Pharmacokinetics/Pharmacodynamics of Antipseudomonal Bacteriophage Therapy in Rats: A Proof-of-Concept Study

Yu-Wei Lin[1*], Rachel Yoon Kyung Chang[2], Gauri G. Rao[3], Brian Jermain[3], Mei-Ling Han[1], Jinxin Zhao[1], Ke Chen[1], Jiping Wang[1], Jeremy J. Barr[4], Robert Turner Schooley[5], Elizabeth Kutter[6], Hak-Kim Chan[2], Jian Li[1*]

[1]Biomedicine Discovery Institute, Department of Microbiology, Monash University, Clayton, Victoria 3800, Australia;

[2]Advanced Drug Delivery Group, School of Pharmacy, The University of Sydney Faculty of Medicine and Health, Sydney, NSW 2006, Australia;

[3]Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina, USA;

[4]School of Biological Sciences, Monash University, 25 Rainforest Walk, Clayton, Victoria, 3800, Australia;

[5]Division of Infectious Diseases and Global Public Health, Department of Medicine, University of California San Diego, La Jolla, California, USA;

[6]The Evergreen State College, Olympia, Washington 98502, USA.

*Joint corresponding authors: Jian Li, telephone: +61 3 9903 9702, fax: +61 3 9905 6450, Email: jian.li@monash.edu and Yu-Wei Lin, telephone: +61 433 973 697, fax: +61 3 9905 6450, Email: yu-wei.lin@monash.edu.

Running Title: PK/PD of Phage Therapy

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Abstract

Objectives: Pandrug-resistant (PDR) *Pseudomonas aeruginosa* is one of the three top-priority pathogens identified by the WHO and bacteriophages have been investigated as an alternative therapy. However, knowledge on the pharmacokinetics/pharmacodynamics (PK/PD) of phage therapy is sparse, limiting its clinical applications. This study aimed to evaluate the PK/PD of an antipseudomonal phage øPEV20 *in vivo* following intravenous administration.

Methods: Healthy Sprague-Dawley rats were given øPEV20 as a single intravenous bolus of ~6, 9, and 11-$\log_{10}$PFU/Rat. Blood was sampled from artery across 72 h. At 72 h, the animals were sacrificed and multiple tissues were harvested for biodistribution study. A PK model was developed using Importance Sampling Algorithm and deterministic simulations with PD models were performed.

Results: A three-compartment model with nonlinear clearance described the exposure of øPEV20 in blood. Model evaluation indicated that the model was robust and parameter estimates were accurate. The median (standard error) values of model-predicted PK parameters for V_C, V_{P1}, V_{P2}, Q_1, Q_2, V_m and K_m were 111 mL/Rat (8.5%), 128 mL/Rat (4.97%), 180 mL/Rat (4.59%), 30.4 mL/h/Rat (19.2%), 538 mL/h/Rat (4.97%), 4.39×10^{10} PFU/h/Rat (10.2%), and 1.64×10^{7} PFU/mL/Rat (3.6%), respectively. The distribution of øPEV20 was not homogeneous with preferential accumulation in the liver and spleen. Deterministic simulations with a PD model confirmed the importance of host immune system in facilitating phage-mediated bacterial elimination.

Conclusions: We developed a robust PK model to describe the disposition of phages in healthy rats. This model may have significant potential in facilitating future preclinical and clinical

PK/PD investigations.

Introduction

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* has been identified by the World Health Organization as one of the 3 top-priority pathogens urgently requiring novel therapeutics [1]. Recently, attention has turned to bacteriophages (phages) as an alternative to conventional antibiotics [2]. Phage therapy was first experimented in 1910s but was largely forgotten in the Western world with the advent of antibiotics in the 1940s [3]. Phage therapy has continued to be refined and is used to treat bacterial infections in the former Soviet Union and Eastern Europe [3]. Recent studies have shown that phage therapy is effective against bacterial infections in mice and patients and, in some cases, is more effective than antibiotics in preventing mortality due to MDR infections [4]. The FDA has recently approved the first human clinical trial of intravenous phage therapy against *Pseudomonas aeruginsa*, and other bacterial species [5-7].

There has been no systematic investigation into phage pharmacokinetics/pharmacodynamics (PK/PD) which has severely limited its clinical utility. This project aimed to assess the bactericidal acitivty of a novel phage (øPEV20) against PDR *P. aeruginosa*, develop a PK model to characterise the disposition of øPEV20 in healthy rats, and conduct deterministic simulations to evaluate its bactericidal acitivty in the presence or absence of host immune system. PK was conducted in a rat model to allow for a better estimation of PK parameters, while the PD study was conducted in a mouse model as it is the most widely used infection model in antimicrobial PK/PD research.

Methods and Material

Bacteria and Bacteriophages

A polymyxin-resistant pandrug-resistant (PDR) isolate *P. aeruginosa* 1121 was employed in the PD studies. Anti-*pseudomonal* øPEV20 was isolated from the sewage treatment plant in Olympia (WA, USA) using *P. aeruginosa* ADDG which was isolated from a cystic fibrosis patient [8]. øPEV20 is a podovirus with a 91-kb genome and a capsid size of 60 nm [8]. øPEV20 was amplified and purified using Phage-on-Tap protocol [9]. *P. aeruginosa* ADDG was employed as a reference bacterial strain to enumerate phage concentration in biological samples using a plaque assay [10].

Animals

All animal experiments were approved by the Monash Animal Ethics Committee, Monash University and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Female Swiss mice (8 weeks, weight 28-35 g, total n=14) were employed for the PD studies, while male Sprague-Dawley rats (300-350 g, n=3 per dose group) were employed for the single-dose PK studies.

Pharmacodynamics of øPEV20

The antimicrobial efficacy of øPEV20 against PDR *P. aeruginosa* 1121 was evaluated in a mouse bacteremia model. On day 0, mice were injected with 50 µL of an early-logarithmic-phase bacterial suspension (*P. aeruginosa* 1121, ~10^{10} CFU/mL) into systemic circulation to achieve an inoculum of ~10^9 CFU/mL. øPEV20 were intravenously administered at 1 h post-inoculation. The dosage regimens involved once daily administration of approximately 2, 3, 4, 5, and 8 $\log_{10}$ PFU/Mouse. Infected mice treated with sterile saline were included as growth

controls. At 0 and 2.5 h post-phage administration, mice were humanely killed and blood was collected via cardiac puncture for quantitation of bacterial cells. The limit of detection was 20 CFU/mL (equivalent to one colony per plate).

PK and biodistribution studies in rats

A single-dose PK study was performed in healthy rats following an intravenous bolus of øPEV20 (~6, 9 and 11-$\log_{10}$PFU/Rat). The jugular vein and caroitid artery of rats were cannulated for intravenous phage administration and blood collection, respectively [11]. Blood samples were collected at 10 and 30 min, and 1, 2, 4, 8, 12, 24, 48, and/or 72 h post-administration. At 48 or 72 h, rats were humanely killed and tissues (i.e. lung, kidney, spleen and liver) were harvested and homogenised in saline. Urine samples were collected during 0-24, 24-48 and/or 48-72 h, and urinary recovery was calculated by dividing phage titer at respective time points by the administered dose. Phage enumeration was performed using a plaque assay [10]. The limit of phage detection (LOD) was 1.70 $\log_{10}$PFU/mL and the limit of phage quantification was 2.20 $\log_{10}$PFU/mL (i.e. 3 PFU per plate).

PK model

The disposition of øPEV20 in blood following intravenous administration in healthy rats was characterised using a nonlinear mixed effects modelling approach. Two- and three-compartment PK models with linear and non-linear clearance were evaluated using the Importance Sampling algorithm (pmethod = 4) in S-ADAPT (version 1.57) facilitated by S-ADAPT TRAN [12]. Model discrimation was performed using log-likelihood ratio test for nested models and AIC for non-nested models. The final model was evaluated using the goodness-of-fit plots and visual predictive checks. Information on the development of PK

model is in the Supplementary Materials.

Deterministic simulations

The final parameters estimated from the current PK model and the previously reported phage-saturation-resistance (PS-R) model were employed to predict the *in vivo* efficacy of øPEV20 in the presence and absence of the immune system [13]. Deterministic simulations were performed using the model-predicted median value of øPEV20 blood concentrations and PD parameters in R (V3.6.4) without inter-animal or residual variabilities. PK parameters were fixed as outlined in Table 1, while PD parameters were fixed to the previously reported values [13] (Table S1). The two models were connected by replacing the first-order phage decay rate constant in the PD model by the current PK model. The following once-daily dosage regimens were simulated: (1) 6-$\log_{10}$PFU/g, (2) 9-$\log_{10}$PFU/g, and (3) 11-$\log_{10}$PFU/g. The initial bacterial inoculum was 7.4×10^7 CFU/g. Simulations were performed under assumptions that (1) øPEV20 exhibited similar absorption and lysis kinetics as PAK_P1 phages against PAK1 [13, 14], (2) phage killing efficacy remained the same between different infection models, and (3) only øPEV20 remained in the central compartment could infect and lyse bacterial cells (i.e. those in the peripheral compartments were inactive).

Results

PD of øPEV20 phage therapy

Figure 1 shows the antimicrobial efficacy of øPEV20 against PDR *P. aeruginosa* 1121 in a mouse bacteremia model. Intravenous administration of øPEV20 at a dose of $>10^4$PFU/mouse

resulted in rapid bacterial killing and >8-log_{10}CFU/mL reduction in bacterial load (compared with the initial inoculum and control at 2.5h) was observed at 2.5h post-treatment. øPEV20 at a dose of <10^4PFU/mouse was ineffective against PDR *P. aeruginosa* 1121.

PK of PEV20 in rats

Figure 2 shows the concentration-time profiles of øPEV20 following intravenous administration of ~6-, 9- and 11-log_{10}PFU/Rat. After treatment, the C_{max} was 2.60±0.26, 6.04±0.62 and 8.22±0.27-log_{10}PFU/mL, respectively. A short distribution phase of 2-4 h was evident in the 9- and 11-log_{10}PFU/Rat groups. Interestingly, there was an observed disproportionate decrease in the CL with increasing phage doses, which demonstrated potential nonlinearity in the elimination of øPEV20 in healthy rats . Our phage PK analysis also revealed that phages preferentially distributed in the liver and spleen of rats at 48 h or 72 h (Figure 3). An extremely minimal proportion of the øPEV20 dose was excreted in the urine with a total recovery of <0.00002% observed across 72 h post-administration for all three doses.

The PK profile of øPEV20 in blood following intravenous administration was well described by a three-compartment model with minor model misspefication (OBJ = 9.73 *versus* >16 for two-compartment model) (Figure 4). The model-estimated PK parameters are presented in Table 1, and model-predicted individual plots are included in Figure S1 in the Supplementary Information. Elimination was saturable, described by a nonlinear clearance and originated only from the central blood compartment. IIV was fixed to 15% for all PK parameters and only

estimated for V_{max}.

The circulation factor was calculated by dividing the observed phage titer by the expected phage titer (Figure 5). The range of circulation factors for the low, medium, and high doses at 10 min was 0.015 to 0.045, 0.017 to 0.303, and 0.379 to 1.25, respectively.

Deterministic simulations

The developed PK model in rats was combined with a previous PD model to evaluate the antimicrobial efficacy of øPEV20 in the presence or absence of the immune system [13]. Deterministic simulations with the PD model predicted that in the absence of the immune system, øPEV20 could infect and control phage-sensitive bacterial subpopulation from amplifying within the first 24-36 h (Figure 6). Importantly, emergence of phage-resistant bacterial subpopulations was predicted. In immunocompetent hosts, phage therapy eradicated phage-sensitive bacterial subpopulation within 36 h, while the immune system prevented the emergence of phage-resistant bacterial subpopulation.

Discussion

In the present study, the PD of intravenously administered øPEV20 was evaluated against PDR *P. aeruginosa* 1121 using a neutropenic bacteraemia model. Consistent with our previous findings [15], øPEV20 exerted significant antimicrobial killing against *P. aeruginosa* 1121 with >6-$\log_{10}$ reduction in bacterial load at 2.5 h post-treatment (Figure 1). This study provides preliminary evidence to support the use of øPEV20 against life-threatening bacteremia

caused by PDR *P. aeruginosa*.

To investigate the PK of øPEV20 in rats following intravenous administration, a PK model was constructed. The three-compartment model adequately described øPEV20 disposition with some minor model misspecfication (Figure 4 and S1; OBJ = 9.73). The central compartment represents a composite volume of systemic circulation as well as the volume of immune cells and organs that rapidly sequester øPEV20. The use of the three-compartment model is consistent with our biodistribution results and previous findings in healthy and neutropenic mice that illustrate a large proportion of phages were distributed into peripheral tissues [16-19]. Phages bind to immune cells (and to some extent erythrocytes), are engulfed by phagocytes, and are uptaken by epithelial cells [18, 20-22]. The estimated central volume of distribution (V_c) in the PK model was larger than the physiological volume of a rat, indicating significant and rapid binding of øPEV20 to immune cells or significant and rapid uptake by organs, immune, and/or epithelial cells [18, 20, 22]. It is highly likely that there is a rapid, saturable process resulting in the sequestering of phages *in vivo*. This was supported by our increasing circulation factor with increasing doses, as larger doses were less affected by this initial saturable process (Figure 5) [18].

The PK of øPEV20 in healthy rats was nonlinear following intravenous administration (Figure 2). The immune system, particularly the mononuclear phagocyte system, has been shown to play an important role in phage clearance [18, 23]. It was hypothesised that phage clearance is mostly the result of phagocytosis of phage particles in the liver and, to a lesser extent, in the spleen [18, 24]; the results of our biodistribution study well support the hypothesis in the

current literature (Figure 3). Phage accumulation was evident in the spleen and liver at 48 and 72 h, given that both organs are important elements of the mononuclear phagocytic system (Figure 3). Because only 0.00002% of phages were recovered in the urine within 72 h, renal secretion plays a negligible role in the elimination, most likely because of the large size (24-200 nm) of the virus particles. The low urinary recovery of phages in rats is consistent with the results of dogs and humans following intravenous administration [25-27]. These results suggest that intravenous administration of phages may not be efficacious for urinary tract infections. Interestingly, phage penetration into bladders is highly dependent on the dose administered, and 10^9 PFU/mouse was the minimum dose required to allow for phage detection in urine following intravenous administration [28]. This dose-dependent behaviour was not observed in healthy rats, since phage recovery in urine was negligible for all doses across the study period. Therefore, the nonlinear clearance is most likely related to the saturation of immune clearance; and in the present study it was well described with the Michaelis–Menten kinetics model (AIC = 23.7). Replacement of Michaelis–Menten kinetics with first-order clearance resulted in a model with a much lower OBJ (AIC = 30.0) and poorer fits. Furthermore, similar to monoclonal antibodies which have a high affinity to ligands, phage binding to immune cells most likely affects their disposition *in vivo* (Figure 2). Future PK studies charactertising the affinity of phages to key immune cells are urgently needed. An in-depth understanding of their binding affinity and clearance mechanisms will help refine our PK model.

Collectively, the PK/PD results suggest that intravenous administration of phages is effective against severe bacteremia caused by PDR *P. aeruginosa*, and phage disposition follows a standard three-compartment kinetic process in the healthy rat model. It should be noted,

however, that phage PK very likely differs in infected individuals because of phage self-replication in susceptible bacterial cells [18, 29]. Further preclinical PK studies are urgently needed to fully characterise phage disposition in infected animals. Such data can be incorporated into the proposed PK model to simultaneously describe the disposition of phages in healthy and infected individuals.

To evaluate the effect of the immunological states of the host on the efficacy of phage therapy, we combined the developed PK model with a previously developed PD model to predict the PD of øPEV20 in the presence or absence of an immune system [13]. Two potential PD models exist: PS-R and heterogenous mixture model (HM-R). The two models differed in the mechanism that underlies the rate of sublinear phage lysis. This rate was accounted for in the PS–R model by including a phage-saturation function. HM-R was not selected for simulations in the present study, because it was developed to describe phage lysis in the presence of spatial heterogeneity in the mouse respiratory tract. Our PK/PD simulations predicted a curative outcome at time >60 h in immunocompetent hosts despite the transient presence of phage-resistant bacteria (Figure 6). Consistent with previous findings, infection clearance requires the synergy of both the phage and host immunity, which is clinically significant because most preclinical studies are conducted in rodents which have immune systems significantly different from that in humans [13]. Interestingly, based on our simulation results, complete eradication of infection could be achieved only >12 h after treatment. This slow clearance timescale was inconsistent with our *in vivo* PD observations in mice, in which >6-$\log_{10}$ reduction in bacterial load was observed 2.5 h after treatment (Figure 1). This inconsistency is most likely a result of interspecies differences in terms of PK and the

assumptions made when performing the simulations. Mechanism-based PK/PD models will provide a superior preditivie power of the antimicrobial efficacy of phage therapy than the previously developed PD model. Considering the critical role of the immune system in the elimination of øPEV20, the phage PK is likely to be different in immune-deficient hosts. Interestingly, no signifcant differences were observed following intravenous administration of phages in healthy and cyclophosphamide-induced neutropenic mice [16]. Systems biology studies are currently conducted in our laboratory to elucidate the mechanism of phage clearance in biological systems.

To the best of our knowledge, this is the first PK model that describes the disposition of phages in blood in rats. With the increasing incidence of disseminated PDR infections and the lack of effective antibiotics, phage therapy is increasinly investigated as a therapeutic alternative. This study highlights the potential therapeutic advantages of phage therapy in treating life-threatening PDR infections. Ultimately, the developed PK model provides an important tool for future phage preclinical PK/PD studies.

Conflict of Interest

The authors declare no conflict of interest. Dr Yu-Wei Lin currently is an employee of Certara, Australia.

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Author contributions

Y-W.L. and J.L. conceived the project and all authors were involved in the design of the experiments. Y-W.L., R.Y.K.C., M-L.H., J.Z., K.C. and J.W. performed the experiments, and Y-W.L., G.R., B.J., J.J.B., R.T.S., E.K. and H-K.C. analysed the results. All authors reviewed the manuscript.

Figure Captions

Figure 1. Efficacy of intravenous administration of øPEV20 against bacteremia caused by PDR *P. aeruginosa* 1121. The limit of detection was 1.30 $\log_{10}$CFU/mL. 0 h corresponds to post-phage administration which is equivalent to 1 h post-bacteria inoculation. 2.5 h corresponds to 2.5 h post-administration which is equivalent to 3.5 h post-bacterial inoculation. Solid lines represent the geometric mean, each symbol represents the observed bacterial load.

Figure 2. øPEV20 concentration-time profiles after intravenous administration of øPEV20 in healthy rats. Each symbol represents the geometric mean ± standard deviation (n = 3). The limit of detection was 1.70 $\log_{10}$PFU/mL.

Figure 3. Biodistribution of øPEV20 following intravenous administration of **(A)** ~9-$\log_{10}$PFU/Rat øPEV20 and **(B)** ~11-$\log_{10}$PFU/Rat øPEV20 in healthy rats at 48 and 72 h, respectively. % recovery was calculated by dividing phage titer at the respective time point by the administered dose. n = 3 rats per dose level per time point.

Figure 4. Visual predictive checks for øPEV20 PK profiles in blood following intravenous administration of **(A)** 6-, **(B)** 9- and **(C)** 11-logPFU/Rat. The solid line represents the median model-predicted concentrations (P50); the broken lines represent the model predicted 10^{th} (P10) and 90^{th} (P90) percentile. The solid dots represent the observed øPEV20 concentration. n = 3 rats per dose level.

315 **Figure 5.** Circulation factor calculated by dividing the observed concentration at 10 min (black)
316 and 30 min (grey) by the expected concentration. The expected concentration was estimated
317 by dividing the administered dose by the hypothetical dilution of phage based on the body
318 weight of the rats. Each symbol represents the mean ± standard deviation (n = 3).

319

320 **Figure 6.** Predicted population densities of øPEV20, sensitive-bacteria, resistant-bacteria and
321 host immunity in the presence or absence of immune system using the developed PK model
322 and a previously developed phage saturation-resistance (PS-R) model. Simulations were
323 performed under the assumption that only øPEV20 remained in the central compartment
324 could infect sensitive-bacterial subpopulation.

325

326 **Table 1.** Estimated PK parameters and the associated standard error expressed as a
327 percentage (SE (%)) for øPEV20 following intravenous administration in healthy rats.

Parameter	Estimated	SE (%)
Q1 (mL/h/Rat)	30.4	19.2
Q2 (mL/h/Rat)	538	4.97
K_m (PFU/mL/Rat)	1.64×10^7	3.6
V_c (mL/Rat)	111	8.5
V_{P1} (mL/Rat)	128	4.97
V_{P2} (mL/Rat)	180	4.59
V_{max} (PFU/h/Rat)	4.39×10^{10}	10.2
SD_{PFU}	0.58	10.9

328

329 The inter-animal variability (IIV) was set to a coefficient of variation of 15% for all parameters
330 except for V_{max} which was estimated to be 34.8% (standard error 60.6%).

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