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Chapter 2

Phage Therapy: The Pharmacology of Antibacterial Viruses

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Abstract

Pharmacology can be differentiated into two key aspects, pharmacodynamics and pharmacokinetics. Pharmacodynamics describes a drug's impact on the body while pharmacokinetics describes the body's impact on a drug. Another way of understanding these terms is that pharmacodynamics is a description of both the positive and negative consequences of drugs attaining certain concentrations in the body while pharmacokinetics is concerned with our ability to reach and then sustain those concentrations. Unlike the drugs for which these concepts were developed, including antibiotics, the bacteriophages (or 'phages') that we consider here are not chemotherapeutics but instead are the viruses of bacteria. Here we review the pharmacology of these viruses, particularly as they can be employed to combat bacterial infections (phage therapy). Overall, an improved pharmacological understanding of phage therapy should allow for more informed development of phages as antibacterial 'drugs', allow for more rational *post hoc* debugging of phage therapy experiments, and encourage improved design of phage therapy protocols. Contrasting with antibiotics, however, phages as viruses impact individual bacterial cells as single virions rather than as swarms of molecules, and while they are killing bacteria,

bacteriophages also can amplify phage numbers, *in situ.* Explorations of phage therapy pharmacology consequently can often be informed as well by basic principles of the ecological interactions between phages and bacteria as by study of the pharmacology of drugs. Bacteriophages in phage therapy thus can display somewhat unique as well as more traditional pharmacological aspects.

Introduction

The words 'drug' and 'chemotherapeutic' often can be used interchangeably. Traditionally this has been especially so when drugs were employed to combat roguely replicating entities such as cancer cells, pathogenic bacteria, and viruses. More generally, a drug can be viewed as a biologically active but non-food substance that provides some benefit to the body. What medical drugs and chemotherapeutics have in common is that both supply therapeutic value, while historically only chemotherapeutics strictly have been 'chemicals'. Drugs, especially defined more generally as medicaments, thus existed prior to the medical use of purified organic chemicals – such as Ehrlich's anti-syphilis chemotherapeutic, 'Salvarsan' (Lloyd et al., 2005; Zaffiri et al., 2012) – and the word 'drug' itself seems to have originated as a reference in French to medicaments sourced from nature, i.e., to dried herbs (Harper, 2020). Here we similarly define 'drug' broadly to include not only purified small molecules such as antibiotics but also less pure or more complex entities such as antibacterial viruses.

Bacteriophages, or phages, are the viruses of bacteria (Calendar and Abedon, 2006; Hyman and Abedon, 2012; Lehman, 2018). As phages can be biologically active within bodies, they also can be considered to be drug-like, i.e., as medicaments. This drug-like activity stems predominantly, though not exclusively, from phages infecting and subsequently killing and lysing body-associated bacteria, and the application of phages to bodies to combat bacterial infections is called phage therapy. With phages serving as drug-like entities, phage therapy therefore can be framed in terms of the science of pharmacology, which is the study of drug-body interactions. Phages as replicating entities, however, also are ecological in their interactions with bacteria (Abedon, 2014b; 2018a). Here therefore we review basic principles of pharmacology as

applicable to phage antibacterial therapy but with emphasis as well on relevant aspects of phage-bacterial ecological interactions.

Pharmacology

Pharmacology is the study of drug functionality. This includes issues of drug design or discovery as well as development of drug delivery strategies that are effective, convenient, minimally invasive, and relatively inexpensive (Goth, 1984; Mycek et al., 2000). As a basic premise, a drug typically will display greater functionality *in vivo* when its concentrations are higher, but at the same time also will display greater negative effects. The general goal then is to sustain drugs at concentrations *in vivo* that result in substantial positive effects while choosing drugs whose negative effects at those same concentrations are minor. In terms of drug choice, pharmacological considerations therefore can be greatly simplified if a drug displays both low toxicities and a relative lack of other side effects at otherwise efficacious *in situ* concentrations. A drug that has a toxic concentration that is substantially higher than its effective concentration in particular can be taken at higher doses and/or with longer intervals between doses than a drug that does not have this property. A drug for which this so-called therapeutic *window* or *index* is smaller, by contrast, may require constant patient monitoring, and particularly so if a drug's side effects are severe (Blix et al., 2010; Pereira and Kelley, 2011; Muller and Milton, 2012).

The science of pharmacology traditionally has been differentiated into two major components, pharmacodynamics and pharmacokinetics. Primary pharmacodynamic effects are the positive impacts of a drug, i.e., efficacy, such as the bactericidal activity of an antibacterial agent. Secondary pharmacodynamic effects instead include negative consequences, i.e., side effects or toxicities (Lees et al., 2004; Marino and Zito, 2020). These side effects often can be emergent properties, however (Curtright and Abedon, 2011). That is, because of the complex interactions between drugs and bodies, it can be difficult to predict the occurrence of drug side effects based solely on drug chemistry or even pre-clinical analysis. Drug development thus typically will necessitate extensive clinical safety determinations before such secondary pharmacodynamic effects can be either identified or ruled out. A great deal of pharmacological research

consequently is devoted to a combination of identifying agents that can potentially deliver primary pharmacodynamic effects and then testing those agents for both efficacy and side effects.

Pharmacokinetics, contrasting pharmacodynamics, is the study of the reaching and sustaining of effective drug concentrations at sites of drug activity (Levison and Levison, 2009). Aspects of pharmacokinetics traditionally have been differentiated into four components, known as absorption, distribution, metabolism, and excretion. Particularly given systemic drug delivery, as opposed to topical application, the 'life cycle' of a drug is one of uptake into the systemic circulation of blood (*absorption*, meaning for the drug to become part of the body), movement out of the blood and into the tissues where it can then have an effect (*distribution*), reductions in blood densities as a consequence of *metabolism* (chemical breaking down of the drug, often by the liver), and *excretion* of otherwise intact drug, typically as effected by the kidneys. Absorption and distribution together describe especially the spatial delivery of a drug to sites in the body where it will display its primary pharmacodynamic effects. A summary of these concepts is provided in Figure 1.

Pharmacological development should be as applicable to phages, as phage-therapy effecting medicaments, as to drugs generally. At the same time, however, a number of characteristics of phages as antibacterial agents seem to have conspired to result in traditionally less formal pharmacological development for phages than has been the case for typical antibiotics. The reasons for this often-reduced phage therapy pharmacological emphasis include simply the long history of phage therapy, the typically low toxicity of phages as therapeutics, and likely as well the potential for phages to increase their numbers *in situ* in the course of effecting their antibacterial activities (Dąbrowska et al., 2018; Dąbrowska and Abedon, 2019). Notwithstanding these useful phage characteristics, approaching phage therapy development from a pharmacological perspective, as this chapter emphasizes, can be helpful toward improving upon phage therapy successes.

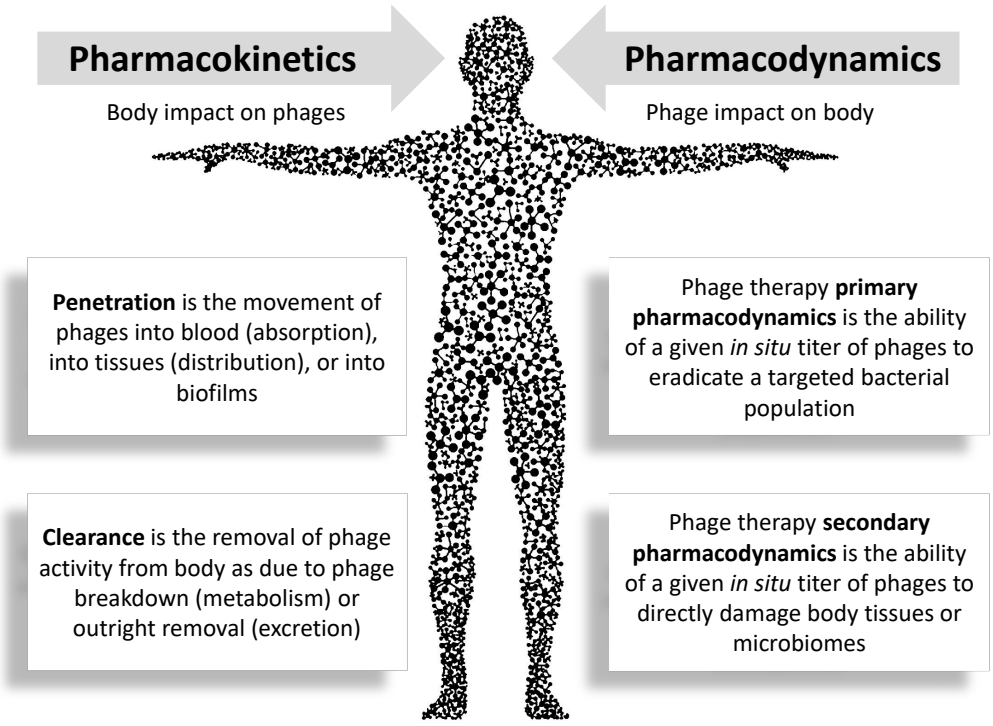

Figure 1. Phage therapy pharmacology. Pharmacokinetics traditionally consists of what are described as absorption, distribution, metabolism, and excretion. These are all things that contribute to drug *in situ* concentrations and are all consequences of a body's impact on a drug (with the concept of 'body' traditionally also including associated microorganisms). For phage therapy, we can also add the ability of phages to replicate, also *in situ*. Pharmacodynamics represents the impacts of a drug on the body, especially as a function of drug *in situ* concentration, and can be distinguished into primary and secondary effects. Primary effects are intended drug effects, such as phage bactericidal activity, while secondary effects are unintended and most notably include toxicities and side effects.

For discussion of advantages of employing phages as antibacterial agents, particularly in comparison to antibiotics, see (Sulakvelidze et al., 2001; Sulakvelidze and Kutter, 2005; Häusler, 2006; Kutter, 2008; Curtright and Abedon, 2011; Loc-Carrillo and Abedon, 2011; Nobrega et al., 2015; Wienhold et al., 2019). For review of the specifics of phage therapy pharmacokinetics, see (Dąbrowska, 2019; Dąbrowska and Abedon, 2019; Matsuzaki and Uchiyama, 2019). For an earlier version of this chapter, presenting additional topics as well as more mathematical detail, see Abedon (2014a) and for access especially to the older phage therapy literature, see Alves and Abedon (2017). Table 1 provides a glossary of terms.

Table 1. Concepts and terms relevant to phage therapy pharmacology.

Term	Definition
Abortive infection	Phage infections that result in death of both the infected bacterium and the infecting phages, i.e., these are bactericidal infections that do not result in virion production; phage abortive infections are often also bacteriolytic but in principle need not be
Absorption	Movement of drug into the blood following dosing; not a*d*sorption
Active penetration	Layer-by-layer phage clearance of bacterial biofilms as mediated by active phage replication, or at least as associated with phage-mediated bacterial lysis
Active treatment	Clearance of bacterial populations *in situ* that is dependent upon auto dosing
Adsorption	Strictly the attachment of a phage to a bacterium but also typically describes as well the local movement of phages to the vicinity of targeted bacteria
Auto dosing	Drug dosing that is *not* a consequence of extrinsic application; phage *in situ* increases in titres that are a consequence of phage replication; a.k.a., self amplification
Bactericidal infection	Phage infection that results in bacterial death, and which also may result in bacteriolysis and virion production but doesn't necessarily
Bacteriolytic infection	Phage infection that results in bacterial death and phage-induced bacterial lysis though does not necessarily always also result in virion production, e.g., see also abortive infection
Bacteriophage translocation	Defined after the concept of bacterial translocation, describes the potential for phages to reach systemic circulation particularly starting from the gut
Body	Body tissues as well as associated microflora
Decimal reduction time (D-value; *D*)	The length of time required to reduce viability of target microorganisms by 90% as a result of antimicrobial application of a given type, density, and under a given set of conditions
Distribution	Drug movement out of systemic circulation and into other body tissues
Dosing	Mechanism initiating the elevation of drug concentrations *in situ*; here "application" and "delivery", both as resulting in dosing, are used synonymously
Efficacy (1)	Positive outcome of an antibacterial procedure, e.g., such as reductions in numbers of targeted bacteria; this is the definition of efficacy used here, and this is rather than definition (2)"

Efficacy (2)	Inherent ability of a drug to produce an effect following binding to its specific target, which for phages would be bacteria killing following adsorption; this definition of efficacy is *not* employed in this chapter other than in this table entry
Excretion	Drug removal from the body as typically mediated by kidneys
Free phage	Phage virions that are mature, functional, and found outside of bacteria, i.e., that state that phages are in when they are able to adsorb bacteria
Inundation threshold	Phage density required to exactly balance bacterial replication with bacterial deaths, the latter including as mediated by phages
Inundative densities	*In situ* phage titres sufficient to achieve adequate bacteria killing over reasonable time frames, though with 'adequate' and 'reasonable' not explicitly defined
Killing titre	Measure of phage density based on bacteria killing ability; assumes adsorption by approximately 100% of the adsorption-competent phage population that is present in the vicinity of a targeted bacterial population
Lytic phage	A phage that, at the end of its infection period, lyses its bacterial host, resulting in the death of its host bacterium and loss of continued infection productivity; as most temperate phages are also lytic phages, the concept of lytic phage contrasts instead with that of chronically (continuously) released phages, e.g., such as the filamentous phage M13
Metabolism	Chemical modification of drugs typically resulting in drug inactivation though in the case of phages can instead result in both activation and auto dosing; inactivation for phages can include as due to immune system action
Minimum effective concentration	That drug density, as measured at the site of drug action, that achieves desired levels and rates of bacteria killing
Minimum toxic concentration	That drug density as measured *in situ* at which toxicity is apparent
Multiplicity of infection (MOI)	Strictly the ratio of *adsorbed* or infecting phages to target bacteria; too often used incorrectly as synonymous with the ratio of *added* phages to target bacteria; by either measure, MOI should never be employed as the sole dosing descriptor during phage therapy
MOI_{actual}	Ratio of adsorbed phages to targeted bacteria; the historical meaning of MOI

MOI_{input}	Ratio of added phages to target bacteria; under conditions of higher bacterial densities MOI_{input} can approximate MOI_{actual}, but this is not the case at lower bacterial densities, or given slower rates of phage virion adsorption
Null	Phage-bacterial interactions that do not result in bactericidal activity
Passive treatment	Clearance of bacterial populations *in situ* that occurs without dependence on auto dosing
Penetration	Phage movement to the vicinity of bacteria including as mediated by bacterial lysis
Pharmacodynamics	Drug impact on bodies
Pharmacokinetics	Body impact on drugs; these are the factors impacting the attainment of drug densities necessary to effect primary pharmacodynamic effects
Pharmacologically emergent property	Especially toxicities and side effects associated with a drug that only become apparent upon clinical testing; pharmacologically emergent properties are a bane of drug development because by definition they only become obvious somewhat late in the drug development process; phages for phage therapy, unlike small-molecule drugs, tend to not display pharmacologically emergent properties
Primary pharmacodynamic effects	The positive impact of drugs on bodies, ideally resulting in efficacy (1)
Professionally lytic	Description of a phage that is both strictly lytic and which is not recently descended from a temperate phage; a virulent mutant of phage λ, for example, may be strictly lytic but would not be professionally lytic; to the extent that the encoding of bacterial virulence factor genes is associated only with temperate phages, then professionally lytic phages are not expected to encode bacterial virulence factor genes
Restrictive infection	Phage infections that result in death of the infecting phage but not in death of infected bacterium; these are infections are null rather than bactericidal, bacteriolytic, or virion productive
Secondary pharmacodynamic effects	Other than efficacy (2) effects, including the negative impact of drugs on bodies, i.e., toxicity, side effects, or destruction of normal flora bacteria
Strictly lytic	Description of a phage that both releases phage progeny via lysis and is unable to display lysogenic cycles; strictly lytic phages are not temperate, and can also be described, e.g., as obligately lytic.

Temperate phage	Phage that is able to initiate non-defective, ongoing, and metabolically active infections that produce no virion progeny but instead generate bacterial lysogens; temperate phages can carry bacterial virulence factor genes and bacterial lysogens typically display an acquired resistance to the associated temperate phage (or phages); note that the term "lysogenic phage" is a misnomer and should never be used
Titre	Phage density, usually as provided in per mL units; titres are the preferred phage dosing unit of measurement, with dosing ideally reported in terms of both volumes applied and titres of phages found within those volumes
Trough concentration	Minimum drug concentration experienced between dosing
Virion production	The maturation and release of progeny phage virions, which for lytic phages are products of bacteriolytic infections

Primary Pharmacodynamics

A drug's most salient feature is its primary pharmacodynamics, i.e., its positive effects. Positive effects will not come about, however, until sufficient drug concentrations are present in target tissues, which in the case of antibacterials are those tissues associated with targeted bacteria. With phages, these sufficient densities can be achieved by either of two approaches (Payne and Jansen, 2003; Cairns et al., 2009; Abedon and Thomas-Abedon, 2010; Letarov et al., 2010). These are (i) as following phage application to bodies (so-called passive treatment) or (ii) via a combination of phage application and *in situ* amplification of phage numbers (so-called active treatment). In this section, we discuss the basics of phage dosing in terms of impact on bacteria such as toward microbiologically positive outcomes. For extensive mathematical treatment of this issue, see Abedon (2011a). For less mathematically intensive explorations of these concepts, see Abedon (2012a; 2018b). Different routes of phage application are considered further below.

Another important consideration of phage therapy primary pharmacodynamics is that of phage host range and associated bacterial resistance (Dąbrowska and Abedon, 2019), though this issue is not addressed here. An additional

consideration, also not further developed here, is the potential for phages to display synergistic, additive, or antagonistic interactions (Chaudhry et al., 2017) with other phages, or instead with other substances that phages may be delivered in association with, i.e., such as antibiotics (Chanishvili et al., 2001; Weber-Dąbrowska et al., 2003; Kutter, 2008; Chanishvili et al., 2009; Cooper et al., 2018; Torres-Barceló and Hochberg, 2016; Abedon, 2019a; Knezevic and Sabo, 2019; Segall et al., 2019; Tagliaferri et al., 2019). Commercially as well as practically, it is also highly relevant that bacterial resistance to antibiotics is not known to result generally in cross-resistance to phages, e.g., (Sulakvelidze, 2005; Międzybrodzki et al., 2012).

Phage therapy primary pharmacodynamic impacts

The primary pharmacodynamic impact of phages, with only few exceptions, occurs during phage infection of targeted bacteria. Exceptions include where phages are employed more as drug delivery vehicles, e.g., (Yacoby and Benhar, 2008; Clark et al., 2012; Peng et al., 2020), in which case only phage adsorption but not infection is required. For the sake of avoiding ambiguity, we therefore note that the words ‘adsorption’ and ‘infection’ for phages should generally not be used interchangeably (Abedon, 2015a). Phage infection of bacteria thus begins with phage adsorption, but this is then followed by phage-genome translocation into the bacterial cytoplasm, and only then does phage infection proper start. Pharmacologically, four often not mutually exclusive outcomes therefore can result from phage application. These we described as null, bactericidal, bacteriolytic, and virion productive (Abedon, 2019a) (Figures 2 and 3), with the fourth category, especially, a pharmacokinetic rather than pharmacodynamic outcome.

Null phage impacts on targeted bacteria

Null, at its simplest, is a phage's inability to adsorb a targeted bacterium. Also in this category, however, are restrictive phage infections, defined here as infections that result in phage inactivation but not in bacterial death (Hyman and Abedon, 2010). Phage restriction occurs such as due to the action of bacterial restriction endonucleases (Korona et al., 1993) or instead CRISPR-Cas systems (Kumar et al., 2015; Strotskaya et al., 2017). Another sort of phage

restriction is seen with secondary adsorption, which is the adsorption by phages to already phage-infected bacteria (Abedon, 2015a) and can result in superinfection immunity or superinfection exclusion (Abedon, 1994; Berngruber et al., 2010; Blasdel and Abedon, 2017; Domingo-Calap et al., 2020). Both are mechanisms that kill adsorbing phages and, if expressed by bacterial lysogens, are associated as well with survival of the phage-adsorbed bacterium. Alternatively is simply the initiation of lysogenic or pseudolysogenic infection cycles by infecting phages, i.e., reductive phage infections (Abedon, 2020b), as these at least in the near term also don't result in bacteria killing. Null phage infections generally are of little utility in terms of phage therapy primary pharmacodynamics.

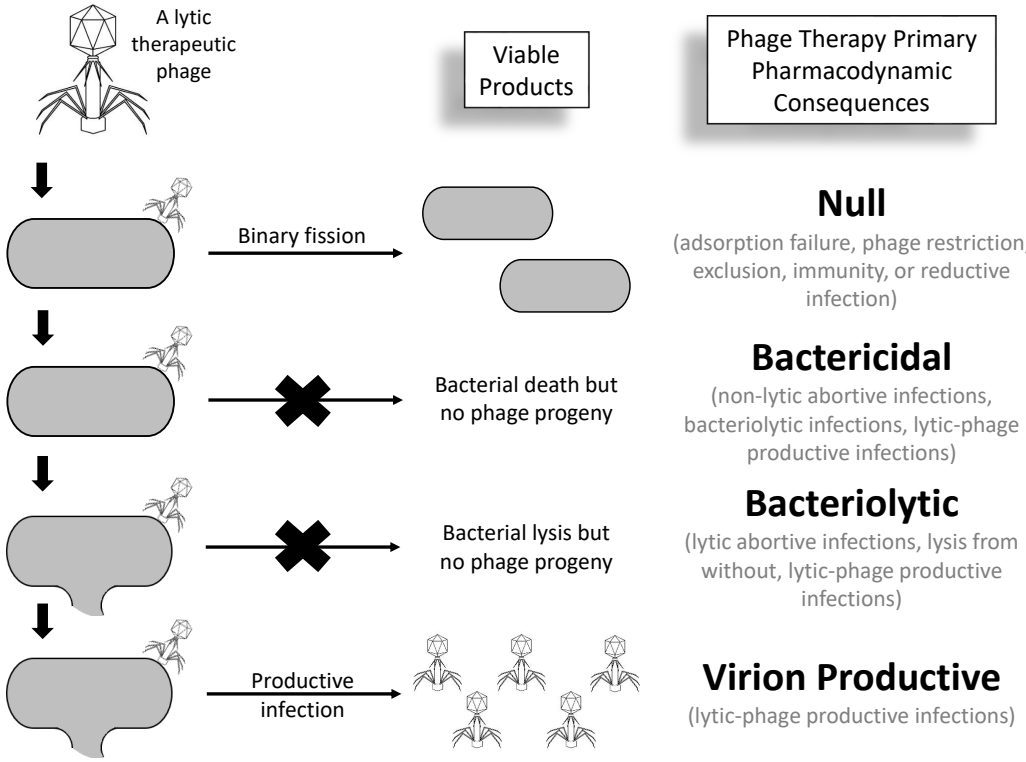

Figure 2. Categories of primary phage therapy pharmacodynamic effects. These are null, bactericidal, and bacteriolytic, which represent increasing phage impacts observed in the course of treatment of targeted bacterial populations. Virion production is shown as well, though this is a pharmacokinetic rather than pharmacodynamic effect.

Bactericidal phage impacts

Phage bactericidal activity results, of course, in bacterial death. This death may not always be accompanied by bacteriolytic activities, however, such as should abortive infection mechanisms interfere early with phage gene expression. More typically, though, phage bactericidal activity during phage therapy is associated with phage bacteriolytic activity, and also with the release of new phage virions. Notwithstanding these additional phage infection activities, phage bactericidal activities should be viewed as the minimum impact that phages must have on targeted bacteria to achieve primary phage therapy pharmacodynamic results, e.g., as associated with passive treatments.

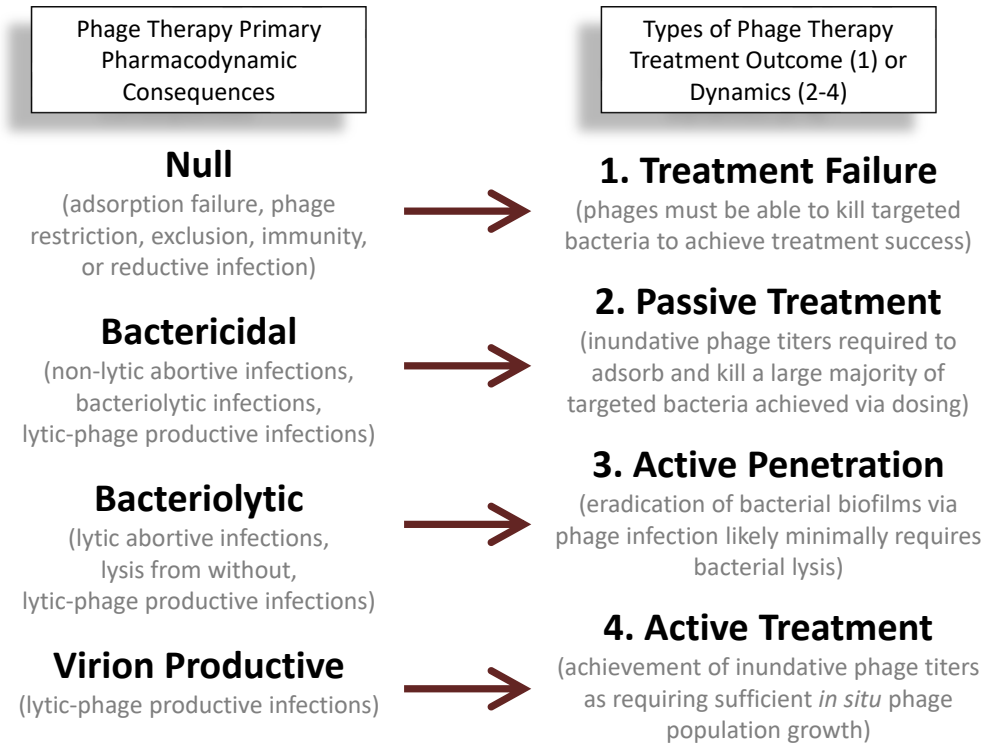

Figure 3. Various categories of phage treatment approaches or consequences.

Bacteriolysis

Phage bacteriolytic activity under all circumstances will be associated as well with phage bactericidal activity against the phage-infected bacterium. The converse is not always true, however, as bacterial death can occur without accompanying bacterial lysis, e.g., such as when phages are engineered to kill but not lyse bacteria (Goodridge, 2010). Of course as highly familiar to those with interest in phage biology, bacteriolytic activity also often is associated as well with release of new phage virions from phage-infected bacteria. If lysis occurs but new virions are not released, then we again can use the term, abortive infection. Contrasting the more typical 'lysis from within' (Young, 1992), 'lysis from without' is also a phage bacteriolytic activity, in this case one which can be induced by high multiplicity phage adsorptions, but that also, by definition, is not associated with new phage virion production nor even necessarily with phage infection (Abedon, 2011d). In either case this lysis is a pharmacodynamic effect as it results in conversion of morphologically intact bacteria into no longer intact bacteria.

We can speculate that bacteriolysis may be especially important toward phage-mediated removal of bacterial biofilms (Abedon and Thomas-Abedon, 2010). This may be because lysed bacteria should be less susceptible to secondary phage adsorption than still-intact bacteria, thereby potentially allowing accelerated diffusion of phages to underlying layers of biofilm bacteria. Phage adsorption even to not-yet-phage-infected biofilm bacteria will likely also delay phage virion penetration into those same biofilms (Abedon, 2017d). We are not aware, however, of proof that bacteriolytic activity is essential for phage-mediated biofilm eradication, such as could possibly occur following high multiplicity application of engineered lysis-defective phages. Nevertheless, phages that retain bactericidal but not bacteriolytic activities possibly may be less suited to treatment of bacterial biofilms than phages retaining both. To the extent that the lysis of bacteria might aid in phage movement, such as penetration into biofilms, then this lysis can be viewed as contributing to phage therapy pharmacokinetics as well as pharmacodynamics.

Virion production

For lytic phages, virion release from phage-infected bacteria is always associated with bacteriolytic and thereby also bactericidal activities. Phage virion production thus can be used as a surrogate indicator of bactericidal and bacteriolytic phage activities, e.g., as may be assessed via one-step growth experiments (Hyman and Abedon, 2009; Kropinski, 2018). Notwithstanding these associations with bacteria killing and lysis, new virion production as occurring *in situ* should be viewed as a pharmacokinetic rather than a pharmacodynamic phage property, as it does not directly impact bacteria though does directly impact phage numbers found in the body. For further discussion of virion production as a pharmacokinetic concept, see “Metabolism and active treatment” as found under the heading of “Pharmacokinetics”, below.

Using titres to describe phage potential to kill bacteria

Most drugs are dosed in a manner that is intended to avoid exceeding toxic concentrations while at the same time, reaching or exceeding effective densities (Figure 4). For phages, the development of dosing strategies can be greatly simplified due to the low toxicity that is a typical phage characteristic (Abedon and Thomas-Abedon, 2010; Curtright and Abedon, 2011; Olszowska-Zaremba et al., 2012; Górski et al., 2018a; 2018b), meaning that efforts toward preventing phages from reaching relatively high concentrations *in situ* can be unnecessary. To a substantial extent, therefore, and other than in terms of purifying phages away from bacterial lysis products prior to use, emphasis during the design of many phage therapy protocols can be placed on enhancing antibacterial activities rather than on avoiding the induction of side effects. Specifically, to be an effective antibacterial agent, a phage must reach sufficient densities (titres) such that the rate of phage adsorption to bacteria is faster than the rate of bacterial replication and, ideally, quite a bit faster (see, that is, Inundation thresholds…, below). Bacteria, as a consequence of exposure to these sufficient phage densities, are killed, and this killing preferably occurs over relatively short intervals.

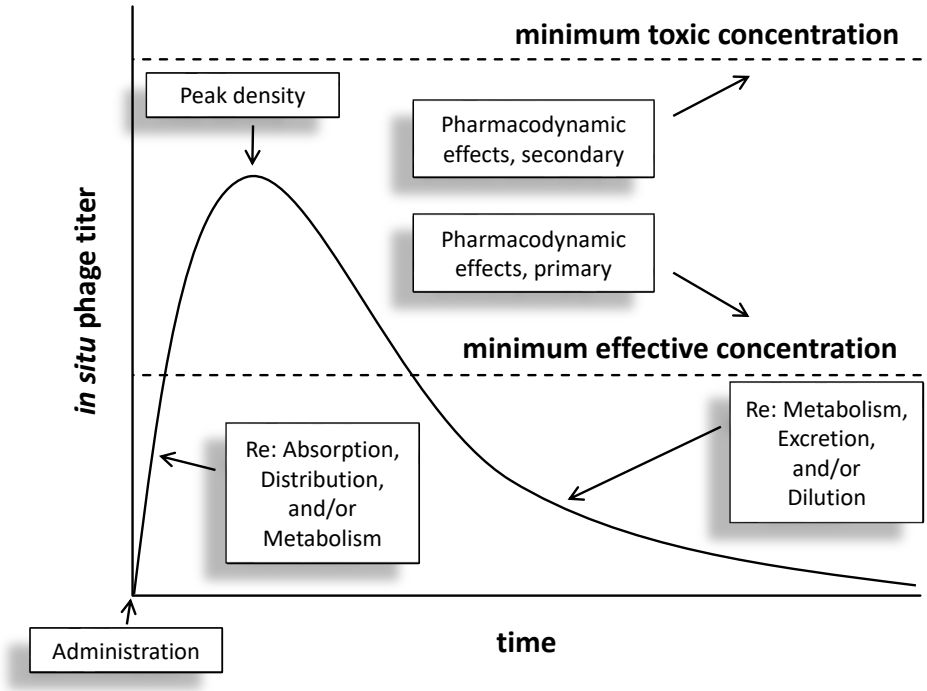

Figure 4. A representative drug time course highlighting pharmacodynamic issues (as explicitly indicated) and pharmacokinetic issues (absorption, distribution, metabolism, and excretion). Metabolism for phages, as indicated to the left (as resulting in 'activation'), is phage replication and is relevant particularly given active treatment. Metabolism, as indicated to the right (as resulting in 'inactivation'), is immune-system sequestration or inactivation of phage particles. Minimum effective concentrations and toxic concentrations refer to bacteria killing efficacy and side effects, respectively.

Most drugs also are dosed in a manner that is based on quantities of drug applied. This can be certain concentrations in combination with certain volumes (such as specific millilitres of milligrams per millilitre) or instead just absolute numbers, such as milligrams, though either often is dosed as a function of body mass as well. For phages we typically describe measures of phage concentration as titres, e.g., virions/mL, or operationally as plaque-forming units/mL. What is crucial, however, is the resulting phage titres as found *in situ*. For local phage delivery (Morozova et al., 2018b), such as the application of excess

volumes of phage-containing buffer to the surface of wounds, just keeping track of what phage titres have been applied can be sufficient. Especially this can involve, for example, the soaking of gauze bandages or the application of phage-containing gels. For systemic delivery, however, *in situ* titres would be a function of numbers of phages applied but also the mass (weight) of the body that has been dosed. At a minimum and regardless, phage titres (and volumes) or instead absolute phage numbers as associated with dosing should always be reported as accurately and unambiguously as possible (Abedon, 2017c).

MOI_{actual}

Assuming that there is a good bactericidal match between applied phages and target bacteria, then the extent of phage adsorption to individual bacteria should be the primary determinant of degrees of bacteria killing (Abedon, 2016). We can describe the extent of phage adsorption as an MOI_{actual}, which stands for *actual* multiplicity of phage infection (MOI_{actual}), or what more accurately can be described as a multiplicity of phage *adsorption*. Explicitly, this is the ratio of numbers of phages that have adsorbed to numbers of adsorbable bacteria. Thus, if on average 10 phages have *adsorbed* per available bacterium, then we would describe MOI_{actual} as being equal to 10.

Note that MOI_{actual} is not defined solely as a function of the titre of phages that have been *added* to targeted bacteria (i.e., so-called MOI_{input}), as time lags are expected to exist between the addition of phages to bacteria and the adsorption of those phages to bacteria. Nevertheless, what value of MOI_{actual} may be achieved, as well as how quickly it may be achieved, should be an explicit function of phage titres. This dependence is due to the rate that individual bacteria become adsorbed by phages being a direct function of how many phages are present within a volume, i.e., the phage titre, and particularly as are found in a bacterium's immediate vicinity (Abedon, 2012b; 2012c; 2017a) (Table 2). The more phages which are present, that is, the higher the titre, then both the faster that a given MOI_{actual} may be reached and the higher the MOI_{actual} eventually achieved.

Titre-based descriptions of phage-mediated bacteria killing include what can be labeled as inundation thresholds and decimal reduction times (Payne and Jansen, 2001; Abedon and Thomas-Abedon, 2010; Abedon, 2011a; 2011b; 2013), i.e., as discussed below. These calculations – describing or based on what *in situ* phage titres have been achieved, whether those titres are theoretically estimated or actually measured – should be used as phage dosing guides. That is, it is useful to be aware of how many phages may be required, *in situ*, in the immediate vicinity of target bacteria, to achieve preferred bacteria-eradication goals.

Table 2. Some calculations regarding phage titres, multiplicities, and bacteria killing.

P[1]	D (min)[2]	D (hours)	M (100 min)[3]	N_t/N_0[4]	M (100 min)[5]	N_t/N_0[6]	M (100 min)[7]	N_t/N_0
10^1	10^8	~10^6	2.5×10^{-6}	1	10^{-6}	1	10^{-7}	1
10^2	10^7	~10^5	2.5×10^{-5}	1	10^{-5}	1	10^{-6}	1
10^3	10^6	15000	0.00025	1	0.0001	1	10^{-5}	1
10^4	10^5	1500	0.0025	1	0.001	1	0.0001	1
10^5	10000	150	0.025	0.98	0.01	0.99	0.001	1
10^6	1000	15	0.25	0.78	0.1	0.90	0.01	0.99
10^7	100	1.5	2.5	0.082	1	0.37	0.1	0.90
10^8	10	0.15	25	10^{-11}	10	0.00005	1	0.37
10^9	1	0.015	250	0	100	0	10	0.00005
10^{10}	0.1	0.0015	2500	0	1000	0	100	0
10^{11}	0.01	0.00015	25000	0	10000	0	1000	0

1. Phage titre, here per mL. A potentially inundative phage density is 10^8 virions/mL.
2. Decimal reduction time, i.e., the time it takes to reduce the number of unadsorbed bacteria tenfold, here assuming a phage adsorption rate constant of $k = 2.5 \times 10^{-9}$ mL^{-1} min^{-1}. D is calculated as $-\ln(0.1)/Pk$. Resulting numbers have been rounded for the sake of increasing clarity, where P is phage titre.
3. M meaning 'Multiplicity of adsorption' as equivalent to multiplicity of infection, actual, i.e., MOI_{actual}, which is the ratio of adsorbed phages to adsorbable bacteria. This is calculated in this column as seen after 100 min of phage adsorption, holding phage titres constant over time, assuming that bacteria do not lyse, and that adsorption is not saturable. Thus, M is calculated in this column as $Pkt = Pk100$ (Abedon, 2016), where t is time in minutes.

4. Fraction of unadsorbed bacteria for a given phage multiplicity of adsorption, *M*. Resulting reductions in bacterial numbers are as equivalent to the impact of a killing titre of value found one column to the left. This is equal to e^{-M}, as based on the *M* value also as calculated to the left (thus, e^{-Pk100}). This then is the fraction of bacteria that are still alive after 100 min of phage adsorption given constant phage titres of *P* (first column).
5. Calculations also can be made if not holding phage titres constant, instead allowing free phages to decline as a function of adsorption to bacteria present at a concentration of *N*. This is done using the formula, $P(1-e^{-Nkt})/N$, assuming that bacteria do not eventually lyse (Abedon, 2011a). Here we assume that $N = 10^7$ bacteria/mL. With lower bacterial concentrations, e.g., 10^6/mL, then *M* will be larger (for $N = 0$, see two columns to the left), whereas with larger bacterial concentrations then *M* will be smaller (see two columns to the right).
6. This column also calculates the fraction of bacteria that remain unadsorbed after 100 min. These numbers are smaller than as previously indicated (two columns to the left) due to free phage losses stemming from adsorption to bacteria, resulting in reductions in *P* over time. Bacterial lysis still is not assumed, however, as then calculations become somewhat more complicated. If lysis were allowed then phage adsorption could be assumed to occur to a greater extent as rates of free phage depletion over time would decline, assuming that phages cannot adsorb lysed bacteria. Thus, more bacteria would be adsorbed over time as more free phages at any given time would remain available to adsorb bacteria. Not considered as well is that phage-induced bacterial lysis usually would be associated with the release of additional, newly produced virions.
7. Same as two columns to the left except that *N* is set as equal to 10^8 bacteria/mL rather than 10^7/mL.

Inundation thresholds and inundative densities

For phage therapy, an ‘inundation threshold’ may be defined as the minimum phage density that can keep a bacterial infection from getting worse. More precisely, it is the phage density that can keep a bacterial culture from becoming more concentrated (Payne and Jansen, 2001; Abedon and Thomas-Abedon, 2010; Letarov et al., 2010). This, in turn, is equivalent to the phage density that achieves bacteria killing at a rate that equals and thereby offsets that of bacterial replication. In other words, and ecologically, when phage titres equal this inundation threshold, then co-located phage-susceptible bacteria neither increase in numbers, despite an intrinsic capacity to do so (i.e., bacteria are not in stationary phase), nor decrease in numbers despite the presences of phages.

Explicitly, when phage titres are equal to their inundation threshold, then for every new bacterium generated *in situ* by binary fission, one bacterium will be lost to phage adsorption. Though this on its face would appear to be a good thing, as bacteria therefore cannot increase in numbers, in practice this inundation threshold can be useful only toward understanding what phage titres would be highly *inadequate* to achieve effective phage therapy (Figure 5). That is, achievement of phage titres equal to the inundation threshold will be insufficient to result in effective phage therapy since not only do phages in phage therapy need to prevent bacteria from increasing in numbers, they also need to *reduce* numbers of already existing bacteria, and do so in a timely manner (Abedon, 2011a; 2017a). More generally, what this means is that the rate that phages will impact bacteria should be a function of phage titres, with relatively high phage titres resulting in net reductions in numbers of targeted bacteria, relatively low phage titres allowing net increases in numbers of bacteria, and the inundation threshold found exactly in between, allowing neither increases in numbers of bacteria nor causing decreases.

A phage therapy MIC?

The concept of an inundation threshold might be viewed as equivalent to minimum inhibitory concentrations (MICs) for antibiotics (Cairns and Payne, 2008). That is, an MIC is that concentration of an antibacterial agent that results in the maintenance of a constant bacterial concentration over time, neither reducing nor allowing an increase in bacterial concentration (Figure 5). This analogy between inundation thresholds and MICs is problematic, however, as stemming from a combination of irreversible single-hit killing by phages (Bull and Regoes, 2006; Abedon, 2011a; 2014a) and the potential for phages to replicate in the presence of targeted bacteria. In other words, while phages at inundation threshold titres should indeed represent the *minimum* concentration that will inhibit bacterial replication within a culture, in practice it is effectively impossible to hold phage titres at this density (Abedon, 2011a).

Inundation thresholds thus at best can only approximate MIC-type determinations (Wiegand et al., 2008). Such an approximation may be achieved if phage titres happen to greatly exceed bacterial densities (thereby with free

phages not rapidly *irreversibly* lost to phage adsorption) and if the phages used are unable to replicate (and thereby are unable to increase in numbers over time). Furthermore, and especially if determined in liquid cultures, MIC-type determinations for phages will tend to give rise to false negatives if bacteria are able to readily mutate to phage resistance, as is often the case. Thus, inundation thresholds as an approximation of a phage MIC might have meaning in concept, but much less meaning in practice.

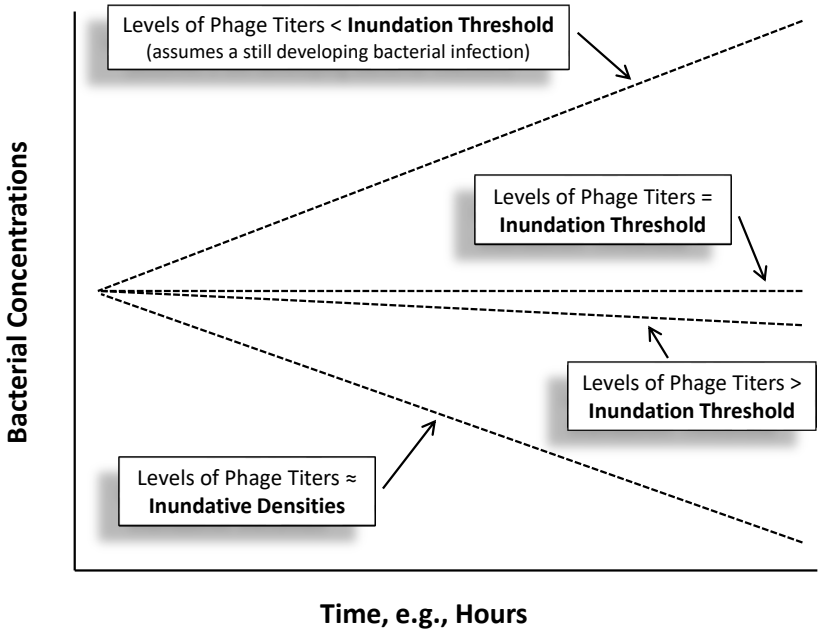

Figure 5. Inundation threshold and inundative density impacts on concentrations of target bacteria over time. Note that only inundative densities, here by definition, reduce bacterial densities substantial over time. In addition, the concept of inundation threshold is meaningless if bacterial concentrations within an infection have reached a steady state, i.e., in which bacterial numbers are no longer increasing independent of phage presence (contrasting the top curve where without treatment bacterial concentrations are assumed to instead be increasing as a function of time). Phage concentrations are described here in titres rather than multiplicities of infection, and the concept of multiplicity of infection is inappropriate to describe either phage inundation thresholds or phage inundative densities.

Not inundative densities

For a vigorously growing bacterial infection, 10^7 phages/mL can be calculated as an inundation threshold, assuming a bacterial doubling time of 30 min and a phage adsorption rate constant of 2.5×10^{-9} mL^{-1} min^{-1} (Abedon, 2014a). That is, given this bacterial doubling time, for a constant phage titre of about 10^7 phages/mL [$\approx \ln(2)/(30 \times 2.5 \times 10^{-9})$], then every time a bacterial division occurs, so too will a phage adsorption occur, thereby keeping bacterial numbers constant over time.

As noted, a phage density that simply keeps bacterial cultures from increasing in size should be viewed as a product of *inadequate* phage dosing (see also Table 2), since by definition inundation thresholds are phage titres that can control but cannot reduce numbers of replicating bacteria. This furthermore would be the case even if the number of phages applied were to greatly exceed the number of bacteria present, i.e., as representing a high MOI_{input} (Kasman et al., 2002; Abedon, 2016). For example, if 10^7 phages/mL are applied to 10^5 bacteria/mL, again assuming that bacteria are replicating with a doubling time of 30 min and phages are adsorbing at a per-virion rate of 2.5×10^{-9} mL^{-1} min^{-1}, then bacteria will not be expected to decline appreciably in number over time, and this is so even though the ratio of added phages to bacteria in this case would be equal to 100. Change the numbers instead to 10^7 and 10^4 and still the same conclusion would be reached, even though now the ratio would be 1,000-to-1 instead of 100-to-1.

To address this inadequacy of inundation thresholds as a guide to phage dosing, we introduce instead the concept of phage inundative densities (see Figure 5, and Table 2). Rather than simply keeping the bacterial culture constant in size, or indicating what phage titres must be exceeded to net reduce those numbers over some potentially very long span of time, an inundative density instead is a phage titre that not only substantially reduces bacterial numbers over time, but does so relatively rapidly. This concept we discuss in greater detail below, including positing that inundative phage densities generally would be equal to roughly 10^8 phages/mL or more.

An additional consideration is that determination of inundation thresholds for phages during actual phage treatments is not straightforward. This is because this calculation explicitly is based on a combination of rates of bacterial replication and phage adsorption, neither of which is easily determined *in situ*. Rather than employing inundation thresholds or MICs toward better appreciating phage therapy pharmacodynamics, we suggest instead the use of killing titre and decimal reduction time determinations.

Killing titres are measures of the bactericidal densities of phages found in phage stocks or within formulated products (Stent, 1963; Carlson and Miller, 1994; Carlson, 2005; Abedon, 2011a; 2020a). Killing titres are a more practical as well as more direct measure of what phage densities can be required to adequately reduce bacterial numbers versus instead, e.g., inundation thresholds (Figure 5). This claim of greater utility comes with the caveat, however, and that is that killing titres don't take into account the issue of ongoing bacterial replication. Therefore, phage adsorption must be relatively fast for killing titre calculations to reasonably hold. That in turn means that densities of targeted bacteria must be fairly high, i.e., somewhat in excess of 10^7 bacteria/mL, or instead that the numbers of phages that fail to adsorb following exposure to target bacteria must be explicitly taken into account.

Determining killing titres

To determine a killing titre, one mixes a phage population with a bacterial population in such a manner that, ideally, all or nearly all phages adsorb, and under conditions or over time spans in which bacterial replication is minimal. Measurement is made of bacterial viability both before and after phage adsorption. The ratio of still-viable bacteria present after phage adsorption (N_t) to that number present before phage exposure (N_0) is then assumed to be equal to e^{-M}, where M is the ratio of adsorbed phages to bacteria present, i.e., MOI_{actual}. In other words, a Poisson distribution of phage adsorption to bacteria is assumed where the fraction of not adsorbed bacteria is equal to e^{-M} (Abedon and Katsaounis, 2018).

Rearranging, $M = -\ln(N_t/N_0)$. From this calculation, it should be clear that the greater the fraction of bacteria that one wants to kill, i.e., the smaller that N_t is relative to N_0, then the greater the number of phages that must adsorb, on average, to each bacterium that is present in a culture. Thus, in other words, the greater that MOI_{actual} must be. For example, for $N_t/N_0 = 10^{-1}$, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6}, the latter meaning, i.e., that only 1 in one-million bacteria survive, then M must be equal to 2.3, 4.6, 6.9, 9.2, 11.5, or 13.8 adsorbed phages/bacterium, respectively. Be cautious, though, as at higher killing titres such as in excess of $M = 10$, then phage-resistant bacterial mutants can be present in numbers that are equivalent to or higher than those of unadsorbed phage-sensitive bacteria, N_t. See Table 2 for additional killing titre calculations.

Insufficient phage penetration to bacteria?

Note that killing titre calculations assume equivalent rates of phage penetration to all targeted bacteria. That assumption is unlikely to hold, however, given environmental or bacterial infection heterogeneity. Making assumptions of equivalent rates of phage penetration to targeted bacteria – should equivalent penetration in fact not be the case – can be problematic as this can lead to underestimations of what ratios of adsorbed phages to bacteria, i.e., MOI_{actual}, may be necessary to achieve sufficient bacteria killing *in situ*. That is, longer phage adsorption times, as a proxy for slower phage penetration, will require higher phage titres to achieve similar ratios of adsorbed phages to adsorbable bacteria. To some degree this issue may be mitigated, though, if phage replication can generate these higher phage numbers, particularly in the immediate vicinity of otherwise less phage-available bacteria, e.g., as might occur during active phage penetration into bacterial biofilms (Abedon, 2017a).

Notwithstanding these various issues, killing titre calculations can be useful toward estimating what fraction of bacteria ought to be killed by the application of a given phage titre to a bacterial culture, including perhaps especially to bacterial biofilms (Abedon, 2011b; 2019a; 2020a). That is, you should be able to calculate how many bacteria should survive if all added phages adsorb ($= e^{-M}$). If the number of bacteria surviving is larger than as calculated, i.e., greater than e^{-M}, then not all added phages may be reaching targeted bacteria even if all

phages are adsorbing. On the other hand, if the number of surviving bacteria is smaller than expected (< e^{-M}), then that may be a consequence of *in situ* phage replication generating more phages than were originally dosed with, i.e., as indicating active treatment.

A yet additional issue, related to that of phage penetration to bacteria and as already noted above, is that killing titre calculations do not explicitly take into account *rates* of phage impact on bacteria. This is because killing titres are determined by how many phages *adsorb* to bacteria, but phage adsorption itself generally takes time. To take into account this time issue, we suggest instead calculations of phage decimal reduction times.

Decimal reduction time

The rate at which phage virions will acquire bacteria is a function of a combination of the intrinsic adsorption properties of individual phages (their adsorption rate constant), phage densities (titres), and the availability of bacteria to these phages, the latter as measurable as well in terms of phage adsorption rate constants (Abedon, 2020a). We thus focus in this section on phage adsorption properties and titres, assuming for convenience that all bacteria are equally available to all treatment phages. If that latter assumption is false, then that is a pharmacokinetic rather than pharmacodynamic concern, that is, it is an issue of the ability of phages to reach targeted bacteria rather than phage impact on those bacteria once they have been reached. Alternatively, phage adsorption rates can vary as a function of phage host range, a pharmacodynamic issue (Dąbrowska and Abedon, 2019).

Notwithstanding these latter considerations, one way of appreciating the impact of especially phage densities and intrinsic adsorption rates on phage therapy pharmacodynamics is in terms of what can be described as a decimal reduction time or D-value. D-value, or *D*, is the time that it takes to reduce the viability of a bacterial population by one log, here as a consequence of phage adsorption. For example, this could be from 10^6 bacteria/mL down to 10^5. Assuming a constancy in phage densities, and also that bacteria are not substantially replicating over the course of reduction times, then $D = -\ln(0.1)/Pk$, where k is

the phage adsorption rate constant, *P* is phage density (titre), and 0.1 represents the decimal reduction. *D*, again, is in units of time.

D-value example calculations

Employing the same phage adsorption rate constant used above to illustrate inundation thresholds, i.e., 2.5×10^{-9} mL^{-1} min^{-1} (Stent, 1963), note that the titre of 10^7 phages/mL would result in a D-value of approximately 92 min (as rounded to 100 in Table 2). Thus, it would take over 350 min, or about six hours under ideal conditions and without bacteria replicating, to reduce the viability of a bacterial culture approximately 10,000-fold. This reduction is found to be $e^{-9.2}$-fold when calculating killing titres, that is, where 9.2 = *M* = MOI_{actual}. This in turn is approximately *M* = 10, a multiplicity that we return to subsequently.

At 10^5 phages/mL, by contrast, that same degree of reduction in bacterial viability would take instead approximately 25 days (not indicated in Table 2 but equal to about 37,000 min or ~600 hours), again assuming that phage titres remain constant and bacteria are not replicating. If faster reductions in bacterial densities are desired, if not all bacteria are equally available to phages (Barrow and Soothill, 1997), or if phage densities should decline over the course of treatment (Table 2), then supplying higher phage numbers would be necessary to achieve the same calculated rates of bacteria killing. See Abedon (2011a; 2011b) for additional discussion.

10^8 phages/mL as a potentially inundative density

From the above calculations, we can see that bacteria killing may not be sufficiently fast if phage densities are not sufficiently high. By ‘sufficiently high’ we mean phage densities that are near to or in excess of about 10^8 phages/mL, contrasting the calculations of ~10^7 phages/mL as an inundation threshold under the same conditions. Derivation as well as experimental application of this idea – that it is phage density that determines rates of bacterial adsorption by phages along with a utility of phage application at titres in the vicinity of 10^8/mL – can be found in a number of places, e.g., (Abedon, 1990; 1999; Payne and Jansen, 2001; Kasman et al., 2002; Goodridge, 2008; Bigwood et al., 2009; Hyman and Abedon, 2009; Abedon and Thomas-Abedon, 2010; Hagens and Loessner,

2010; Abedon, 2011a; 2011b; 2011c); see also (Spouge, 1994; Payne and Jansen, 2000; Abedon, 2018b). That is, for $k = 2.5 \times 10^{-9}$ mL^{-1} min^{-1}, as above, then 10^8 phages/mL corresponds to a D-value of approximately 10 min, while titres of 10^9 phages/mL will instead have a D-value of 1 min, and as noted for 10^7 phages/mL it instead is 100 min (Table 2).

In cases where excessively rapid bacterial lysis might be a concern, e.g., such as due to endotoxin release in the course of phage treatment of Gram-negative infections of the blood, then lower phage titres and therefore lower D values instead might be appropriate. That statement comes with the caveat, however, that it may not always be possible to control just what titres phages can attain *in situ* nor how rapidly cultures may be lysed by phages. Nevertheless, to achieve sufficient bacteria killing over reasonably short time frames, then sufficient phage titres must be present *in situ* to result in sufficiently short decimal reduction times (again, see Table 2). Such 'sufficient' phage titres are what we describe here as inundative, that is, inundative densities of phages, which we suggest can be in the range of at least 10^8 phages/mL titres. What phage numbers need be supplied in the course of dosing, then, to result in inundative densities of phages *in situ*?

Supplying sufficient phage titres

As generally we are concerned not only with killing bacteria but also killing them over reasonable time frames, introduction of a time component to calculations of phage impact on bacteria is important. That is, bacteria typically will not be adsorbed instantaneously by phages. Nevertheless, for phage therapy sooner phage adsorption generally is preferable (minutes or at most hours) to greatly delayed phage adsorption (days or even weeks). It is this time component that is supplied by decimal reduction time calculations. Indeed, as illustrated by decimal reduction time calculations, those phage titres that can achieve adequate bacteria killing over reasonable time frames we have described as 'inundative'. Just what constitutes 'adequate' or 'reasonable' may be subject to debate, however, as well as vary with circumstances. With these considerations in mind, in this section, we discuss further the concept of inundative densities.

The problem of slow adsorption

Reduction of 10^6 bacteria/mL to a density of 10^{-6} bacteria/mL – a drop in density to 10^{-12} of the original – will require a minimum of 3×10^7 phages/mL. Where does this number come from? To achieve that much bacteria killing then MOI_{actual} must be equal to roughly 30, i.e., $M \approx 30$. That is, $N_t/N_0 = e^{-M}$, where N_t is the ending bacterial concentration and N_0 is the bacterial concentration prior to phage addition. Thus, N_t/N_0 is the fraction of bacteria that survive following phage adsorption, which in the example is equal to 10^{-12}, and $-\ln(10^{-12}) = 28 \approx 30$, i.e., where $30 \times 10^6 = 3 \times 10^7$. Therefore, 3×10^7 phages must fully adsorb 10^6 bacteria to reduce that number of bacteria to 10^{-6}.

Alternatively, such substantial bacteria overkill may not be necessary to the extent that phage-mediated disruption of bacterial infections is followed by immune system clearance (Levin and Bull, 2004; Leung and Weitz, 2017; Roach et al., 2017; Abedon, 2019b). This phage density of 3×10^7/mL, however, corresponds to an initial *D*-value, as seen prior to phage losses due to bacterial adsorption, of approximately 30 min, assuming again a phage adsorption rate constant of 2.5×10^{-9} mL^{-1} min^{-1}. This means that, if conditions for bacterial acquisition are poorer than anticipated, such that *D* instead, e.g., equals 1 hour, then this phage density, sufficient as it could be for bacteria overkill, may in fact *not* result in timely bacteria killing. That is, in this latter example, it would take 12 hours to reduce the bacterial density 10^{-12}. That time frame may or may not be adequate for a given application.

Perhaps consistent with that concern of slowness of phage adsorption, note that some efforts to model phage therapy mathematically, as reviewed by Gill (2008), have employed adsorption rate constants of much lower magnitude than that used in the above calculations. For example, these appear to have been adsorption rate constants of ~10^{-11} mL^{-1} min^{-1} or lower, which is ~40-fold smaller than 2.5×10^{-9} mL^{-1} min^{-1}. Confusingly, these values are often expressed in per hour rather than per min units, i.e., such that a constant of 10^{-9} mL^{-1} h^{-1} is equal to approximately 2×10^{-11} mL^{-1} min^{-1}. With a phage density of 3×10^7/mL and an adsorption rate constant of 10^{-11} mL^{-1} min^{-1}, then the calculated D value is 5 days! Thus, in the above scenario the 10^{-12} reduction would require 60 days of

constant phage adsorption as well as assumptions of a lack of compensating bacterial binary fission.

Consistently as well, phage densities of the reviewed simulations often reach approximately 10^{10}/mL before substantial declines in bacterial densities are seen (Gill, 2008), which can serve as an example of how slower phage acquisition of, or penetration to individual bacterial cells can negatively impact phage therapy efficacy (Barrow and Soothill, 1997). Also consistently, Levin and Bull (1996) note in their model that with lower phage adsorption rates, bacteria are more likely to exceed their "lethal threshold", that is, to kill the patient prior to infections being brought under control following phage application. Thus, in short, a seemingly quite adequate MOI_{input} such as 3×10^7/mL will not necessarily always translate into timely bacteria killing, and this explicitly is because it can take time, and sometimes substantial amounts of time, before MOI_{input} comes to approximate the more pharmacodynamically useful MOI_{actual} (Abedon, 2016) (Table 2).

Indeed, MOI_{input}, or even phage inundative densities, are more pharmacokinetic than pharmacodynamic descriptors, representing what numbers of phages have been made available toward bacteria killing rather than what number of phages are, at a given time, actually effecting that killing. It is the achievement instead of an MOI_{actual} that is directly relevant to phage therapy primary pharmacodynamics. Nevertheless, we are defining here inundative densities as phage titres that over reasonable time frames are able to achieve adequate MOI_{actual} values, such as 10 adsorbed phages per adsorbable bacterium. To achieve this, if working with phages displaying unusually slow intrinsic rates of phage adsorption (small adsorption rate constants), then phage titres representing inundative densities simply must be correspondingly higher, e.g., 40-fold higher given 40-fold slower adsorption.

The problem of free phage losses

A further concern is that D-value calculations are based on an assumption that phage titres will remain constant over time. This is rather than titres declining as a consequence of phage adsorption to bacteria or especially as due to bacteria-

independent mechanisms of phage decay, the latter, e.g., such as due to the action of immune systems (Dąbrowska et al., 2018; Dąbrowska and Abedon, 2019). A utility of phage replication *in situ* thus is that it could contribute to a maintenance of phage titres in the face of these various mechanisms of titre decline. If bacterial densities are too low, however, then substantial increases in phage densities will not be able to proceed as a consequence of *in situ* phage replication. Thus, phage replication after dosing might help *in situ* phage titres to either reach or sustain inundative densities, though only if bacterial densities are sufficiently high to support that replication.

These statements are consistent with a scenario posited by Payne and Jansen (2000) in which too early phage application during the development of a bacterial infection, followed by substantial phage decay, could give rise to what they describe as a "failure threshold time". This is defined as a time before which numbers of targeted bacteria have grown to be sufficiently high to support adequate phage population growth. One can view this perspective, however, as also pointing to a problem of supplying too few phages rather than supplying those phages too early. Furthermore, it is an open question as to when, clinically, one in fact might catch a bacterial infection too early in its development to support active treatment, since bacterial infections often are fairly advanced before they are brought to a physician's attention. A possibly relevant scenario, though, could be during prophylactic phage treatment. Under such circumstances, an important consideration would be to supply as well as retain sufficient phage densities that newly forming bacterial infections are brought under control early in their development (Abedon, 2010; 2011b). Generally, such early control would require that phages have been supplied in sufficient numbers that inundative densities are retained despite free phage losses over time, as bacterial densities by definition in this case would be insufficient to support substantial phage replication especially early following the occurrence of bacterial contamination.

10^8 phages/mL again as a potentially inundative density

For these various reasons, striving for phage densities in the vicinity of 10^8 phages/mL, or higher, and then sustaining those phage densities over the

course of treatment – whether through passive or active means – should be the default dosing goal in phage therapy protocols. Lower densities should instead be employed or sought only to the extent that they are demonstrably and consistently effective in reducing bacterial densities to desirable extents. Substantially higher densities may also be employed should 10^8 phages/mL be found to be inadequate in achieving desirable levels of bacteria killing, though with the caveat that an alternative explanation for poor efficacy, to that of too few phages, can be poor phage choice, e.g., such as stemming from insufficiently high adsorption rate constants.

Some of these issues are explored in Table 2. There we see that 10^8 phages/mL may achieve a D value of 10 min, assuming no substantial reductions in phage numbers over time, whereas the D value given 10^7 phages/mL of the same phages is 100 min. Perhaps more telling, after 100 min MOI_{actual} is expected to reach 25 given a constant 10^8 phages/mL, versus 2.5 for 10^7 phages/mL. That means that at 10^8 phages/mL an MOI_{actual} of 10 would be reached in 40 min, versus 400 min given 10^7 phages/mL. If phages decline in number over time, which as shown in Table 2 is due to phage adsorption to target bacteria, then so too does the MOI_{actual} decline. Declines in phage numbers to adsorption, however, should only be a concern when employing phages that are unable to replicate *in situ*, as bacterial densities that are sufficient to substantially reduce phage numbers over time due to adsorption should also be sufficient to substantially support phage population growth as well. Nonetheless, for replication-competent phages, declines in phage numbers that are due to bacteria-independent mechanisms will still have an impact on what MOI_{actual} can be achieved over a given time frame. So too, as noted, will smaller phage adsorption rate constants reduce the number of phages that will come to adsorb over a given time span. Thus, while achieving 10^7 phages/mL *in situ* might appear under some circumstances to at least be adequate for a given phage treatment, that adequacy to a degree is dependent on phages finding and then adsorbing targeted bacteria quickly and virions declining in numbers only slowly. A more conservative dosing goal, and particularly so if phage penetration to some targeted bacteria is slower than it is for others, therefore could be one that is higher than 10^7 phages/mL, e.g., such as 10^8 phages/mL.

Keep in mind that it is also always important to monitor for toxicity during animal testing. This is particularly so given increased levels of dosing since the standard pharmacodynamic assumption is that greater drug densities will be associated with greater drug toxicity. This assumption, though, may be less relevant when using well-purified phage products given the generally low toxicity of phage therapy virions. Also, and as noted, what generally is key in terms of *in situ* phage titres are those titres found in the immediate vicinity of target bacteria rather than necessarily as found on average more systemically (Abedon, 2017a). Thus, phage titres of 10^8/mL need not always be achieved more globally during phage treatments but instead only more locally, i.e., as may be readily achieved especially via more direct phage application to more localized bacterial infections (Table 3).

Striving for substantial killing of bacteria

A perhaps reasonable dividing line, following phage application, between substantial and insubstantial *reductions* in bacterial densities, whether *in vitro* or *in vivo*, is approximately 10,000-fold (Kasman et al., 2002). Experimentation that fails to achieve this level of bacteria killing might be deemed to be inadequate unless clinically satisfactory endpoints nonetheless are achieved, e.g., such as a consequence of concurrent immune-system action. Experimentation in which neither clinically positive outcomes nor substantial reductions in bacterial densities are observed, however, should not be used as evidence either for or against the potential for phage therapy to control a specific bacterial infection, unless higher phage density 'positive controls' are also attempted.

Examples of such bacteria-killing positive controls would be 10^9, 10^{10}, or even higher phage titres (phages/mL), as achieved within the vicinity of target bacteria *in situ*. In other words, one should not simply supply what may be phage doses that do not achieve inundative phage densities *in situ* and then from that declare that phage therapy generally has not been successful. Another way to state this is that given that phage treatments tend to display low toxicities, then especially during *in vitro* testing, and presumably during animal testing as well, it behooves researchers to establish dose-response

relationships. Researchers particularly should not inadvertently or intentionally ignore higher phage dosing unless lower phage doses already provide desirable efficacies especially under more stringent or realistic conditions, e.g., such as toward the treatment of chronic bacterial infections (Abedon, 2019b).

Pharmacokinetics

Pharmacokinetics, as noted, can be described as the body's impact on a drug. What that really means is that the concentration and location of a drug within a body will be impacted by the anatomy and physiology of that body. What that further means is that to properly function, a drug must become present in sufficient quantities within the immediate vicinity of target tissues. For antibacterial agents, this is in the immediate vicinity of target bacteria. The extent to which phages can attain as well as maintain those necessary quantities, or concentrations – what above we have described as inundative densities – is a function of their pharmacokinetics. This means that phages must become associated with the body (absorption), move within the body to the location of their target bacteria (distribution), and once reaching those bacteria change into a form that is explicitly antibacterial (metabolism). All the while they must avoiding becoming chemically inactivated (also metabolism) or otherwise being removed from the body (excretion) (for summaries, see the left side of Figure 1 and also Figure 4).

Given the diversity of phages, the diversity of dosing routes, and the diversity of infection properties, study of the pharmacokinetics of phages represents a vast undertaking. Specifically, just as the pharmacokinetics of every drug must be individually studied, including for every condition that the drug may be used to treat, so too the pharmacokinetics of every phage type that may be used for phage therapy might be individually studied for every dosing route and bacterial infection type. Ultimately, for treatment with phages as a whole, undertaking such an effort however becomes daunting. Therefore, general principles by necessity must be sought, some of which are our focus here.

In this section we start with metabolism especially in terms of phage 'activation', consider the ultimate product of pharmacokinetic processes, i.e., *in situ* phage

titres, and then briefly address issues of phage absorption and distribution. For additional, recent reviews of phage therapy pharmacokinetics, see (Dąbrowska, 2019; Dąbrowska and Abedon, 2019; Matsuzaki and Uchiyama, 2019).

Metabolism and active treatment

Drug accumulation within body tissues involves diffusion and other movement processes. Pharmacokinetically, these are drug absorption and distribution (Figures 1 and 4). Alternatively, mechanisms exist by which certain drugs, such as the anti-tuberculosis drug, isoniazid (Then and Sahl, 2010), become *activated* in the course of pharmacokinetic metabolism. That is, isoniazid becomes chemically modified within the body – specifically by targeted bacteria – to its pharmacodynamically functional form. Thus, for some drugs it isn't good enough for them simply to have entered the body. Instead, to become active they must also be subject to pharmacokinetic metabolism.

Phage infection as a pharmacokinetic metabolism

Phages too can be chemically altered within the body. Most obviously, this can result in their inactivation, such as in the course of interacting with a body's immune system. Perhaps less obviously, the process of phage infection of bacteria itself represents a series of chemical alterations first of the phage particle and then as occurring over the course of phage gene expression as well.

As microbiomes pharmacologically are considered to be aspects of bodies, phage replication as a biochemical process – as occurs in association with microbiome bacteria – too should be considered to be a product of pharmacokinetic metabolism (Abedon, 2014b). In addition, however, so too are phage bactericidal activities products of pharmacokinetic metabolism as well, i.e., as also resulting from phage gene expression. While phage bactericidal activity nonetheless explicitly is pharmacodynamic, the point here is that its generation instead is pharmacokinetic, i.e., as a product of metabolism. For phage replication by contrast, both its generation (metabolism) and consequence (*in situ* changes in numbers of phages) are pharmacokinetic

aspects (Figure 6). This *in situ* phage replication is explicitly required for so-called *active* phage treatments.

Bactericidal activity as a product of pharmacokinetic processes also is required for active treatment, but so too it is essential for passive treatment, the latter as considered above as well as discussed further in a subsequent section. See Figure 7 for a summary of the relationship between these various phage pharmacological activities and active vs. passive phage treatments.

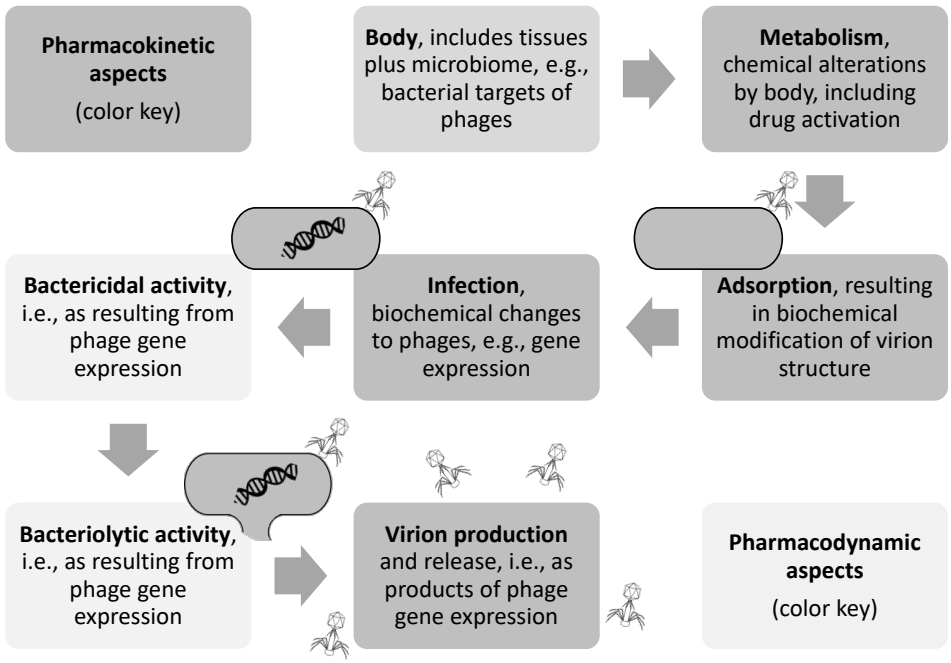

Figure 6. Phage infections and pharmacology. Target bacteria found within bodies pharmacologically are considered aspects of the body. Pharmacokinetic metabolism is the chemical alteration of pharmacological agents. Chemical and biochemical alterations of phages occur during their adsorption and infection in association with body bacteria. Bactericidal and bacteriolytic activities are primary pharmacodynamic consequences. Resulting virion production, as a contributor to changes in phage numbers instead is a pharmacokinetic product of this metabolism. If phages are inactivated upon infection (not shown), then that too would be a consequence of pharmacokinetic metabolism. Also, bacterial lysis, if it can contribute to phage penetration to bacteria, such as into bacterial biofilms, also would contribute to a phage therapy pharmacokinetic aspect, i.e., that of phage movement to target bacteria.

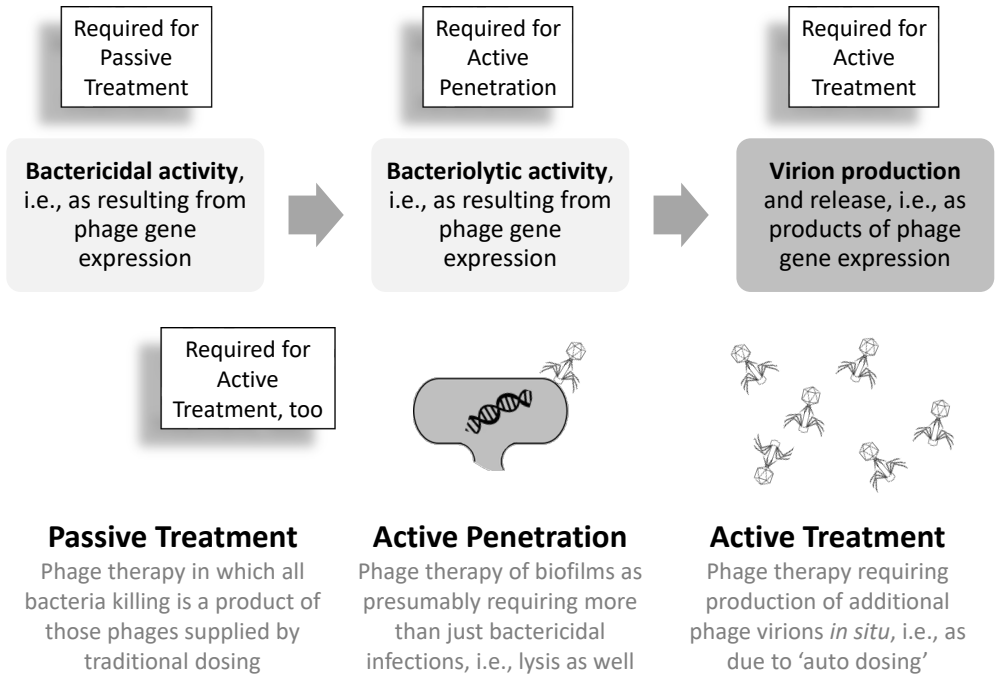

Figure 7. Restatement of relationship between different pharmacological phage activities and categories of phage treatment. While bactericidal and bacteriolytic activities are pharmacodynamic as phage impacts on bacteria (as an aspect of the body), virion production is pharmacokinetic, as bacteria impact on phage. As indicated, bactericidal activity is required for passive treatment, both bactericidal and virion production activities are required for active treatment, and bacteriolytic activity likely is either necessary or useful for phage penetration into bacterial biofilms, and also is required for the release aspect of virion production.

A replicating drug but with resource requirements

Replication is decidedly unusual for a drug. Nonetheless, similar *in situ* drug amplification in densities can occur simply through dosing by conventional means. That is, by administering more drug, including via automatic dosing in response to *in situ* conditions, drugs too can increase in concentrations within the body. The increases in drug concentrations following conventional dosing, versus this phage "auto dosing" (Abedon and Thomas-Abedon, 2010; Loc-Carrillo and Abedon, 2011), can however result in changes in concentrations in distinctly different locations within the body. Phage self-amplification specifically

will tend to occur precisely in the vicinity of phage antibacterial activity. That is, active treatments can allow the generation of inundative phage densities, especially where they may be most needed, rather than requiring that such densities be reached throughout the body (Abedon, 2017a). In addition, in those circumstances where phages are not delivered to a bacterial infection directly (e.g., locally) but rather indirectly (i.e., systemically), this ability of phages to increase their numbers though auto dosing can mean that phages need not pharmacokinetically penetrate to bacteria already possessing inundative densities for phage treatments to be successful.

Whether actively or passively supplied, ultimately phage therapy efficacy is dependent on the generation, in the vicinity of target bacteria, of peak phage concentrations that are sufficient, i.e., which are inundative, to result in both substantial and timely bacterial eradication (Abedon, 2011a; 2012a; 2017a). In terms of active treatments, strictly lytic phage populations explicitly will need to go through repeated rounds of bacterial adsorption, infection, and lysis to achieve necessary titres. Therefore, anything that speeds up adsorption, decreases the time spent infecting (shorter latent periods), or increases the yield from infections (larger burst sizes) should to a first approximation increase these rates of phage population growth (Levin and Bull, 2004; Abedon et al., 2009). Phage survival is also important (Abedon, 2009; Chan and Abedon, 2012), as discussed further below as well as touched upon above, and see also Abedon (2014a) for a listing of various complications on these processes. Rapid phage population growth, however, is only one component of active treatments.

Also as touched upon above, successful active treatment is also absolutely dependent on concentrations of target bacteria: If there are not enough bacteria present, less than the so-called proliferation threshold (Payne and Jansen, 2000; Payne and Jansen, 2001; 2003; Abedon and Thomas-Abedon, 2010; Abedon, 2011a) or what others refer to simply as a threshold (Brüssow, 2007; Gill, 2008; Letarov et al., 2010), then phage population growth and therefore active treatment simply will not work (Levin and Bull, 2004; Bigwood et al., 2009; Capparelli et al., 2010). Active treatments also require that phages replicate to sufficient titres. In particular, unless sufficient densities of target bacteria are

present, then inundative phage densities will not be reached no matter how rapidly a phage population may replicate, and therefore active treatments again will not be successful. Nonetheless, what active treatment can supply to phage therapy protocols is the presentation of large numbers of phages to bacteria that otherwise are difficult to directly deliver phages to, though this is true only so long as there are sufficient numbers of bacteria present that physiologically are sufficiently able to support phage population growth.

Phage in situ *concentrations (titres)*

What phage titres, as found *in situ*, are sufficient for achieving phage therapy efficacy? Ultimately this is a somewhat unresolved question. Thus, as noted above, perhaps 10^8 phages/mL could constitute a reasonable minimum target phage density under many circumstances (Abedon, 2018b), especially as achieved in the immediate vicinity of target bacteria (Abedon, 2017a). This titre might then be described as approximating a minimum effective phage concentration (Figure 4), i.e., as equivalent to an inundative phage density (above). What specific *in situ* phage titre constitutes a minimum effective concentration therefore should vary depending on the rate at which elimination of bacteria is desired, with killing over minutes requiring higher phage densities than killing that instead is envisaged to involve hours or days of treatment. Phage densities ideally would then remain at or higher than their minimum effective concentration throughout much of a course of treatment, again explicitly as found in the immediate vicinity of targeted bacteria (Abedon, 2017a). This section considers various aspects of the occurrence and maintenance of such phage *in situ* titres.

Passive treatment, with some consideration of active treatment

With passive treatment, phage adsorption need result only in the killing of target bacteria for therapeutic outcomes to be successful, that is, rather than also the production of additional phages (Figure 7). From Barrow and Soothill (1997), p. 271, "…sufficiently large dose of phage could be given that might be able to overwhelm the bacterial pathogens without any significant phage multiplication taking place." That is, with passive treatment, by definition (Payne and Jansen, 2000; 2001; 2003), doses supplied must be sufficiently large that a good

approximation of all target bacteria become phage adsorbed without relying on *in situ* phage replication to bolster phage numbers. Phages must also adsorb bacteria reasonably quickly, i.e., see inundative density, above, with the more phages supplied then the faster bacteria will be adsorbed (Table 2). Passive treatment thus is dependent on four criteria: (i) phages reaching target bacteria in sufficient numbers, (ii) phages adsorbing the bacteria they reach, (iii) phages killing the bacteria they've adsorbed, and (iv) all of this occurring over reasonable time frames.

Though these criteria seemingly are at least modestly stringent, in fact they can be less strict in terms of phage properties than requirements for active treatment, since the latter by definition also requires adequate levels of phage population growth. Passive treatments thus can demand only relatively low levels of 'performance' on the part of the phages employed, i.e., just adsorption and killing. Furthermore, passive treatment can be effected by phages that have been engineered to kill bacteria without lysing them (Goodridge, 2010). On the other hand, the need for phages to reach targeted bacteria in adequate numbers can be *more* difficult with passive treatment than for active treatment (Abedon, 2017a). This is because with passive treatment every phage necessary to effect bacterial inundation must be supplied via external dosing rather than generated *in situ*.

Fortunately for the utility of phage therapy, naturally occurring phages often will retain a capacity to display auto dosing whether passive treatment is being attempted or not. Thus there usually will exist a possibility of near-immediate augmentation of passive dosing that is a consequence of active phage replication, presumably resulting in "a 'margin of safety' toward attaining phage therapy efficacy" (Abedon and Thomas-Abedon, 2010). These issues point to an importance of performing adequate *in vitro* growth as well as host range determinations with all potential therapeutic phages even if only passive treatment is envisaged, with determinations of phage growth characterization more relevant the more that active treatment is relied upon. For additional consideration of phage choice for phage therapy, see (Gill and Hyman, 2010; Łobocka et al., 2014).

Multiple or continuous dosing

Various factors can conspire to make it necessary to supply more than just that minimum number of phages which otherwise are thought to be adequate to achieve treatment success. One way of augmenting phage numbers, a means that should be assumed to be required unless it can be experimentally demonstrated to not be necessary (Abedon, 2012a), is to supply phages either in multiple doses (Summers, 2001; Capparelli et al., 2007) or continuously (Kutter et al., 2010). In Georgia and Poland for example, where phage therapy of humans has been routinely employed for decades (Kutter et al., 2010; Abedon et al., 2011), multiple or continuous dosing is typical. For example, treatment of intestinal ailments using the formulation Intestiphage involves ingestion of many tens of millilitres multiple times per day. Multiple dosing, similarly and elsewhere (Belgium) was found to be effective in a case of a human patient treated intravenously with phages every 6 hours due to a difficult sepsis (Jennes et al., 2017). See as well, e.g., (Fish et al., 2016; Schooley et al., 2017; Aslam et al., 2019b; Dedrick et al., 2019; Bao et al., 2020).

A further issue is the appropriate duration of multiple or continuous dosings, which in some instances, particularly against chronic infections, can take weeks (Ślopek et al., 1983; 1987; Duckworth and Gulig, 2002). Quoting d'Hérelle (d'Hérelle and Smith, 1930), p. 176:

> The number of administrations essential to induce a therapeutic effect varies with the type of condition under treatment; in acute infections or in infectious conditions that have not become fully chronic, a single administration or, perhaps, two administrations should overcome the organisms. In chronic conditions, on the other hand, it may be necessary to continue bacteriophage therapy over a relatively long period.

More generally, from Adembri and Novelli (2009), p. 523: “Microbiological and clinical success are less likely when suboptimal exposure and/or incorrect duration of antimicrobial therapy occur”.

An exception to the suggestion that multiple or continuous dosing might always be assumed to be necessary can be found in the treatment of non-human subjects such as industrially raised chickens (Kosznik-Kwaśnicka et al., 2019). In this case limitations of convenience or of economic feasibility can be severe, resulting in greater impetus to design treatments in which a single dose is sufficient (Carvalho et al., 2010). Under those circumstances, dosing with higher phage titres also may be more feasible from a regulatory or safety perspective (Sulakvelidze and Pasternack, 2010; Kosznik-Kwaśnicka et al., 2019). Also, it is important to keep in mind that single doses *can* be effective even in the clinic (Wright et al., 2009; Chan et al., 2018), and potentially more effective than multiple dosing with too low phage titres (Jault et al., 2019). Nonetheless, single-dose phage therapy is not necessarily *always* more effective nor necessarily *always* more feasible than multiple or continuous dosing. As Lampert et al. put it (1935, p. 443), “We consider our successful results to be due in large measure to the technique of application which we employ routinely, and which is based upon direct contact of the bacteriophage with the infected tissues by means of generous daily applications.” See Abedon (2011a; 2014a) for further consideration of phage frequency of dosing.

Peak and trough phage concentrations

For a standard drug, its peak concentration, or density, is a consequence of conventional dosing alone. Peak concentrations or titres for phages, however, can also be a consequence of *in situ* phage replication. While peak drug concentrations typically are referred to in terms of plasma concentrations, for phages peak concentrations often can be more relevantly described in terms of concentrations within other tissues. The reason for this distinction is that conventional drugs will typically achieve higher concentrations in blood than they will achieve following distribution outside of the blood, unless drugs are preferentially concentrated in specific tissues. Engineered phages that display tissue-targeting peptides or proteins too can potentially accumulate in tissues by

affinity only (Górski et al., 2015), though not necessarily highly effectively (Hodyra-Stefaniak et al., 2019). Highest *in situ* concentrations for naturally occurring phages, by contrast, should be achieved especially at the site of infection due to the phage ability to replicate in association with target bacteria (Abedon, 2015b; 2017a).

Contrasting peak drug concentrations are instead trough concentrations (Czock et al., 2009). Specifically, the goal of multiple dosing should be to sustain drug concentrations at or above those required to achieve efficacy, and for at least some antibacterials, this should be as high as possible (Cerf et al., 2010). Thus, if there exists a minimum *effective* concentration of phages that is necessary to achieve a reasonable degree of bacteria killing at a reasonable rate (i.e., inundative densities), then the success or at least duration of phage therapy treatments likely will be a function of the length of time over which phage concentrations meet or exceed this density, however those densities are achieved. That is, trough phage densities, particularly as found in direct association with targeted bacteria, ideally will not fall substantially below minimum effective phage concentrations (Figure 4).

A related consideration is the question of what might contribute to an *upper* limit on peak phage densities. Should they, for example, be limited by safety concerns, regulatory considerations, economic issues, phage replication performance *in situ*, or even due to declines in antibacterial efficacy of ever greater levels of dosing? Generally for phages, however, the answer to this question is not known.

Absorption and Distribution

The ability of drugs to move between as well as within the different “compartments” making up bodies is crucial to their *in situ* activity. For many drugs this movement will consist of uptake into the blood stream (absorption) along with movement into other tissues (distribution). For phages, though, the terms translocation and penetration to a degree have been used synonymously with absorption and distribution, respectively. The phage ability to pass from compartment to compartment within bodies, such as from the gastrointestinal

lumen into the blood, for example, has been described by Górski et al. (2006) in their review of the subject as “Bacteriophage translocation”; see also Olszowska-Zaremba et al. (2012). In a more narrow sense, phage translocation is a process of phage movement through an epithelial cell, as recently demonstrated *in vitro* by Nguyen et al. (2017) on cell layer cultures. Penetration can also be used to describe phage movement into localized infections, such as infiltration into biofilms, or into animal tissues in general (Dąbrowska et al., 2005). Distribution, or penetration, also can refer to movement from extracellular to intracellular compartments, though the phage potential to impact intracellular bacterial infections has not been substantially explored (Broxmeyer et al., 2002; Danelishvili et al., 2006; Capparelli et al., 2007). Issues of gastrointestinal (enteric) delivery of phages along with bacterial translocation will not be extensively addressed here, but see Dąbrowska and Abedon (2019) for review. We concentrate instead on a subset of topics pertaining to phage absorption and distribution, including examining the extent to which phage therapy researchers should even be concerned with these issues.

Limitations to phage therapy pharmacokinetics

The pharmacokinetics of chemotherapeutics can differ among the species being treated, e.g., mouse vs. human, and also in terms of the health of the recipient (healthy volunteers vs. patients). Phage therapy so far has not been studied sufficiently robustly to greatly understand pharmacokinetic issues in even one system, however, much less comparatively. Indeed, such determinations are not fully worked out even for standard chemotherapeutic antibacterials (Adembri and Novelli, 2009; Czock et al., 2009). Furthermore, phage therapy may be unusual among antibacterial approaches in that there in fact appears to be a tradition of not always emphasizing especially pharmacokinetic analyses during the development of treatments (Dąbrowska et al., 2018; Dąbrowska and Abedon, 2019).

One example where even the most basic of phage therapy pharmacokinetic properties have not been well worked out is for *per os* delivery, especially that results in phage absorption into the blood. This appears to be possible (Górski et al., 2006; Olszowska-Zaremba et al., 2012; Nguyen et al., 2017), at least

under some not yet well specified circumstances, but has not been found to be effective under other circumstances. For example, phage absorption following oral delivery is perhaps less effective for some phages such as certain T4-like and other large phages (Krylov, 2001; Bruttin and Brüssow, 2005; Denou et al., 2009). Some further generalizations also may yet exist, e.g., it appears that the higher the oral phage dose then the higher the probability of active phages coming to be found in blood. As with the pharmacokinetics of drugs more generally, the phage potential for translocation from the gut also might differ depending on the health state of the subject (Letarov et al., 2010). Nevertheless, our potential to predict the degree that a given oral dose of a given phage under a given set of circumstances will be absorbed by a patient is at best limited. A systematic review of such pharmacokinetic issues was recently presented by Dąbrowska (2019) and see also (Dąbrowska and Abedon, 2019; Matsuzaki and Uchiyama, 2019).

A related issue is that phages in phage therapy tend to be diverse, often differing greatly even among the phages sharing a particular target pathogen. Consequently, data collected for any one phage that impacts any one bacterial pathogen may not be greatly applicable even to other phages impacting that same pathogen. These same general issues also exist for antibiotics, however, i.e., an inability to exhaustively characterize their pharmacokinetics in all relevant systems. The number of antibiotics for which pharmacokinetic information is desired, though, is seemingly far smaller than is the case with phages (Harper and Kutter, 2009). The phage therapy literature, that is, is full of research groups isolating novel phage strains that are then relevantly characterized primarily in terms of their pharmacodynamics, i.e., efficacy but also toxicity. To fully appreciate phage therapy pharmacokinetics, however, all phage types used for treating a given pathogen and disease would need to be equivalently and comparatively studied across all relevant systems, e.g., mouse and human as well as healthy and diseased, though this is both unlikely to happen and, at least arguably, may not even be entirely necessary.

As a consequence of the diversity of phages that potentially could be used therapeutically, one could readily argue that performing detailed

pharmacokinetic analyses of all phages of interest to phage therapy, under all relevant circumstances, would either represent a daunting task or instead be something that should represent a lower priority than pharmacodynamic determinations. The latter point may be particularly valid to the extent that concerns of phage absorption and distribution are less relevant given phage application directly to bacterial infections – such as topical application to wounds and lungs or application by injection directly to sites of infection – and so long as phages are capable of amplifying their numbers to effective densities once they have reached target bacteria. Thus, while at the same time pharmacokinetic issues may be more challenging to address for phage therapy as a whole, nonetheless under many treatment scenarios pharmacokinetic issues also may be less important to address than simply issues of efficacy and possible toxicity.

How much does phage inactivation really matter?

In this modern era of phage therapy research, roughly the mid-1990s and later (Abedon, 2017b), a fair amount of emphasis has been placed on the potential for bodies to reduce phage numbers *in situ*. Emphasis predominantly has been on what can be described pharmacokinetically as metabolism, and especially the impact of immune systems on continued phage presence. Though it has long been known that the adaptive immune system, especially antibodies, can inactivate phages (Bradley and Watson, 1963; Nelstrop et al., 1968; Ochs et al., 1971; Huff et al., 2010; Hodyra-Stefaniak et al., 2015), it was later found that the mononuclear-phagocyte system, a.k.a., the reticulo-endothelial system can be important as well (Inchley, 1969; Merril et al., 1996; Nilsson et al., 2004; Merril, 2008). Indeed, a standard concern outside of the phage therapy community seems to be that phages simply will be inactivated by the body's immune system prior to the achievement of efficacy. This potential, however, is inconsistent with numerous examples of seeming phage therapy efficacy found throughout the phage therapy literature (Chanishvili, 2012; Abedon, 2015c; 2018a; Morozova et al., 2018b; Abedon, 2019b; El Haddad et al., 2019; Melo et al., 2020), and indeed would constitute an argument against the use of non-human protein-based drugs generally. In addition, and importantly, there are conceptual as well as empirical arguments against the relevance of these concerns regarding phage therapy.

The conceptual arguments stem from issues of treatment duration and location. Adaptive immunity, at least for individuals who at first are naïve to the phages employed, should require up to weeks to fully develop, resulting in little expectation of substantial impact on efficacy if phage treatments are sufficiently short in duration. Alternatively, the mononuclear phagocyte system is an aspect of innate immunity so will not require substantial physiological development to have a negative impact on phage persistence. Nevertheless, this system is an aspect of microorganism removal from the blood, thus suggesting especially that topical or other more localized phage delivery (Table 3) should not be extensively impacted by this mechanism, nor have a substantial impact on those phages which have left the blood into other tissues, i.e., as following their pharmacokinetic distribution.

Table 3. Efficacy studies involving direct or nearly direct phage application to infections *in vivo*.[8]

Treatment Target[9]	Model Animal-Treatment Studies	# (%)	Human-Treatment Studies	# (%)
Bacteremias	(Capparelli et al., 2007; Oduor et al., 2016a; Oduor et al., 2016b)[2]	3 (4)	(Duplessis et al., 2018; Aslam et al., 2019b)	2 (7)
Burn infections	(Kumari et al., 2011; Chadha et al., 2016)	2 (3)	(Jikia et al., 2005; Marza et al., 2006; Rose et al., 2014; Jault et al., 2019; Patel et al., 2019)	5 (17)
Ear infections	(Marza et al., 2006; Hawkins et al., 2010)	2 (3)	(Weber-Dąbrowska et al., 2006; Wright et al., 2009)	2 (7)
Eye infections (injection or corneal surface treatment)	(Fukuda et al., 2012; Furusawa et al., 2016; Kishimoto et al., 2019)	3 (4)	(Fadlallah et al., 2015)	1 (3)
Gastrointestinal delivery	(Tanji et al., 2005; Capparelli et al., 2006; Raya et al., 2006; Sheng et al., 2006; Niu et al., 2008; Rozema et al., 2009; Bach et al., 2009; Jamalludeen et al., 2009; Mai et al., 2010; Stanford et al., 2010; Maura et al., 2012; Jaiswal et al., 2013; Abdulamir et al., 2014; Jaiswal et al., 2014; Jun et al., 2014; Mai et al., 2015; Nale et al., 2015; Sarker et al., 2016; Nikkhahi et al., 2017; Yen et al., 2017; Zhao et al., 2017; Vahedi et al., 2018; Bhandare et al., 2019; Dallal et al., 2019; Dissanayake et al., 2019; Xue et al., 2020)	26 (36)	(Sarker et al., 2017; Corbellino et al., 2019)	2 (7)
Injection into or near local infections	(Wills et al., 2005; Gill et al., 2006; Capparelli et al., 2007; Bhowmick et al., 2009; Alam et al., 2011; Chhibber et al., 2013; Trigo et al., 2013; Basu et al., 2015; Kishor et al., 2016; Breyne et al., 2017; Chhibber et al., 2017; Yin et al., 2017; Albac et al., 2020; Geng et al., 2020)[3]	14 (19)	(Chan et al., 2018; Ferry et al., 2018)	2 (7)

Instillation or irrigation in an infection's vicinity		0	(Khawaldeh et al., 2011; Schooley et al., 2017; Bao et al., 2020)	3 (10)
Lower respiratory tract delivery	(Debarbieux et al., 2010; Morello et al., 2011; Henry et al., 2013; Cao et al., 2015a; Cao et al., 2015b; Dufour et al., 2015; Yang et al., 2015; Gu et al., 2016; Pabary et al., 2016; Wang et al., 2016; Waters et al., 2017; Cha et al., 2018; Chang et al., 2018; Forti et al., 2018; Hua et al., 2018; Abd El-Aziz et al., 2019; Anand et al., 2019; Dufour et al., 2019; Jeon et al., 2019; Jeon and Yong, 2019; Ji et al., 2019)	21 (29)	(Kutateladze and Adamia, 2008; Hoyle et al., 2018; Aslam et al., 2019a; Maddocks et al., 2019)	4 (14)
Nasal or sinus treatment	(Drilling et al., 2014; Verstappen et al., 2016)[4]	2 (3)		0
Skin infection		1 (1)	(Zhvania et al., 2017)	1 (3)
Wound infections (other than burn wounds)	(Mendes et al., 2013; Seth et al., 2013; Shivaswamy et al., 2015; Kusradze et al., 2016; Regeimbal et al., 2016; Sarhan and Azzazy, 2017; Yin et al., 2017; Chhibber et al., 2018)	8 (11)	(Rhoads et al., 2009; Fish et al., 2016; Fish et al., 2018; Morozova et al., 2018a; Gupta et al., 2019; Patel et al., 2019; Tkhilaishvili et al., 2019)	7 (24)
Totals		72 (100)		29 (100)

8. Studies listed are limited to those published 2005 or later, in English, and in which mammals were treated.
9. Not explicitly shown is the study by Międzybrodzki *et al.*, 2009, in which phages were applied topically to humans, potentially in a variety though nonetheless not explicitly described contexts.
10. With Capparelli *et al.*, 2007, both phages and bacteria were supplied via i.v. Oduor *et al.*, 2016a and 2016b, were probably i.v. treated with phages but this is not stated explicitly
11. With Capparelli et al., 2007, it may be inferred that phages and bacteria were injected into similar locations toward abscess formation and treatment, respectively.
12. Verstappen et al., 2016, probably treated with phages intranasally but this is not stated explicitly.

Empirically, studies have looked for associations between the presence of neutralizing antibody in the blood and phage therapy outcomes. These studies, however, have failed to find much of a correlation to either presence or absence of phage therapy efficacy (Łusiak-Szelachowska et al., 2014; 2017). For recent reviews of phage-immune system interactions, see (Dąbrowska et al., 2018; Dąbrowska, 2019; Dąbrowska and Abedon, 2019; Van Belleghem et al., 2019; Żaczek et al., 2019).

Phage movement into and out of the blood

Movement of phages into blood, i.e., absorption, is an important issue for a variety of reasons. First, it is a primary concern in the pharmacokinetic analysis of drugs in general. That is, essentially the default pathway that drugs take through the body in terms of systemic delivery can be broken down into application that is followed by absorption and only then distribution. Second, blood has the utility of being easily sampled, though plasma drug levels are not always the most meaningful measure of drug pharmacokinetics (Levison and Levison, 2009). Nonetheless, time courses of drug movement especially into and out of the blood can be easily obtained, particularly from larger animals and without animal sacrifice. The result not only is greatly reduced investigation expense but also ready determination of this aspect of drug pharmacokinetics in humans. Three, for targets that are limited to within systemic circulation, drug access to the blood is of course of primary concern. Lastly, to study drug distribution, that is, out of the blood and into other tissues, it is essential to first deliver a drug to the blood. For a general review of these various issues of phage movement within the body, see (Dąbrowska, 2019; Dąbrowska and Abedon, 2019). Nevertheless, absorption is relevant to phage therapy efficacy only to the extent that phages either cannot or are not delivered to sites of bacterial infection more directly.

There exist a variety of pathways by which absorption can be initiated, the most straightforward of which is intravenous (i.v.) delivery. Such parenteral delivery not only results in direct application to systemic circulation but also in principle can be applied at such a rate so as to achieve steady state plasma levels (Levison and Levison, 2009). I.v. application, especially, should be employed for the study of phage distribution – movement out of the blood into different locations – that is unless there is suspicion that alternative absorption protocols could provide qualitatively different phage distribution patterns. This point is also relevant for issues of decline in phage concentrations in the blood, where if phages are not first delivered directly to the blood then it may be difficult to distinguish slow phage movement into the blood from fast phage movement out of the blood (Uchiyama et al., 2009).

While i.v. delivery provides an obvious simplification of absorption, particularly in terms of the study of subsequent distribution, it is not necessarily always ideal for phage delivery, though it certainly can be useful when appropriate. Concerns include its overly invasive nature, the rapidity with which absorption is achieved (though nonetheless this can also be as slow as desired), concerns associated with carryover of contaminating bacterial toxins such as endotoxin directly into the blood (Carlton, 1999; Boratyński et al., 2004; Merril et al., 2006; Gill and Hyman, 2010), and a lack of convenience, particularly in limiting phage delivery to clinical settings. As a consequence, alternative approaches to phage absorption have been developed by various groups. See, though, Speck and Smithyman (2016) for arguments in favor of the use of i.v. delivery of phages for phage therapy and Table 4 for a listing of numerous phage therapy efficacy studies involving i.v., as well as other means of indirect phage dosing.

In terms of subsequent distribution, the more body compartments a drug must pass through to reach its site of activity, then the more steps during which drug concentration can be reduced (Levison and Levison, 2009). These reductions can be due simply to dilution or instead as a consequence of “translocation” inefficiencies. As a result, fewer phages may reach their target upon distribution than are required for treatment success, which in a worst-case scenario could mean no phages at all reaching target bacteria. In addition, effective concentrations such as for chemical antibiotics even following successful absorption can be difficult to achieve due poor blood circulation such as to burns (Ahmad, 2002), osteomyelitis, or diabetic ulcers (Stone, 2002). Therefore, the greater the number of phages that must be delivered to target bacteria to achieve desired levels of bacteria killing or control, then either the more directly phages must be applied to infections (Morozova et al., 2018b) (Table 3), the more phages that must be supplied to the patient overall, or both. Treatment failures, that is, can be due to insufficient numbers of phages encountering target bacteria rather than because of poor intrinsic phage potential to clear bacterial infections. It is crucial for researchers, therefore, to employ pharmacologically rigorous follow up should they observe disappointing phage therapy performance. In particular, though not necessarily easily determined,

there can be a need to know whether adequate numbers of dosed phages have reached target bacteria or, instead, whether local phage replication might have been inadequate to compensate.

Table 4. Efficacy studies involving indirect phage application, i.e., as toward systemic delivery *in vivo*.[13]

Application Approach[14]	**Model Animal-Treatment Studies**	**# (%)**	**Human-Treatment Studies**	**# (%)**
Intramuscular (i.m.)	(McVay et al., 2007; Bull et al., 2012; Ibrahim et al., 2016; Chhibber et al., 2017)[15]	4 (7)		0
Intraperitoneal (i.p.)[16]	(Matsuda et al., 2005; Vinodkumar et al., 2005; Wang et al., 2006a; Wang et al., 2006b; Capparelli et al., 2006; McVay et al., 2007; Chhibber et al., 2008; Nishikawa et al., 2008; Uchiyama et al., 2008; Vinodkumar et al., 2008; Kumari et al., 2009a; Kumari et al., 2009b; Zimecki et al., 2009; Carmody et al., 2010; Kumari et al., 2010; Sunagar et al., 2010; Zimecki et al., 2010; Hung et al., 2011; Tothova et al., 2011; Jun et al., 2014; Shivshetty et al., 2014; Takemura-Uchiyama et al., 2014; Pincus et al., 2015; Singla et al., 2015; Chai et al., 2016; Guang-Han et al., 2016; Regeimbal et al., 2016; Chadha et al., 2017; Cheng et al., 2017; Green et al., 2017; Yin et al., 2017; Can et al., 2018; Gelman et al., 2018; Jasim et al., 2018; Wang et al., 2018; Chen et al., 2019; Jeon and Yong, 2019; Kaabi and Musafer, 2019; Leshkasheli et al., 2019; Morris et al., 2019; Tang et al., 2019; Yuan et al., 2019; Alvi et al., 2020)[17]	43 (80)		0
Intrarectal		0	(Letkiewicz et al., 2009; Corbellino et al., 2019)	2 (12)
Intravenous (i.v.)	(Danelishvili et al., 2006; Oechslin et al., 2017; Prazak et al., 2019)	3 (6)	(Fadlallah et al., 2015; Jennes et al., 2017; Schooley et al., 2017; Duplessis et al., 2018; Aslam et al., 2019b; Dedrick et al., 2019; Gilbey et al., 2019; Law et al., 2019; Maddocks et al., 2019; Nir-Paz et al., 2019)	10 (59)

Per os	(Hung et al., 2011)	1 (2)	(Leszczyński et al., 2006; Weber-Dąbrowska et al., 2006; Międzybrodzki et al., 2009; Zhvania et al., 2017; Corbellino et al., 2019)[18]	5 (29)
Subcutaneous (s.c.)	(McVay et al., 2007; Gupta and Prasad, 2010)[19]	2 (4)		0
Transdermal	(Rastogi et al., 2017)	1 (2)		0
Totals		54 (100)		17 (100)

13. Studies listed are limited to those published 2005 or later, in English, and in which mammals were treated.
14. See Table 3 for a summary of the use of more direct phage application strategies.
15. Bull et al., 2012, specifically indicate that phages were inoculated into “opposite thighs” relative to application of the bacterial challenge
16. Studies are included in this list whether or not the bacterial challenge was also i.p. delivered, though that mostly was not the case. It also has not escaped our attention the extreme discrepancies seen between pre-clinical and clinical use of the i.p. route of phage application
17. Jeon and Yong, 2019, probably i.p. treated with phages but this is not stated explicitly.
18. While the Międzybrodzki et al., 2009, study is it presented as an efficacy study, it nevertheless piggybacked upon phage therapy treatments intended to be efficacious.
19. For McVay et al., 2007, it is not clear that phages were not applied in the immediate vicinity of the s.c. bacterial challenge, though the relatively poor efficacy especially in comparison with i.p. phage application is suggestive that such co-location of bacterial challenge and phage treatment was not the case.

Conclusion

Pharmacology is the study of drug impact on bodies as well as body impact on drugs, where body is defined broadly to include normal microbiota and bacterial pathogens as well as normal body tissues. With phage therapy, however, pharmacology is complicated due to the potential for phages to replicate and also due to the large size and complexity of their virions. The latter, for example, makes the phage ability to translocate across certain tissues both somewhat surprising and less predictable. Though the phage potential to replicate *in situ* is a conceptual complication on their pharmacology, alternatively this ability can serve to reduce the importance of greatly understanding certain other aspects of their pharmacology, such as more quantitative measures of phage competence

to move between body compartments. More important perhaps than even their ability to replicate, however, is that the use of phages appears to be fairly safe, usually generating at worst only minimal side effects. Notwithstanding this phage potential to amplify their numbers *in situ* and seeming ability to do so rather safely, it is desirable for the sake of improvement of efficacy, regulatory approval, and simply rigorous experimentation that pharmacological considerations not be ignored during phage therapy development. In short, it is important to know *in situ* and over time where in bodies phages are found, at what titres, and to what if any antibacterial or toxic effects. It is especially from a better understanding of these basic pharmacological measures that the effectiveness of phage therapy approaches may be improved.

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