Bacteriophages and Lytic Enzymes

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Both phages and their lytic enzymes are now widely considered as safe and have now progressed to clinical phase II to show clinical efficacy as pharmaceutical.

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1. Phages and Phage-Inspired Antibiotics

Ever since their discovery, bacteriophages have inspired to be used as antibacterial therapeutics. Whereas initially the use of intact phages has been considered for therapy, intensive research of phage biology has nowadays yielded several other avenues of investigation towards the development of novel antibacterials as well. Indeed, during their replication cycle phages interfere at all stages with the bacterial integrity and viability, providing different clues for novel antibacterials.

Many phages are equipped with polysaccharide depolymerases in their tail fibers or tail spikes. When initiating a phage infection cycle, these enzymes degrade capsule polysaccharides (CPSs), O-polysaccharide chains of lipopolysaccharide (LPS) molecules or extracellular polysaccharides (EPSs) that contribute to a biofilm matrix [1]. Treatment of mice infected with *Klebsiella pneumoniae* with a capsule-specific depolymerase led to complete survival without significant clinical signs of illness, whereas the lack of treatment resulted in a high lethality (87.5%) [2]. Moreover, isolated phages equipped with putative depolymerases or their isolated depolymerases successfully rescued mice and *Galleria mellonella* larvae infected by a hypervirulent *K. pneumoniae* strain, which is a hyper-producer of capsular polysaccharide [3][4][5][6][7]. Additionally, *Escherichia coli* K1, K5 and K30-specific depolymerases were successfully evaluated in a mouse thigh model to treat infections [8]. Depolymerases are proposed to function as antivirulence compounds through the degradation of a major bacterial virulence factor. Encapsulated *K. pneumoniae* cells exposed to recombinant capsular depolymerases become more prone for complement-mediated killing in serum and phagocytosis, resulting in a reduced virulence in a *Galleria mellonella* larvae infection model [4]. In addition, capsule removal by depolymerases can increase the in vivo efficacy of standard-of-care antibiotics [9].

An important class of phage-encoded enzymes with antibacterial potential are phage lytic enzymes [10][11]. To eject the phage genome into the host cell, phages locally degrade the cell wall with a first phage lytic enzyme, called virion-associated peptidoglycan hydrolase. This enzyme creates a local hole in the peptidoglycan layer for transfer of the genome, but its association with the phage particle structure avoids extensive damage of the peptidoglycan layer taking place. A second phage lytic enzyme is produced at the end of the replication cycle. This protein is produced as a soluble, free enzyme and is called an endolysin. At a genetically programmed time point, (pin)holins release the endolysin to the periplasm or activate previously secreted endolysin molecules. These endolysins then extensively degrade the peptidoglycan layer from within, resulting in a sudden osmotic lysis of the bacterial cell and dispersion of the newly matured phage particles [12]. The potential of phage lytic enzymes as antibacterials for use in medicine and food conservation was first described in 2001 and 2000, respectively [13][14].

Besides intact phages and phage-encoded enzymes, also small chemical molecules mimicking growth-inhibitory phage-host interactions have been proposed as novel antibacterials. From the early stage of infection, such phage-host interactions take place to control the host cell machinery and to redirect the cellular resources for phage production. These interactions are often mediated by small proteins, which are among the earliest expressed ones [15]. Thirty-one phage proteins were identified in 26 *Staphylococcus aureus* phages with a growth-inhibitory effect [16]. The specific interaction between a phage protein and the bacterial target DnaI was used to screen for small molecules, mimicking the effect of the phage protein. Insights in the basic biology of phage-host interactions are now drastically accumulating [17][18][19][20], offering further clues for small molecule design.

The first phage-borne depolymerases are now evaluated preclinically, while phage-inspired antibiotics based on phage-host interactions are in the discovery phase with a single small molecule hit selected. In contrast, both phages and lytic enzymes have been demonstrated to be efficient and safe in extensive preclinical studies [21][22][23][24][25]. In addition, the safety of specific phages and lytic enzymes has been proven in human case studies and completed clinical trials phase I. One phase I/II and one phase I trial has been completed with static phage-containing medicinal products (drugs in US), and three phase I trials for phage lytic enzymes. Different clinical phase II or II/III trials have been initiated for phages and phage lytic enzymes. Phage-containing medicinal products (drugs) and recombinant phage lytic enzymes are thus the most advanced phage(-derived) products on the clinical development path for use in human medicine. Both phages and phage lytic enzymes were withheld in a recent pipeline review of alternative antibacterials [26]. Phage lytic enzymes were classified as the alternative with the highest potential on effective implementation for antibacterial therapy. Wild type and engineered phages were also scored high for their potential impact, but ranked lower for their technical feasibility.

2. Phages versus Their Lytic Enzymes as Antibacterials: An Old and Young History

Phages were discovered in the early 20th century. They have been investigated for application in phage therapy shortly after their discovery. The earliest experiments were performed by Felix D'Herelle in 1918, treating a 12-year-old boy with severe dysentery. Twenty years later, the first commercial companies, L'Oréal in Europe and Eli Lily Company in the United States, produced phage preparations for human therapy [27]. The discovery and global use of classical antibiotics as first-line antibacterials led to a large extent, to the abandoning of phage therapy in the west. In contrast, the use of phage therapy persisted in the USSR, even when mass antibiotic production was established in the USSR by 1950. Phage research especially found ground in Georgia, where the Eliava institute (Tbilisi) was founded, which is nowadays still a global key site for phage therapy [28]. The renewed interest in the west is triggered by the global call for novel treatments of bacterial infections because of the spread of multi-drug resistant bacterial isolates and an insufficiently filled development pipeline of new antibiotics. The prospect of phage therapy remains a matter of intense debate between proponents and opponents, awaiting clinical efficacy data.

Phage lytic enzymes have been studied since the late 50 s of the 20th century, with the first biochemical characterizations of the endolysin of streptococcal phage C1 [29], the endolysins of *E. coli* phages from the T-series (T1 up to T7), selected by Max Delbrück in an effort to focus the global work of phage researchers on a standard set of phages [29][30][31][32], and *E. coli* phage λ [33]. The T4 lysozyme was a model protein for the study of protein folding [34]. In addition, the lytic activity of endolysins from phages infecting Gram-positive bacteria was thankfully used to lyse Gram-positive cells for the study of wall carbohydrate and protein components or to produce protoplasts. Only since the beginning of the 21st century, the interest in the use of phage lytic enzymes as enzyme-based antibiotics or "enzybiotics" has emerged [13][35], and the first companies started focusing on phage lytic enzymes about 10 years later.

In spite of the longer history of phage therapy, reflected by a high number of studies addressing phage therapy compared to therapy with phage lytic enzymes, the (pre)clinical evaluation of phage lytic enzymes has obviously advanced faster. While both classes of antibacterials are clearly different from existing classes of antibacterials, the standards used in the preclinical analysis of small molecule antibiotics could be more easily translated to the preclinical evaluation of phage lytic enzymes compared to phages. Furthermore, the availability of a platform for protein production, engineering and formulation into different dosage forms for an increased number of proteinaceous products (enzymes, hormones and monoclonal antibodies) registered annually to the market will facilitate entry of the phage lytic enzymes to the market [36]. Some of the unique features of phages that may leverage the therapeutic potential (discussed below) also represent hurdles that have to be tackled, resulting in a slower process featured by gradually proceeding insights to develop phages as successful antibacterials.

3. Bacteriophages can Replicate and Evolve

The replicative and evolvable nature of phages has been highlighted as a unique feature in terms of therapy. After infection of—and replication in—a bacterial host, a multifold of new phage particles (burst size) are produced. Yet, it has been shown that the bacterial cell number should be higher than the proliferation threshold to sustain an active multiplication. This proliferation threshold is a function of the rate at which a phage meets a bacterium, the burst size of the phage, and phage decay through inactivation or removal by the reticulo-endothelial system of spleen and liver. In other words, phage amplification is only able to compensate for phage decay above this threshold. Below this threshold, the doses of phage particles must be sufficiently high (a multiplicity of infection of 10) to ensure killing of every cell without relying on self-replication [37][38]. When active replication takes place, phages are also able to evolve by the accumulation of stochastic mutations. In combination with natural selection, this will result in co-evolved phages that respond to the

development of resistance by the target bacterium. Indeed, bacteria have evolved an extensive array of mechanisms to protect themselves from phage infection, ranging from adsorption inhibition, superinfection exclusion systems, restriction-modification systems, CRISPR-Cas mediated immunity, inhibition of crucial steps of phage multiplication [39] to the recently discovered chemical molecules [40]. Bacteria are not known to have developed natural resistance against phage lytic enzymes. Infected cells are already getting resource-depleted before phage endolysins come into play. Resistance mechanisms against the earliest stages of phage infection therefore appear as most meaningful.

The potential of replication and evolution set phages apart from any other antibacterial, but consequently also apart from any existing regulatory framework that exists for the approval process of clinical trials and eventually their approval as medicinal products. The advantages of replication of phages thus also represent a significant delaying factor in the preclinical and clinical evaluation of their potential. Phage lytic enzymes do not replicate. They must be applied in sufficiently high doses as any other antibiotic to kill the bacteria before they are removed from the body.

4. Engineered Phages and Phage Lytic Enzymes

Using a biological such as a phage or its lytic enzymes in human medicine benefits from different traits that have evolved during natural Darwinian evolution. Typically, engineering efforts aim to perform directed evolution on a lab scale to improve the characteristics of the biological. In casu, synthetic biology and protein engineering are used to increase the therapeutic potential of phages and lytic enzymes, respectively. In fact, these efforts are similar to the extensive chemical engineering of natural antibiotics, resulting nowadays in up to the 4th generation semi-synthetic antibiotics. For phage lytic enzymes, the large, existing toolbox of protein engineering methods can be used. The most commonly used method is domain swapping [11]. Shuffling of the modular composition of phage lytic enzymes comprising cell wall binding and enzymatically active domains allows for improvement of antibacterial properties such as specificity, activity, stability, and solubility. Additionally, fusion of additional modules expands or modulates their activity. Fusion of outer membrane permeabilizing peptides to phage lytic enzymes (Artilysin®) sensitizes Gram-negative pathogens for their bacteriolytic action [41][42]. Addition of a polycationic peptide also increased and accelerated the bactericidal effect, while reducing the required dose, for a streptococcal endolysin [43]. Compared to many other commercially available enzymes, the potential of mutagenesis has merely been exploited for phage lytic enzymes [44].

Phage engineering, especially of lytic phages, has been more cumbersome. Phages have been engineered by a wide range of methods, yet with increasing efficiency along with the emergence of synthetic biology [45][46][47]. Phage engineering has offered a way to produce new variants with expanded host range and, hence, potentially decreasing the number of phages in the cocktail needed to cover bacterial diversity [45][48][49]. It has also provided an approach to attract investment by generating patentable phage variants [49]. Phages have been also engineered to allow killing of other strains and increase efficiency against biofilm forming bacteria by insertion of bacteriocins, enzybiotics, quorum sensing inhibitors, and biofilm degrading enzymes [50][51][52][53][54]. Purification efficiency could also be improved by insertion of purification tags [55]. Longer circulation of the phage in the bloodstream has been achieved by displaying a specific protein [56][57]. Finally, phage engineering has also been used to enhance the cell-internalization efficiency, to achieve targeted delivery [58][59] and to generate non-replicative bacteriophages to control their spread and for an immune-safe product [60][61][62]. The large majority of these engineering efforts are focused to eliminate the hurdles of phage therapy. Though, since phages have a genome and are replicative, the engineering of phages may raise itself additional legislative and ethical concerns related to genetic modification. In contrast, engineered phage lytic enzymes will not elicit these concerns.

References

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