas-mediated phage engineering has also been developing in recent years [1]. For example, a type I-E CRISPR/Cas system has been used to engineer phage T7 [13]. The field of bacteriophage engineering methodology is rapidly developing, increasing opportunities for phage engineering to generate improved phage therapeutics, some of which are highlighted herein.

Engineering phage: temperate versus lytic

For WT phage therapy, obligately lytic phages have generally been favoured over temperate phages because of their ability to potentially kill all sensitive target bacteria and, perhaps most importantly, to avoid the potential for lysogenic conversion [8,14]. However, it may be easier to identify temperate phages with suitable host ranges to treat a bacterial infection of concern, and this may be a useful trade-off [14]. Phage engineering can also be used to counter some of the disadvantages associated with temperate phages. For example, Dedrick *et al.* reported a cocktail of three phages (two of which were temperate phages engineered by BRED to render them obligately lytic) that was recently successfully used to treat a disseminated *Mycobacterium abscessus* infection following lung transplant, in a patient with cystic fibrosis [15].

Phage therapy challenges and engineered phage solutions

Spectrum of activity

One key phage therapy issue is that WT phages often have a narrow host range within their target species, with a resulting narrow spectrum of bactericidal activity [1]. For WT phages, the historical and established route to circumvent limited host range is the use of phage cocktails. This approach has been used with clinical success, such as the highly tailored WT phage cocktail used under emergency use authorisation to treat an antibiotic-resistant *A. baumannii* infection [16]. This tailored cocktail was effectively a 'single-use' therapeutic. However, as a commercial strategy, this tailored approach brings challenges: the cost of goods for one patient are potentially high and add to the complexity of requirements to manufacture each phage to Good Manufacturing Practice (GMP), to store under GMP conditions and then mix phages as required for each individual patient infection. For a broader spectrum WT phage therapeutic suitable for treatment of multiple cases, it is often necessary to increase the number of phages in the cocktail, a strategy that was used in the Phagoburn study, which assessed WT phage therapy in burns patients with *P. aeruginosa* infections [17]. Such cocktails often contain tens of different phages, each of which

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overcomes a phage defence mechanism present in the target bacterial population.

However, although phage cocktails might solve the issue of narrow spectrum of activity, their use brings different potential challenges. Individual phages in the cocktail might exhibit different pharmacokinetic and pharmacodynamic (PK/PD) profiles and, thus, might require different doses or dosing regimens; phages might behave differently when co-administered rather than individually administered; manufacturing might need to be tailored to each phage in the cocktail, resulting in significant extra time and cost investments, and associated risks. Additionally, a method for distinguishing and enumerating the biologically active phage particles present for each of the phages in the cocktail is essential. The classical method of phage enumeration estimates titre by plaque assay; however, this requires identification of host strains that distinguish between each of the phages, and the larger the cocktail, the more difficult this becomes. Other techniques involving facile detection of phage genomic DNA do not distinguish between inactive and active phage particles, or naked and phage-packaged DNA. Thus, there is a real need in the field of WT and engineered phage therapy for the development of novel methods for determining phage activity.

There are several mechanisms that dictate the spectrum of activity of a phage, including the loss, diversity, or masking of phage receptors in the bacterial host (adsorption resistance), the presence of phage-encoded repressors leading to superinfection immunity, DNA restriction mechanisms, the bacterial adaptive immune system that is CRISPR/Cas, and the production of a variety of intracellular proteins that cause phage infection to be aborted [18]. Each resistance mechanism offers an opportunity to engineer improved phage therapeutics that then evade it.

For example, adsorption resistance can be overcome by engineering phage using alternative HRD that recognise a broader, or altered, selection of bacterial hosts. This increases feasibility of a single phage therapeutic, or of much smaller phage therapeutic cocktails containing related phage. As an example, SASPject PT3.9 from Phico Therapeutics uses a mix of three engineered phages that target *P. aeruginosa*, for use as an intravenous therapeutic [19]. Unlike Dedrick *et al.*'s three-phage mix [15], Phico's three *P. aeruginosa* phages are 99% identical to each other, being engineered from one lytic phage, changing the HRDs and incorporating a small acid-soluble spore protein (SASP) gene [19]. This strategy is reported as conferring a 90% host range activity against > 500 geographically diverse *P. aeruginosa* clinical isolates [20].

Alternatively, a phage can be engineered such that it is only necessary for the phage to bind to, and inject its DNA into, target bacteria to bring about cell death. This strategy can help the engineered phage to evade some, or all, of the intracellular phage resistance mechanisms that act downstream of phage infection. One way of achieving this is to engineer the phage for use as a gene delivery vehicle, for example for components of the CRISPR/Cas bacterial immune system [1].

There are multiple types of CRISPR/Cas system, but CRISPR-Cas9 and CRISPR-Cas3 in particular have made a significant contribution to the engineered phage approaches in commercial development. Phages engineered to deliver CRISPR/Cas9 systems programmed to target specific bacterial DNA sequences cause lethal cleavage of the bacterial DNA during the phage infection process [1]. This can be applied in many ways: for example, Nemesis Bioscience engineered phages to deliver a conjugative transmid carrying CRISPR/Cas9 targeted to antibiotic resistance gene sequences. Thus, antibiotic-resistant bacteria are targeted for killing, and bacterial survivors have been shown to have lost the antibiotic resistance locus and have antibiotic susceptibility restored [21]. Eligo Bioscience's 'Eligobiotics' uses nonreplicative 'particules' engineered from phage, to deliver a CRISPR/Cas9 payload that targets sequences specific to microbiome target bacteria, including antibiotic-resistant bacteria [22–24]. Locus Biosciences is also developing phage delivery vectors, but using the CRISPR/Cas3 system [25], and a Phase Ib clinical trial using engineered *Escherichia coli* 'crPhage™' for treatment of urinary tract infections has been successfully completed [26].

Phages can also be engineered to deliver toxins: Phico's SASPject technology delivers a gene encoding an antibacterial protein (SASP) to target bacteria [27], where SASP binds to, and inactivates, bacterial DNA in a DNA sequence-independent manner. SASPject PT3.9 targets *P. aeruginosa*, whereas SASPject PT1.2 targets *S. aureus*. PT1.2 has been used in a successful Phase I clinical trial for the nasal eradication of *S. aureus*, including methicillin-resistant *S. aureus* (MRSA) [28].

Phage stability

To have commercial potential, therapeutic phage preparations need to retain biological activity during storage, potentially up to 2 years. This is particularly pertinent when separate phages are mixed to produce a product, for which it is crucial to ensure that the phages do not interact and that all remain individually viable. Instability was a key factor that led to the premature halting of the Phagoburn clinical study: the daily dose of a cocktail of 12 *P. aeruginosa* phages dropped to 10^1–10^2 pfu/ml from the planned 10^6 pfu/ml [17]. An understanding of factors leading to phage stability/instability is an exciting area of research that has the potential to increase the repertoire of efficacious engineered phages available for therapeutic use.

PK/PD properties

Other important considerations for a phage therapeutic are its PK/PD properties [29]. The half-life of the phage at the target site of the infection and its biodistribution profile both impact the efficacious phage dose and the frequency of administration required for successful therapy. Phages can exhibit widely varying half-lives, which can limit their use as therapeutic agents. However, phages can be chemically modified or encapsulated to aid stability during storage and/or administration [1,30]. There are also examples of genetically modified phages that show improved PK/PD characteristics, for example, the 'long-circulating phages' [29]. This is another important area of phage engineering that could help to broaden the scope of phage therapy.

Concluding remarks

As discussed herein, there are multiple approaches to engineering phages for improved 'phage therapy' uses: as antibacterial agents themselves, as virions delivering antibacterial agents, and as delivery

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agents for mechanisms that regenerate or preserve sensitivity to existing conventional antibiotics. Each of these approaches brings their own challenges, whether it is forging new regulatory pathways, or the challenges of producing robust and reproducible manufacturing processes that support a range of routes of administration, through to dealing with potential immune inactivation. Despite the potential advances of engineered phages, some of the issues that face WT phage therapy will also undoubtedly apply here. Additionally, engineered phages that include non-native DNA sequences are genetically modified organisms (GMO) and, as such, might be subject to different regulatory requirements and considerations compared with WT phages, such as considerations of release of a GMO into the environment from treated patients. Engineered phage developers need to work with regulators to develop clear and manageable paths to approval, and work to educate clinicians and industry on their use, to enable the safest and most clinically useful outcomes for patients. The commercial and therapeutic applicabilities of phage therapy still attract controversy, partly because it has yet to be broadly exemplified in properly controlled clinical trials. Multiple robust, and successful, clinical trials are now urgently needed to help realise the full potential of phage therapy. Engineered phages offer the opportunity to improve on the best WT phage that nature can offer, and engineered phage technologies are leading the way in establishing the clinical credentials of phage therapy. Phage engineering methodologies are also providing many options for the creation of novel therapeutics to combat bacterial infections. These improved, engineered phage have the potential to establish a new paradigm for 'treating the untreatable' and combatting antibiotic resistance.

Declaration of Competing Interests

A.M.L.B. is Molecular Biology Manager at Phico Therapeutics Ltd. and an inventor on European patent EP3201323. H.I.M.F. is Founder and CEO of Phico Therapeutics Ltd. and an inventor on European patents EP3201323 and EP3340200.

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