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Establishment of a diverse pheno-genotypic challenge set of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* suitable for use in the murine pneumonia model

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Background: Preclinical murine infection models lack inter-laboratory uniformity, complicating result comparisons and data reproducibility. The European Innovative Medicines initiative-funded consortium (COMBINE) has developed a standardized murine neutropenic pneumonia protocol to address these concerns. While model methods have been standardized, a major obstacle to consistent results is the lack of available bacteria with defined viability and variability. Herein, we establish a diverse challenge set of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* suitable for use in the COMBINE protocol to further minimize experimental inconsistency and improve the interpretability of data generated among differing laboratories.

Materials and methods: Sixty-six *K. pneumoniae* and 65 *P. aeruginosa* were phenotypically profiled against tigecycline (*K. pneumoniae* only), levofloxacin, meropenem, cefiderocol and tobramycin. Fifty-nine isolates were introduced into the COMBINE model to assess the sufficiency of the starting bacterial inoculation, resultant baseline bacterial burden, achievement of ≥1 $\log_{10}$cfu/lung growth at 24 h, time to and percentage mortality. Forty-five isolates displaying desirable minimum inhibitory concentration profiles were subjected to replicate *in vivo* testing to assess target parameters.

Results: 83% of *K. pneumoniae* reached the prerequisite growth at 24 h using a starting bacterial burden ≥7 $\log_{10}$cfu/lung. *P. aeruginosa* isolates grew well in the model: 90% achieved the growth target with a starting bacterial burden of 6 $\log_{10}$cfu/lung. Mortality was negligible for *K. pneumoniae* but high for *P. aeruginosa*. Poor or inconsistent achievement of the 24 h growth target was seen in 11/59 isolates.

Conclusions: With this diverse cache of viable isolates established in the COMBINE pneumonia model, future translational studies can be undertaken to set efficacy benchmarks among laboratories.

Background

Preclinical murine infection models play an integral role in the development of direct-acting antibiotics by assessing efficacy and informing optimized dosing regimens through pharmacokinetic and pharmacodynamic analyses.[1–3] Despite decades of use in supporting drug development, there remains a lack of uniformity in murine models that makes comparisons of results and data reproducibility problematic, calling into question clinical translation.[4–7] While on the surface the historic use of *in vivo* murine pneumonia models may seem more similar than divergent, numerous variables often differ regarding the animals, bacteria, infection procedure, treatment and endpoints. Animals may have differences in strain, sex, age, weight and number per treatment group. The differing phenotypic and genotypic profiles and intrinsic virulence of the bacteria as well as the method of inoculum preparation (i.e. growth stage, bacterial density) can substantially alter the effectiveness of the test agent. Infection procedures vary in immunosuppression, anaesthesia and infection route. Dissimilarities exist in treatment outcomes relating to the time to initiation of treatment, baseline bacterial density, *in vivo* growth characteristics of the bacteria, drug exposure (pharmacokinetic/pharmacodynamic, PK/PD) profile at target site and duration of therapy. Importantly for a pneumonia indication, antibiotic exposures at the target site of infection, pulmonary epithelial lining fluid (ELF), are not consistently characterized. Instead, plasma concentrations are measured as a surrogate with little consideration for interspecies differences in target site penetration between mouse and man.[8]

A standardized global protocol has been established by the Collaboration for prevention and treatment of MDR bacterial infections (COMBINE) consortium in hopes of harmonizing the methods utilized in the murine pneumonia model.[9] While adherence to the COMBINE protocol will assist in narrowing the gap in inter-laboratory variability, a major impediment to consistency in results and subsequent interpretations among these *in vivo* studies is the lack of available bacteria with defined phenotypic and genotypic profiles that reproducibly induce infection under the stipulated constructs of the COMBINE protocol. The goal of the current investigation is to establish a diverse challenge set of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* suitable for use in the COMBINE protocol to further minimize experimental inconsistency and improve the interpretability of endpoint data such as quantitative cfu or pharmacodynamic profiling among differing laboratories.

Materials and methods

Antimicrobial agents

Analytical grade powders were acquired as follows for *in vitro* broth microdilution testing: tigecycline (Supelco, lot no. LRAD6890), levofloxacin (Sigma Aldrich, lot no. BCCF6845), meropenem (Supelco, lot no. LRAD1014) and tobramycin (Supelco, lot no. LRAC7886). For cefiderocol, commercial vials were reconstituted for *in vitro* broth microdilution testing (Shionogi, lot no. 0021).

Bacterial isolates and *in vitro* susceptibility testing

K. pneumoniae and *P. aeruginosa* isolates were sourced from the isolate repository at the Center for Anti-Infective Research and Development (CAIRD) (Hartford, CT, USA), the CDC (Centers for Disease Control and Prevention) and FDA (Food and Drug Administration) Antibiotic Resistance Isolate Bank (CDC Bank) (Atlanta, GA, USA), the Paul Ehrlich Institute (PEI) (Berlin, Germany) and the Leibniz Institute (DSMZ) (Brunswick, Germany). These contemporary clinical isolates, including those from COMBINE consortium sites PEI and DSMZ, were selected to encompass a variety of genotypic and phenotypic profiles resulting in a wide distribution of minimum inhibitory concentrations (MICs) against tigecycline, levofloxacin, meropenem, cefiderocol and tobramycin. These five agents have been selected because they represent differing drug classes: tetracycline-derivative, fluoroquinolone, β-lactam, siderophore-conjugate and aminoglycoside. In addition to differing mechanisms of action/resistance, this set of compounds represents divergence in both their pharmacokinetic and pharmacodynamic profiles, thus serving as ideal benchmarking therapies for the future assessment of novel agents in this humanized translational model.

Before experimentation (both *in vitro* and *in vivo*), each isolate was sub-cultured twice on Trypticase soy agar with 5% sheep blood (Becton Dickinson and Co., Sparks, MD, USA) and incubated at 37°C for ∼16 h. In total, 66 *K. pneumoniae* and 65 *P. aeruginosa* isolates were tested by broth microdilution on separate days in triplicate or until a modal value was achieved in accordance with Clinical and Laboratory Standards Institute guidance before introduction into the murine neutropenic pneumonia model.[10] Tigecycline was only tested against *K. pneumoniae* isolates due to its lack of activity against *P. aeruginosa*. *Enterococcus faecalis* 29212 (levofloxacin MIC range 0.25–2 mg/L; meropenem MIC range 2–8 mg/L) and *Escherichia coli* 25922 (tigecycline MIC range 0.03–0.25 mg/L; cefiderocol MIC range 0.06–0.5 mg/L; tobramycin MIC range 0.25–1 mg/L) were used as quality control strains on each day of study.[11] Colony counts were performed from the control well of every replicate in accordance with CLSI methods.[10] If the colony count and/or the QC was out of range, the MIC data were discarded and repeated.

Ethics

Animals were maintained and used in accordance with National Research Council recommendations. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Hartford Hospital (Assurance no. A3185-01).

Laboratory animals and the neutropenic pneumonia model

The model followed the COMBINE protocol with laboratory-specific detailed methods as follows.[9] Specific pathogen free CD-1, female mice 6–8 weeks old were acquired from Charles River Laboratories, Inc. (Raleigh, NC, USA). All animals were allowed to acclimatize for 72 h before any study procedures and were housed in groups of six at controlled room temperature in HEPA-filtered cages (Innovive, San Diego, CA, USA). Study rooms were maintained with diurnal cycles (12 h light/12 h dark) and food and water were provided *ad libitum*.

A predictable degree of renal impairment was produced using 5 mg/kg of uranyl nitrate administered intraperitoneal on day −3.[12] Bacterial colonies from the overnight culture plate were suspended in NS to a McFarland target of 2.5 and further diluted in saline to produce the final inoculum. Mice were anaesthetized using inhaled isoflurane, manually restrained upright and infected with 50 µL of bacterial suspension via the nares. Each bacterial inoculation suspension was used within 30 mins of initial preparation and was serially diluted and plated to confirm the cfu/mL.

Two hours after bacterial inoculation, 3–6 mice per isolate were sacrificed via CO_2 asphyxiation followed by cervical dislocation to determine initial bacterial burden in the model. An additional six mice per isolate were euthanized 24 h later, or on earlier infection-related mortality or loss of the righting reflex. Lungs from each mouse were harvested aseptically and homogenized. Homogenates were serially diluted and plated on Trypticase soy agar with 5% sheep blood and incubated at 37°C for ∼16 h before cfu enumeration. Inoculum bacterial suspension cfu/mL, baseline bacterial burden cfu/lungs, cfu/lung at 24 h, time to and percentage mortality over the 24 h post-inoculation period before scheduled harvest time were quantified for each isolate.

Selection of isolates for initial and replicate *in vivo* testing and data analysis

For consideration of inclusion in the *in vivo* testing system, a modal MIC value had to be established for each organism using the gold standard, broth microdilution technique. Isolates that displayed a >8-fold spread (i.e. three doubling dilutions) in the MIC distribution from the modal value were considered to have highly variable phenotypic profiles and were excluded from subsequent *in vivo* studies. Test isolates considered for replicate *in vivo* testing were required to display a consistent inter-mouse starting bacterial burden, defined as having all samples in the group within 1 $\log_{10}$ cfu/lung of each other, based on the quantitative assessment at 2 h after inoculation. Test isolates considered for replicate *in vivo* testing were also required to display sufficient growth at 24 h, defined as ≥1 $\log_{10}$ cfu/lung relative to the starting bacterial burden. Last, test isolates considered for replicate *in vivo* testing were also required to display limited infection-related mortality over the initial 8 h post-inoculation period as acute mortality due to overwhelming sepsis during this timeframe does not allow for the discrimination of drug related activity.

The sole purpose of this multi-month, repetitive assessment of isolates was to define the reproducibility of the target model parameters with the standardized employed COMBINE methods as reported. Therefore, the performance of each isolate was individually assessed based on its ability to reproducibly be recovered 2 h after intranasal inoculation and grow over the 24 h study period while not causing overt mortality in the initial hours after inoculation. All data are displayed using descriptive analysis.

Table 1. MICs determined by broth microdilution against *Klebsiella pneumoniae* isolates tested *in vivo*

		MIC (mg/L)													
Isolate origin	Isolate ID	TGC modal	TGC range	No. of replicates	LVX modal	LVX range	No. of replicates	MEM modal	MEM range	No. of replicates	FDC modal	FDC range	No. of replicates	TOB modal	TOB range	No. of replicates
CAIRD	Kp 266	0.5	[a]	3	>32	[a]	3	4	[a]	3	1	[a]	3	64	64->64	3
CDC Bank	49	4	[a]	4	>32	[a]	3	>64	[a]	3	4	1–8	12	>64	[a]	3
CDC Bank	68	2	[a]	3	>32	[a]	3	>64	32->64	3	4	[a]	3	>64	[a]	3
CDC Bank	87	2	[a]	3	>32	[a]	3	0.25	≤0.063–0.25	8	4	[a]	3	0.25	0.25–0.5	3
CDC Bank	98	0.5	[a]	3	>32	[a]	3	16	[a]	3	0.5	[a]	3	>64	[a]	3
CDC Bank	106	2	[a]	3	>32	[a]	3	>64	[a]	3	8	4–8	3	>64	[a]	3
CDC Bank	129	1	0.5–1	3	>32	[a]	3	16	[a]	3	2	[a]	3	32	[a]	3
CDC Bank	160	0.25	[a]	3	0.063	0.063–0.125	3	8	8–16	3	0.125	0.125–0.25	3	0.25	0.25–0.5	3
CDC Bank	504	0.5	0.25–0.5	3	0.5	[a]	3	8	[a]	4	0.25	0.25–0.5	3	8	8–16	3
CDC Bank	522	0.5	[a]	3	32	[a]	3	64	[a]	3	8	[a]	3	1	[a]	3
CDC Bank	523	1	[a]	4	4	4–8	4	64	32–64	4	2	[a]	4	16	8–16	4
CDC Bank	542	4	2–4	3	>32	[a]	3	2	[a]	3	>32	[a]	3	>64	[a]	3
CDC Bank	548	2	[a]	3	>32	[a]	3	16	8–16	3	1	1–4	5	0.25	0.25–0.5	3
CDC Bank	550	1	[a]	3	>32	[a]	3	64	64->64	3	2	[a]	3	32	32–64	3
CDC Bank	553	0.125	0.125–0.5	9	16	8–16	3	2	[a]	3	0.125	[a]	3	>64	[a]	3
CDC Bank	555	2	[a]	3	>32	[a]	3	>64	[a]	3	4	4–8	3	>64	[a]	3
CDC Bank	558	0.5	0.5–1	3	8	8–16	3	2	[a]	3	0.5	0.25–0.5	3	>64	[a]	3
CDC Bank	560	1	1–2	3	>32	[a]	3	>64	[a]	3	16	8–16	3	>64	[a]	3
CDC Bank	831	8	4–8	4	4	[a]	4	≤0.063	[a]	3	0.5	[a]	3	8	[a]	3
CDC Bank	848	4	[a]	3	>32	[a]	3	64	[a]	3	4	[a]	3	>64	[a]	3
CDC Bank	851	0.25	0.25–0.5	3	0.063	[a]	3	≤0.063	[a]	3	0.063	≤0.031–0.25	15	16	[a]	3
CDC Bank	860	2	[a]	3	32	32->32	3	4	4–8	3	4	[a]	3	4	2–4	3
PEI	Kp C1.104	0.5	[a]	3	≤0.063	[a]	3	≤0.063	[a]	3	0.25	0.25–0.5	3	0.5	0.25–0.5	6
PEI	Kp C1.111	0.5	[a]	3	0.063	≤0.063–0.25	5	≤0.063	[a]	3	0.13	0.13–0.25	3	0.25	0.25–0.5	6
PEI	Kp C1.112	0.25	0.25–0.5	3	≤0.063	≤0.063–0.125	3	≤0.063	[a]	3	0.25	0.13–0.25	3	0.5	0.25–0.5	6
PEI	Kp C1.113	0.25	0.25–0.5	3	≤0.063	≤0.063–0.125	3	≤0.063	[a]	3	0.25	0.13–0.25	3	0.25	[a]	6
PEI	Kp C1.147	1	[a]	3	32	32->32	3	32	16–32	3	2	1–2	3	>64	[a]	3
PEI	Kp C1.151	1	[a]	3	>32	[a]	3	>64	[a]	3	2	1–2	3	32	16–32	3
DSMZ	30104	0.25	[a]	3	0.063	0.063–0.125	3	0.125	<0.063–0.125	3	0.25	[a]	3	0.125	[a]	4

[a]The range is equivalent to the mode as all replicates had the same MIC value.

Kp, *Klebsiella pneumoniae*; TGC, tigecycline; LVX, levofloxacin; MEM, meropenem; FDC, cefiderocol; TOB, tobramycin.

Results

Broth microdilution MIC determination

MIC values were determined for all 131 *K. pneumoniae* and *P. aeruginosa* against the five tested antibiotics (tigecycline tested only against *K. pneumoniae* isolates). Of the total isolate number, 59 isolates meeting the definition of low phenotypic variability were advanced into the *in vivo* model. The number of replicates needed to determine a modal value ranged from 3 to 15. Modal MICs, range of MIC values, and number of broth microdilution replicates used to determine the mode for each isolate introduced into the *in vivo* model are presented for all *K. pneumoniae* and *P. aeruginosa* strains in Tables 1 and 2, respectively. Available genotypic data from the CDC and FDA Antimicrobial Resistance Isolate Bank and the ERACE-PA surveillance programme are provided separately (Tables S1 and S2, available as Supplementary data at *JAC* Online) as well as select isolates that were run on the BioFire® FilmArray® Pneumonia Panel.[13,14]

Klebsiella pneumoniae model performance

Targeting a baseline bacterial burden of 6–6.5 $\log_{10}$ cfu/lung using 16 *K. pneumoniae* isolates, the mean ± SD inoculum, baseline and 24 h were 7.42 ± 0.19 $\log_{10}$ cfu/mL, 6.28 ± 0.26 $\log_{10}$ cfu/lung and 5.22 ± 1.12 $\log_{10}$ cfu/lung, respectively. Only two (13%) (CDC 560 and CDC 106) isolates achieved the requisite mean growth of ≥1 $\log_{10}$ cfu/lung for viability in the pneumonia model, albeit with considerable variability (Table S3). Increasing the baseline $\log_{10}$ cfu/lung target to 6.5–7 in 11 isolates yielded mean ± SD inoculum, baseline and 24 h of 7.89 ± 0.09 $\log_{10}$ cfu/mL, 6.84 ± 0.13 $\log_{10}$ cfu/lung and 6.88 ± 1.04 $\log_{10}$ cfu/lung, respectively (Table S3). Despite increasing the starting bacterial burdens by 0.5 log, only one (9%) (DSMZ 30104) isolate reached growth of ≥1 $\log_{10}$ cfu/lung. Indeed, pushing the starting bacterial burden up to 7–7.5 $\log_{10}$ cfu/lung was necessary to consistently achieve viability in the model (Figure 1). Across the 29 isolates studied at the highest inoculum, 25 (83%) reached the minimum 24 h growth threshold and mean ± SD inoculum, baseline and 24 h of 8.40 ± 0.25 $\log_{10}$ cfu/mL, 7.29 ± 0.19 $\log_{10}$ cfu/lung and 8.45 ± 1.35 $\log_{10}$ cfu/lung. Data for individual isolates at each tested inoculum are presented in Table S3. There was negligible (<1%) mortality before scheduled harvest times using these *K. pneumoniae* isolates, regardless of inoculum used. Among the 25 *K. pneumoniae* that met the predefined inclusion criteria for replicate *in vivo* testing, 21 were subsequently tested numerous times (ranging from 2 to 12 replicates). Of note, the other four isolates (Kp 266, CDC 49, CDC 68 and Kp C1.104) performed well in the model when tested in singlet, but had similar phenotypic and genotypic profiles to the other isolates and as such were not explored further. Based on poor or inconsistent growth at 24 h, the following (n = 6) isolates were determined unsuitable for use in the COMBINE murine neutropenic pneumonia model: CDC 98, CDC 522, CDC 550, CDC 553, CDC 860 and Kp C1.111.

Pseudomonas aeruginosa model performance

Among the 30 isolates tested in the pneumonia model, the mean ± SD inoculum suspension cfu/mL and baseline bacterial burden cfu/lung were 7.49 ± 0.24 and 5.93 ± 0.34, respectively (Figure 2). Most (27/30%–90%) fulfilled the growth threshold of ≥1 log cfu/lung at 24 h. Despite targeting the lower end of starting bacterial burden endorsed in the COMBINE protocol, percentage mortality over the 24 h post-inoculation period was profound with median (IQR) of 96% (35%–100%). Time to mortality across all isolates varied widely with median (IQR) of 16 h (14–24 h) as shown in Figure 3. Inoculum bacterial suspension cfu/mL, baseline bacterial burden cfu/lungs, cfu/lung at 24 h, time to and percentage mortality over the 24 h post-inoculation period for each *P. aeruginosa* isolate tested in the model are shown numerically in the supplement (Table S4). Among the 27 isolates that met the pre-specified criteria for replicate *in vivo* testing, 24 were examined numerous times (ranging from 2–6 replicates). Importantly, the other three isolates (CDC 511, Pa 88826, PSA INT 4–100) performed well in the model during initial *in vivo* assessment, but similarly to some *K. pneumoniae* isolates mentioned previously, had phenotypic and genotypic profiles that were already well represented by other isolates in the cohort and as such were not further explored in the model. Based on poor or inconsistent growth at 24 h, the following (n = 5) isolates were determined unsuitable for use in the COMBINE murine neutropenic pneumonia model: CDC 234, CDC 256, CDC 356, CDC 358 and CDC 439.

Discussion

International harmonization of preclinical PK/PD infection models provides an opportunity to improve clinical translation and inter-laboratory reproducibility. In this current study, our phenotypic profiling efforts were initiated with 131 isolates against five selected reference compounds. Fifty-nine displaying low phenotypic variability were advanced into the *in vivo* environment to determine viability in the COMBINE murine neutropenic pneumonia model. Forty-five of the strains displaying *in vivo* viability in initial studies were subjected to repeat assessment to understand inter-day variability. These 45 isolates encompass a large assemblage of β-lactamases and efflux and porin mutations that will allow for the benchmarking of novel compounds with diverse mechanisms of action/resistance in this *in vivo* model. As this translational model is utilized to evaluate pharmacodynamics, the most important bacterial-derived distinctions for suitable isolates are its phenotypic/genotypic profile, sufficient *in vivo* growth and limited mortality over the first 8 h post-inoculation period where the potential for drug efficacy is unlikely to be recognized due to overwhelming sepsis. While the characterization of other bacterial attributes such as virulence genes/function, sequence types and capsular serotypes may be undertaken, these assessments are not generally done for isolates used in pharmacodynamic models. This translational model and the selection of viable isolates as previously defined is similar to the clinical setting where clinical microbiology laboratories routinely define phenotypic profiles but do not characterize additional bacterial-derived factors such as virulence genes, sequence types and capsular serotypes for the care of patients. Although this additional testing has not been undertaken in the current study, the incorporation of the rate and extent of mortality in the isolate selection process is an assessment of the collective expression of all these factors in the background of the test organisms.

These data provide vital insights into the viability and variability of the COMBINE neutropenic pneumonia model.

Table 2. MICs determined by broth microdilution against *Pseudomonas aeruginosa* isolates tested *in vivo*

		MIC (mg/L)										
Isolate origin	Isolate ID	LVX modal	LVX range	No. of replicates	MEM modal	MEM range	No. of replicates	FDC modal	FDC range	No. of replicates	TOB modal	TOB range	No. of replicates
CDC Bank	111	16	16–32	3	64	[a]	3	0.25	0.25–0.5	3	>64	64–>64	5
CDC Bank	234	8	[a]	3	2	[a]	3	0.5	0.25–0.5	3	64	[a]	5
CDC Bank	250	32	32–>32	3	>64	[a]	3	4	[a]	3	>64	[a]	3
CDC Bank	256	0.5	[a]	3	4	[a]	3	≤0.031	≤0.031–0.063	3	0.5	[a]	3
CDC Bank	272	8	8–16	3	4	2–4	3	0.063	[a]	3	>64	[a]	3
CDC Bank	354	8	[a]	3	4	[a]	3	0.125	0.063–0.125	3	>64	[a]	3
CDC Bank	356	0.5	0.5–1	3	64	64–>64	3	1	1–2	3	32	32–>64	7
CDC Bank	358	2	[a]	3	16	16–32	3	0.063	[a]	3	32	[a]	3
CDC Bank	439	32	[a]	3	32	[a]	3	0.25	0.125–0.25	4	>64	[a]	3
CDC Bank	443	>32	[a]	3	64	32–>64	5	1	0.125–2	13	>64	[a]	3
CDC Bank	458	1	1–2	3	2	0.5–2	6	0.5	0.125–0.5	6	2	[a]	3
CDC Bank	459	8	8–16	3	16	16–32	3	0.063	[a]	3	2	2–8	8
CDC Bank	511	0.5	[a]	3	4	[a]	4	0.063	[a]	4	1	0.5–1	5
CDC Bank	516	0.5	[a]	3	64	[a]	3	1	0.125–2	12	0.5	[a]	5
CDC Bank	767	32	32–>32	3	>64	[a]	3	8	4–8	4	>64	[a]	3
CDC Bank	771	>32	[a]	3	>64	[a]	3	4	1–4	5	>64	[a]	3
PEI	Pa 88198	4	[a]	3	2	[a]	3	0.25	0.125–0.5	12	0.5	0.25–1	8
PEI	Pa 88276	1	[a]	3	1	0.5–4	8	0.5	0.25–4	13	1	[a]	6
PEI	Pa 88342	1	0.5–1	3	1	0.5–4	10	0.5	0.25–1	7	1	0.5–1	6
PEI	Pa 88356	2	1–8	9	32	32–64	3	4	[a]	3	2	1–4	8
PEI	Pa 88826	1	[a]	3	1	1–2	3	0.063	≤0.031–0.063	3	0.5	[a]	6
PEI	Pa 89268	8	[a]	3	1	[a]	3	0.25	0.063–0.25	8	0.5	0.125–0.5	8
DSMZ	DSM50071	1	[a]	3	2	2–4	3	0.125	0.063–0.25	7	2	*	3
CAIRD	PSA INT-2–41	>32	[a]	3	16	[a]	3	16	[a]	4	>64	[a]	5
CAIRD	PSA INT-4–99	32	[a]	3	8	[a]	3	8	8–16	3	>64	[a]	3
CAIRD	PSA INT-4–100	1	0.5–1	3	4	[a]	3	0.125	[a]	3	0.5	[a]	5
CAIRD	PSA INT-5–19	32	[a]	3	>64	[a]	3	0.125	0.125–0.25	3	>64	[a]	5
CAIRD	PSA INT-5-35	2	[a]	3	16	[a]	3	0.5	0.125–0.5	10	0.5	[a]	3
CAIRD	PSA INT-12-18	16	[a]	3	8	[a]	3	1	1–4	5	16	[a]	3
CAIRD	PSA US-4-27	32	[a]	3	64	[a]	3	16	[a]	3	>64	[a]	3

[a]The range is equivalent to the mode as all replicates had the same MIC value.
LVX, levofloxacin; MEM, meropenem; FDC, cefiderocol; TOB, tobramycin.

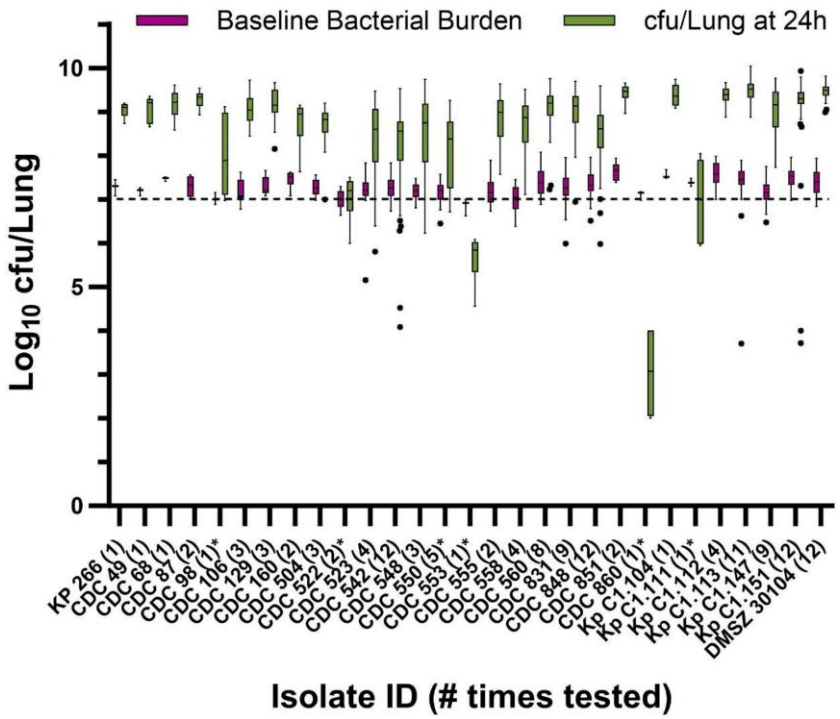

Figure 1. *Klebsiella pneumoniae* baseline bacterial burden and *in vivo* growth in the COMBINE murine neutropenic pneumonia model. The dashed line represents the target baseline burden of 7 $\log_{10}$ cfu/lung. Outlier mice were determined by Tukey's test and are displayed as individual dots. Asterisk denotes isolates unsuitable for the model.

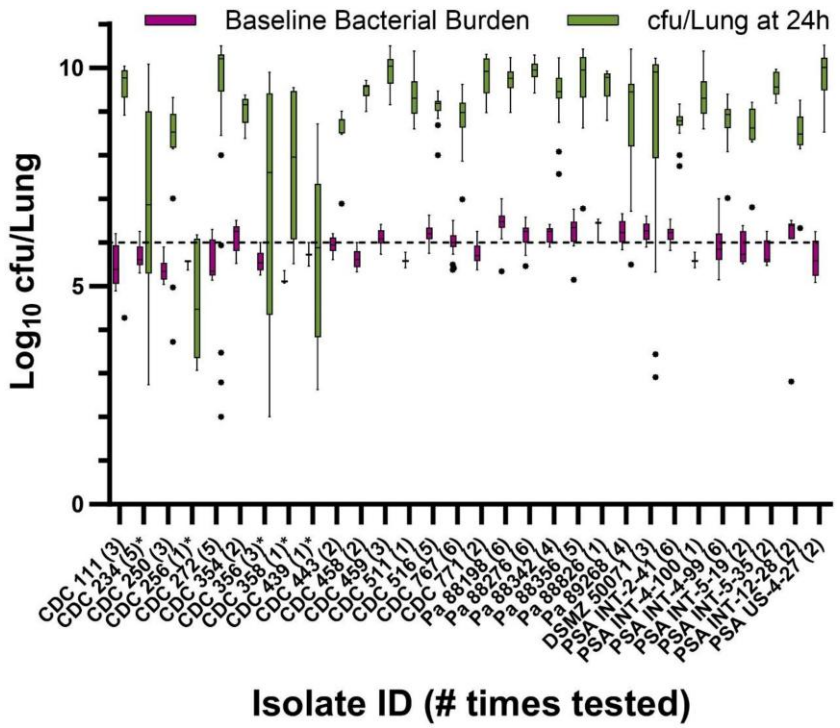

Figure 2. *Pseudomonas aeruginosa* baseline bacterial burden and *in vivo* growth in the COMBINE murine neutropenic pneumonia model. The dashed line represents the target baseline burden of 6 $\log_{10}$ cfu/lung. Outlier mice were determined by Tukey's test and are displayed as individual dots. Asterisk denotes isolates unsuitable for the model.

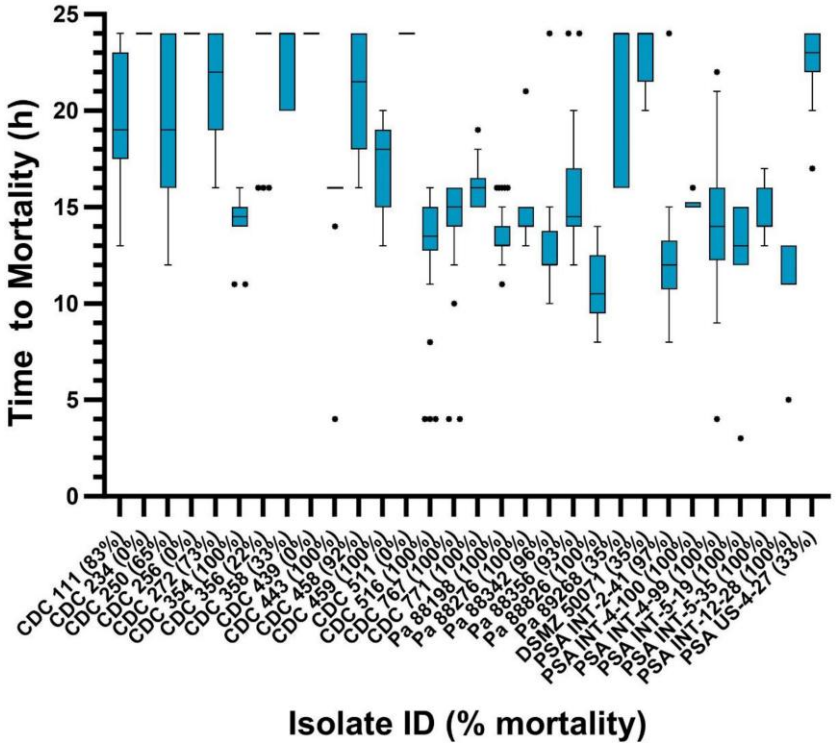

Figure 3. Time to and percentage mortality of *Pseudomonas aeruginosa* isolates in the COMBINE murine neutropenic pneumonia model. Outlier mice were determined by Tukey's test and are displayed as individual dots. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Importantly, sensitivity to the starting bacterial burden for *K. pneumoniae* isolates was demonstrated, as even half-log differences were shown to improve or impede isolates from establishing viable growth in the model. As such, starting bacterial burdens for *K. pneumoniae* isolates may need to target the upper end or even exceed the COMBINE proposal of 6–7 $\log_{10}$ cfu/lung. Conversely, *P. aeruginosa* strains tended to grow well at the lower end of the described starting bacterial burden range. Despite this, mortality was profound before the scheduled harvest time 24 h post-inoculation. Although not directly assessed, starting *P. aeruginosa* isolates at the upper end of the defined COMBINE range could plausibly enhance the rate of mortality such that even PK/PD optimized antibiotic exposures may be unable to produce meaningful reductions in cfu/lung due to enhanced severity of the infection and overwhelming sepsis. Additionally, among the 45 isolates that were tested multiple times (up to 12) *in vivo* on separate experiment days, only six had inter-run categorical differences in reaching 1 $\log_{10}$ cfu/lung of net growth at 24 h, underscoring model reproducibility and stability.

This paper lays the groundwork for establishing a benchmark of expected results against a broad array of *K. pneumoniae* and *P. aeruginosa* by robustly describing expected viability and variability of many model elements against key target pathogens inclusive of broth microdilution MICs, required inoculums, initial bacterial burden, growth at 24 h, time to and percentage mortality. With the stability of the test isolates and model now established, further explorations can focus on the use of this translational PK/PD model with the five selected clinically available therapies to provide a foundation suitable for benchmarking novel therapeutic compounds. These derived data in conjunction with the availability of the isolates to investigators via a central repository will serve as a resource for organism selection when using the COMBINE murine pneumonia model methodology. This paper serves to identify suitable isolates for the model, providing the framework as part one of a four-part series describing the activity and variability in the model of human-simulated exposures of both plasma and pulmonary ELF for tigecycline, levofloxacin, meropenem, cefiderocol and tobramycin against a selection of the isolates defined herein. With established clinical use of these compounds for the treatment for Gram-negative pneumonia, humanized exposures in the murine model will allow for back-translation of anticipated efficacy and provide meaningful benchmarks for cfu/lung assessments of novel compounds under development.

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Transparency declarations

A.J.F. and A.M.P. have none to declare. E.M.D. is an employee of the study funder, CARB-X. D.P.N. is a consultant, speaker bureau member or has received research funding from Abbvie, CARB-X, Cepheid, Innoviva, Merck, Pfizer and Shionogi.

Supplementary data

Tables S1 to S4 are available as Supplementary data at *JAC* Online.

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Quantitative performance of humanized serum and epithelial lining fluid exposures of tigecycline and levofloxacin against a challenge set of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in a standardized neutropenic murine pneumonia model

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Background: Lack of uniformity in infection models complicates preclinical development. The COMBINE protocol has standardized the murine neutropenic pneumonia model. Herein we provide benchmark efficacy data of humanized exposures of tigecycline and levofloxacin in plasma and epithelial lining fluid (ELF) against a collection of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

Methods: Following the COMBINE protocol, plasma and ELF human-simulated regimens (HSRs) of tigecycline 100 mg followed by 50 mg q12h and levofloxacin 750 mg once daily were developed and confirmed in the murine neutropenic pneumonia model. Tigecycline HSRs were tested against seven *K. pneumoniae* isolates. Levofloxacin HSRs were assessed against 10 *K. pneumoniae* and 9 *P. aeruginosa*. The change in cfu/lung over 24 h for each treatment was calculated. Each isolate was tested in duplicate against both the plasma and ELF HSRs on separate experiment days.

Results: Tigecycline 1.8 and 3 mg/kg q12h achieved humanized exposures of serum and ELF, respectively. Levofloxacin 120 and 90 mg/kg q8h led to *f*AUC exposures in plasma and ELF similar to in humans. Both tigecycline regimens were ineffective across the MIC range. Levofloxacin regimens achieved multilog kill against susceptible isolates, and no appreciable cfu/lung reductions in isolates with an MIC of ≥32 mg/L. Differences in cfu/lung were evident between the levofloxacin plasma and ELF HSRs against isolates with MICs of 4 and 8 mg/L.

Conclusions: Administering HSRs of tigecycline and levofloxacin based on both serum/plasma and ELF in the COMBINE pneumonia model resulted in cfu/lung values reasonably aligned with MIC. These data serve as translational benchmarks for future investigations with novel compounds.

Background

Preclinical pharmacokinetic/pharmacodynamics (PK/PD) models remain a cornerstone of antibiotic development, enabling proof-of-concept efficacy studies and providing valuable insight for the projection of clinical dose selection.[1] Unfortunately, extensive interlaboratory discrepancies in the methodology of commonly utilized preclinical models predominate the landscape, particularly with pneumonia models.[2] The collaboration for prevention and treatment of MDR bacterial infections (COMBINE) consortium have proposed a standardized global protocol for the preclinical murine neutropenic pneumonia model in hopes of aligning fundamental elements across laboratories.[3] Novel therapeutics can benefit from direct comparison with the established standard of care at the preclinical stage of drug development. Establishing benchmark data against a collection of phenotypically and genotypically diverse isolates when treated with humanized exposures of standard-of-care antibiotics across multiple classes in the standardized model can assist in making these comparisons and ensuring model stability and reproducibility across time and location. Importantly for a pneumonia indication, humanized exposures at the target site of infection, pulmonary epithelial lining fluid (ELF), as opposed to plasma, provide an even more translational benchmark. Many investigations in the murine pneumonia model still utilize plasma targets, mostly due to the simplicity of study design, despite potentially

unreliable translational application of these targets in the context of agents with discordant ELF penetration between humans and mice. As such, the magnitudes of plasma PK/PD indices required for efficacy determined in a murine pneumonia model may fail to predict the clinical outcome.[4]

Previous efforts have established an isolate collection of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* suitable for this purpose. These isolates have been phenotypically characterized using broth microdilution to determine modal MIC values, and their viability and the growth stability of untreated controls in the standardized murine neutropenic pneumonia model have been reported.[5] This manuscript details the development of human-simulated regimens (HSRs) of tigecycline and levofloxacin in both plasma and pulmonary ELF in the model and describes the change in cfu/lung with these regimens against Gram-negative isolates.

Materials and methods

Antimicrobial agents

Commercial vials of tigecycline were reconstituted for *in vivo* studies and diluted further in 0.9% saline to deliver weight-based dosing to the mice. For levofloxacin, analytical grade powder (Sigma–Aldrich) was acquired and dissolved in sterile water with the addition of 0.1 M NaOH.

Bacterial isolates

K. pneumoniae and *P. aeruginosa* isolates were sourced from the isolate repository at the Center for Anti-Infective Research and Development (CAIRD) (Hartford, CT, USA), the CDC and FDA Antibiotic Resistance Isolate Bank (CDC Bank) (Atlanta, GA, USA), the Paul Ehrlich Institute (PEI) (Berlin, Germany), and the Leibniz Institute (DSMZ) (Brunswick, Germany). The phenotypic and known genotypic profiles of these isolates were previously determined and are provided in Table 1.[5] Prior to experimentation, each isolate was subcultured twice on Trypticase soy agar with 5% sheep blood (Becton Dickinson and Co., Sparks, MD, USA) and incubated at 37°C for approximately 16 h. Bacterial colonies from the overnight culture plate were suspended in normal saline to a McFarland target of ∼2.5 and further diluted in saline as necessary to produce the final inoculum.

In vivo murine neutropenic pneumonia model

The details of the COMBINE protocol and model specificities have been previously published.[3] In brief, specific-pathogen-free CD-1, female mice 6–8 weeks old were acquired from Charles River Laboratories, Inc.

Table 1. Phenotypic and known genotypic profiles of *K. pneumoniae* and *P. aeruginosa* isolates

			MIC (mg/L)		
	Isolate origin	Isolate ID	TGC	LVX	Known genotypic information
K. pneumoniae	CDC Bank	523	1	4	aadA1, aph(3′)-Ia, dfrA1, KPC-2, sul1
	CDC Bank	542	4	>32	aac(6′)-Ib, aadA2, catA1, dfrA12, EMRD, KDEA, mph(A), oqxA, oqxB, SHV-12, sul1
	CDC Bank	558	0.5	8	aac(3)-IId, aac(6′)-Ib-cr, aadA1, aadA2, armA, ARR-2, catA1, CTX-M-15, dfrA12, dfrA14, EMRD, ere(A), fosA5, KDEA, OXA-181, SHV-26, sul1, sul2, tet(A), tet(R)
	CDC Bank	560	1	>32	aac(3)-IId, aac(6′)-Ib-AKT, aadA1, armA, ARR-2, ble-MBL, catA1, catB4, cmlA5, CMY-4, CTX-M-15, dfrA1, EMRD, ere(A), fosA, KDEA, mph(E), msr(E), NDM-1, oqxA, oqxB20, OXA-1, OXA-9, sat2, SHV-100, sul1, TEM-1A
	CDC Bank	831	8	4	aac(3)-IIa, aph(3′)-Ib, aph(6)-Id, catB4, CTX-M-15, dfrA14, OXA-1, QnrB1, SHV-187, sul2, TEM-1B, tet(A), tet(R)
	CDC Bank	848	4	>32	aac(3)-IIa, aac(6′)-Ib-cr, catB4, CTX-M-15, dfrA1, Omp35, OmpK35, oqxA, oqxB, OXA-1, OXA-48, QnrS1, SHV-11, sul1, TEM-1B, tet(A), tet(R)
	PEI	Kp C1.112	0.25	≤0.063	Unknown
	PEI	Kp C1.113	0.25	≤0.063	Unknown
	PEI	Kp C1.147	1	32	CTX-M, OXA-48-like
	PEI	Kp C1.151	1	>32	KPC
	DSMZ	30104	0.25	0.063	Unknown
P. aeruginosa	CDC Bank	354	N/A	8	aadA11, ant(2′)-Ia, aph(6)-Id, dfrB1, strA
	CDC Bank	459	N/A	8	OXA-50
	CDC Bank	516	N/A	0.5	KPC-2
	CDC Bank	767	N/A	32	GES-20
	PEI	Pa 88198	N/A	4	Unknown
	PEI	Pa 88276	N/A	1	Unknown
	PEI	Pa 88342	N/A	1	Unknown
	CAIRD	PSA INT-2-41	N/A	>32	aac(6′)-Ib, aac(6′)-Ib-cr, aadA6, aph(3′)-IIb, $bla_{CTX-M-2}$, $bla_{OXA-488}$, bla_{PDC-35}, catB7, fosA, qacE, sul1
	CAIRD	PSA INT-4-99	N/A	32	aac(6′)-Ib-cr, aadA6, aph(3′)-IIb, bla_{OXA-14}, $bla_{OXA-488}$, bla_{PDC-35}, catB7, cml, cmlA1, fosA, qacE,sul1

LVX, levofloxacin; MEM, meropenem; FDC, cefiderocol; TOB, tobramycin; N/A, not applicable.

(Raleigh, NC, USA). All animals were allowed to acclimatize for 72 h prior to any study procedures and were housed as groups of six at controlled room temperature in HEPA-filtered cages (Innovive, San Diego, CA, USA). Study rooms were maintained with diurnal cycles (12 h light/12 h dark) and food and water were provided *ad libitum*. Prior to inoculation, neutropenia was induced by administering 150 mg/kg of intraperitoneal (IP) cyclophosphamide (0.2 mL) on Day −4 and 100 mg/kg on Day −1. In addition, a predictable degree of renal impairment was produced using 5 mg/kg uranyl nitrate administered IP (0.2 mL) on Day −3. Mice were anaesthetized using inhaled isoflurane, manually restrained upright, and infected with 50 µL of bacterial suspension via the nares. Inoculums were prepared to ~10^8 and 10^7 cfu/mL for *K. pneumoniae* and *P. aeruginosa*, respectively, to deliver 10^7 and 10^6 cfu/lung, respectively, which are necessary for model performance as previously described.[5] Dosing was initiated 2 h after inoculation.

PK studies

Ex vivo tigecycline serum protein-binding studies

All PK studies undertaken to develop the HSRs (including protein-binding studies) were performed in mice infected with *K. pneumoniae* (CDC 851), and mice were handled as described above. Escalating single doses of tigecycline (2.25, 4.5 and 9 mg/kg) were administered subcutaneously to determine tigecycline serum protein binding. Triplicate pooled serum and ultrafiltrate samples were collected at 1 h (5 mice per replicate, 15 mice per dose) and stored at −80°C until concentration determination. Whole blood was allowed to clot and then subsequently centrifuged at 4°C at 3000×**g** for 10 min. Serum was separated (total serum) and 900 µL was added to an ultrafiltration device (Centrifree®, Merck Millipore Ltd., Ireland) and centrifuged using a fixed rotor at 10°C at 2000×**g** for an additional 45 min to obtain protein-free ultrafiltrate. The triplicate free and total tigecycline concentrations were averaged and then free fractions were calculated using: free fraction = $\text{Concentration}_{\text{ultrafiltrate}}/\text{Concentration}_{\text{total serum}}$.

Human-simulated exposure PK studies

PK studies were undertaken to establish HSRs that approximate $f\text{AUC}_{0-24}$ exposures achieved after IV administration of tigecycline 100 mg loading dose followed by 50 mg q12h and levofloxacin 750 mg q24h in humans. Separate HSRs were developed for serum (tigecycline), plasma (levofloxacin) and pulmonary ELF (both). The blood matrices for tigecycline and levofloxacin of serum and plasma, respectively, were selected to align with human PK studies.[6,7] At predefined timepoints, groups of six mice had blood sampled via retro-orbital bleeding (two timepoints, under anaesthesia with isoflurane 2%–3% v/v in 100% oxygen via inhalation) and cardiac puncture (one terminal timepoint). Proparacaine hydrochloride ophthalmic solution 0.5% was applied to the eye (1–2 drops) after blood sampling via retro-orbital bleeding. The volume of blood collected was 0.15 mL per sample via retro-orbital bleeding with subsequent fluid replacement using 0.2 mL normal saline given IP. Unlike protein-binding studies, samples were not pooled and the exposure in each individual mouse was assessed. Tigecycline free serum concentrations were determined from the total utilizing the protein-binding percentage determined presently. Levofloxacin free plasma exposures were estimated from the total by assuming 30% protein binding, which has shown to be consistent across humans and multiple animal species, including mice.[8,9] Following blood collection by cardiac puncture, but prior to cervical dislocation, bronchoalveolar lavage (BAL) fluid was collected from the mice at the same timepoints. A catheter was inserted into the trachea of the mice, and lungs were lavaged with four aliquots of 0.4 mL of normal saline. For ELF concentration determination, urea correction was applied to BAL fluid concentrations. Due to the lack of albumin in BAL fluid, total ELF concentrations were considered free (unbound). *f*AUC for each dose was determined using the trapezoid method and multiplied by the number of doses throughout the 24 h experiment to determine $f\text{AUC}_{0-24}$. Previously reported tigecycline and levofloxacin serum/plasma HSR regimens developed in alternative murine models were used as a baseline.[10,11] Mathematical modifications were made to the baseline regimens as necessary to achieve translational *f*AUC exposures and repeat confirmatory PK studies were undertaken for both serum/plasma and ELF. While HSRs were developed and confirmed prior to *in vivo* efficacy studies, additional PK studies were undertaken on the same day as *in vivo* efficacy studies using the same methods and predefined timepoints to assess interday and inter-isolate variability.

The sample size calculation was performed using nQuery Advisor based on the following: (i) the mean % coefficient of variance (CV) of the PK parameter for typical antibiotics is usually less than 30%; and (ii) a two-sided 95% CI with 80% probability will have an interval that extends no more than 1 SD from the observed mean. As a result, the sample size of six mice per timepoint is sufficient for the assessment of drug disposition.

In vivo efficacy studies

Controls were sacrificed just prior to antibiotic initiation (0 h controls) and 24 h later. All groups contained six mice except for 24 h controls, which contained 4–6 mice. Plasma (or serum) and pulmonary ELF HSRs were administered over 24 h (or equal volume of normal saline in controls), then animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Lungs from individual animals were aseptically harvested and homogenized in normal saline. Homogenized tissue was then serially diluted and 50 µL was plated for cfu/lung quantification (lower limit of quantification of 1×10^2 cfu/lung). The change in cfu/lung over 24 h for each treatment was calculated relative to the initial bacterial burden. Each isolate was tested in duplicate against both the plasma and ELF HSRs on separate experiment days to incorporate interday model variability. The raw data from individual experiments were combined and analysed together. Tigecycline serum and ELF HSRs were tested against seven *K. pneumoniae* isolates with tigecycline MICs previously determined by broth microdilution ranging from 0.25 to 8 mg/L. Levofloxacin plasma and ELF HSRs were assessed against 10 *K. pneumoniae* isolates (levofloxacin MIC range: ≤0.063 to >32 mg/L) and 9 *P. aeruginosa* isolates (levofloxacin MIC range: 0.5 to >32 mg/L).

Sample size was calculated as follows: (i) for typical antimicrobial agents, optimal dosing regimens usually produce approximately 2–3 log decrease in bacterial density with 40% CV; and (ii) in order to have an observed mean that deviates from the true mean by no more than 1 SD using a two-sided 95% CI with 80% probability, $n=6$ datapoints are required.

Analytical procedures

All compounds were analysed using a Waters Acquity UPLC H-Class system with tandem TQ-XS mass spectrometer (LC-MS/MS) equipped with an Acquity UPLC BEH C18, 1.7 µm, 2.1×50 mm column. All reagents were obtained from commercial sources and used without further purification.

Concentrations of levofloxacin in BAL and murine K_2EDTA plasma were determined with validated UPLC methods using levofloxacin-d_8 as the internal standard. The concentration range for both levofloxacin methods was 0.01–100 µg/mL. Mean interday CV for low, medium and high values of levofloxacin in saline were 10.4%, 7.2% and 12.3%, respectively. Mean interday CV for low, medium and high values of levofloxacin in murine K_2EDTA plasma were 6.3%, 5.2% and 7.6%, respectively.

Concentrations of tigecycline in BAL and murine serum were determined with validated UPLC methods using tigecycline-d_9 as the internal standard. The concentration range for the tigecycline in the BAL method was 1–2500 ng/mL, while the concentration range for the tigecycline in

serum method was 0.05–25 μg/mL. Mean interday CV for low, medium and high values of tigecycline in saline were 8.5%, 4.2% and 7.5%, respectively. Mean interday CV for low, medium and high values of tigecycline in murine serum were 9.9%, 11.7% and 9.8%, respectively.

Urea concentrations in BAL and murine K_2EDTA plasma and serum were determined with a validated UPLC method using standards in saline (5–500 μg/mL) and [^{13}C]-urea as the internal standard. The internal standard was diluted with 3:4 acetonitrile/water to yield a 950 ng/mL solution of [^{13}C]-urea for protein precipitation. Compounds were monitored using an ESI probe in positive acquisition mode. The quantitative mass transition for urea was 61.0 → 43.8. The quantitative mass transition for [^{13}C]-urea was 62.0 → 45.0. For the preparation of all standards and samples, 630 μL of protein precipitation solution was added to a well plate containing 30 μL of standard or sample. Nominal concentrations of the low, medium and high quality controls for urea in saline were 7.5, 75 and 400 μg/mL, respectively. Mean interday CV for low, medium and high values of urea were 9.6%, 9.1% and 9.4%, respectively.

Ethics and laboratory animals

Animals were maintained and utilized in accordance with National Research Council recommendations. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Hartford Hospital (Assurance #A3185-01).

Results

PK studies

Ex vivo tigecycline serum protein-binding studies

Similar to previous findings, the percentage of tigecycline bound to protein was dose-dependent.[12] Mean percentages of protein binding (±SD) were 15.9% (5.0%), 50.5% (18.5%) and 41.7% (15.1%) at the 1 h timepoint after receiving doses of 2.25, 4.5 and 9 mg/kg, respectively. For the tigecycline serum HSR, all total concentrations in the profile were corrected for binding using 15.9% as the exposures were closest to the 2.25 mg/kg dose.

Human-simulated exposure PK studies

In the COMBINE murine neutropenic pneumonia model, administration of tigecycline 1.8 mg/kg every 12 h delivered in 0.1 mL subcutaneous injections achieved an $fAUC_{0–24}$ of 2.38 mg·h/L after correcting for 15.9% protein binding. This free drug exposure is consistent with findings in patients receiving 100 mg followed by 50 mg every 12 h with ventilator-associated pneumonia (VAP) and exceeds exposures observed in healthy volunteers (Table 2).[13,14] The penetration into ELF was observed to be lesser in mice than in man, necessitating a higher dose of 3 mg/kg every 12 h to achieve an ELF $AUC_{0–24}$ of 5.68 mg·h/L, which is comparable to exposures seen in both VAP patients and healthy volunteers.[13,14] The observed tigecycline concentrations from repeat PK studies performed concurrently with *in vivo* efficacy studies are available in Figure S1 (available as Supplementary data at *JAC* Online).

Administration of 120 mg/kg levofloxacin every 8 h in the model yielded plasma $AUC_{0–24}$ exposure of 145 mg·h/L in mice, which is well matched with the mean plasma exposure (140 mg·h/L) observed in infected patients with acute exacerbation of chronic bronchitis receiving 750 mg orally every 24 h.[6] Assuming 30% protein binding, the resultant plasma $fAUC_{0–24}$ was 102 mg·h/L. Conversely to tigecycline, ELF penetration was greater in infected mice relative to humans with infections. The administration of the levofloxacin plasma HSR (120 mg/kg every 8 h) resulted in ELF $AUC_{0–24}$ exposure of 168 mg·h/L, which is ~40% higher than the median exposure of 119 mg·h/L observed in the ELF of infected humans.[6] Therefore, a reduction in dose to 90 mg/kg every 8 h was required to humanize ELF exposures in mice, with a resulting $AUC_{0–24}$ of 129 mg·h/L (Figure 1). Data derived from repeated PK studies of the levofloxacin HSRs undertaken alongside efficacy studies are presented in Figure S2.

In vivo efficacy studies

Tigecycline serum and ELF HSR efficacy

The mean ± SD baseline bacterial burden, and 24 h change in $\log_{10}$ cfu/lung in controls relative to baseline across the seven *K. pneumoniae* isolates tested were 7.34 ± 0.09 and 1.65 ±

Table 2. Tigecycline $fAUC_{0–24}$ exposures in humans and mice receiving HSRs

Tigecycline regimen	Species (matrix)	$fAUC_{0–24}$ (mg·h/L) Mean ± SD
100 mg LD, 50 mg q12h maintenance	Human (serum)	0.88 ± 0.17 (HV)[13]
		3.24 ± 3.09 (VAP)[14]
1.8 mg/kg q12h (serum HSR)	Murine (serum)	2.38
100 mg LD, 50 mg q12h maintenance	Human (ELF)	6.32 (HV)[a]
		7.13 ± 2.61 (VAP)
3 mg/kg q12h (ELF HSR)	Murine (ELF)	5.68

LD, loading dose; HV, healthy volunteer; VAP, ventilator-associated pneumonia.
[a]SD not provided in the reference.

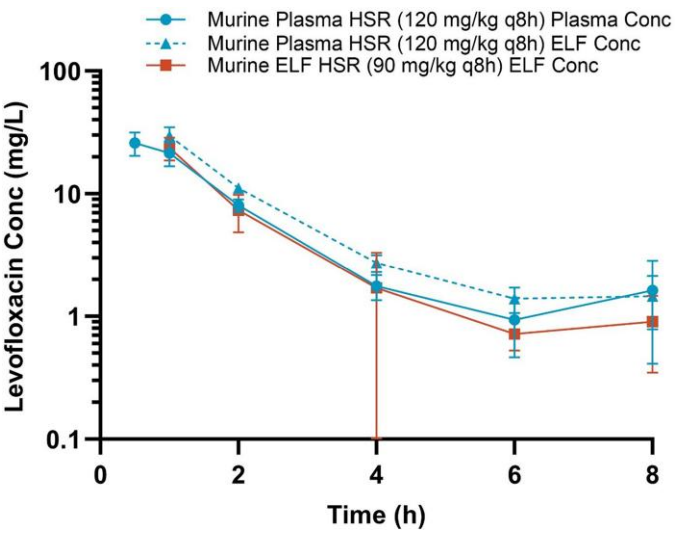

Figure 1. Levofloxacin exposures in the COMBINE murine neutropenic pneumonia model following administration of plasma and ELF HSRs of 750 mg every 24 h. Conc, concentration. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

0.44 $\log_{10}$ cfu/lung, respectively. Inoculum cfu/mL, baseline bacterial burden and growth in 24 h controls are available on an individual isolate basis in Table S1. The 24 h change in $\log_{10}$ cfu/lung after receiving saline control, tigecycline serum HSR and tigecycline ELF HSR for each of the seven isolates is presented in Figure 2. Among all isolates, net growth was observed on both the serum and ELF HSRs, with minimal differentiation between the HSRs when simulating the currently licensed tigecycline dose of 100 mg followed by 50 mg every 12 h.

Levofloxacin plasma and ELF HSR efficacy

K. pneumoniae The mean ± SD baseline bacterial burden and 24 h change in $\log_{10}$ cfu/lung in controls relative to baseline across the 10 *K. pneumoniae* isolates tested were 7.37 ± 0.22 and 1.62 ± 0.40 $\log_{10}$ cfu/lung, respectively. The 24 h change in $\log_{10}$ cfu/lung after receiving saline control, levofloxacin plasma HSR and levofloxacin ELF HSR for each of the 10 isolates is presented in Figure 3. The three isolates categorically susceptible to levofloxacin experienced multilog kill when exposed to either HSR. Among the two isolates with an MIC of 4 mg/L, there was clear differentiation in the efficacy of the plasma and ELF HSR regimens. When the levofloxacin exposure at the target site of infection (i.e. ELF) was matched, the 1 log kill efficacy target was not achieved. When matched solely on plasma exposures, both isolates neared or exceeded 1 log kill. The remaining isolates with an MIC of >4 mg/L experienced mean growth relative to the corresponding baseline bacterial burden, regardless of which levofloxacin HSR was administered.

P. aeruginosa Among the nine *P. aeruginosa* isolates tested in the model against levofloxacin, the mean ± SD baseline bacterial burden and 24 h change in $\log_{10}$ cfu/lung in controls relative to baseline were 6.13 ± 0.22 and 3.10 ± 0.38 $\log_{10}$ cfu/lung, respectively. Figure 4 displays the 24 h change in $\log_{10}$ cfu/lung after receiving saline control, levofloxacin plasma HSR and levofloxacin ELF HSR for each isolate. Multilog killing was seen with the levofloxacin HSR of each matrix in the four isolates with an MIC of ≤4 mg/L (the resistance breakpoint). There was considerable variability in cfu/lung in the two isolates with an MIC of 8 mg/L, with the plasma HSR achieving larger reductions relative to ELF HSR. As expected based on phenotype, the remaining isolates with an MIC of 32 mg/L or greater experienced net growth after administration of either levofloxacin HSR.

Discussion

Harmonization of crucial elements of *in vivo* infection models is imperative for making reasonable comparisons between compounds and laboratories, especially when assessing against the same cohort of isolates. In accordance with the COMBINE protocol for the murine neutropenic pneumonia model, we developed and confirmed serum (tigecycline), plasma (levofloxacin) and pulmonary HSRs (both). While these HSRs were developed using the methodology and drug products as noted herein, confirmatory PK studies should be undertaken prior to future use of our mg/kg dosing schemes as differences in the drug products (i.e. pharmaceutical versus analytical grade), formulations and manufacturers may result in markedly different drug exposures in each of the biological target sites that have been profiled.

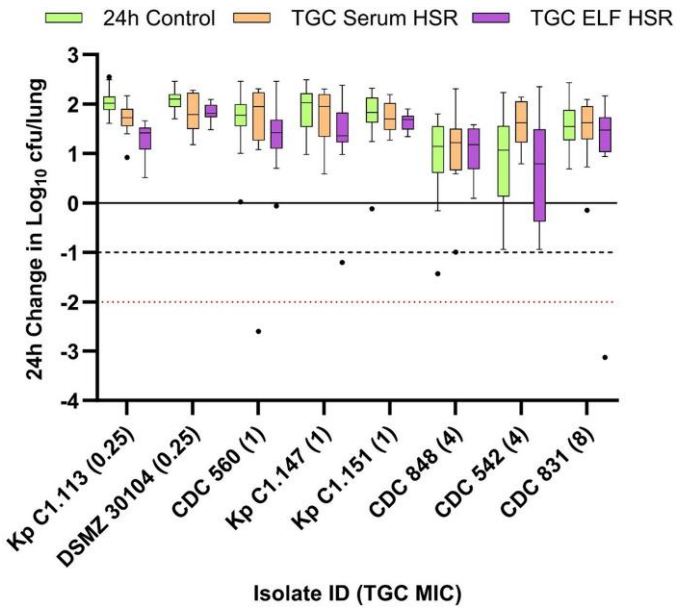

Figure 2. Cfu/lung data following administration of humanized tigecycline (TGC) (100 mg followed by 50 mg every 12 h) serum and ELF exposures in the COMBINE murine neutropenic pneumonia model against *K. pneumoniae* isolates. Boxes represent IQRs, with horizontal lines denoting the medians. Whiskers were determined by Tukey's test and outliers are displayed as individual dots. The solid line denotes stasis; the dashed line denotes 1 log kill; the red dotted line denotes 2 log kill.

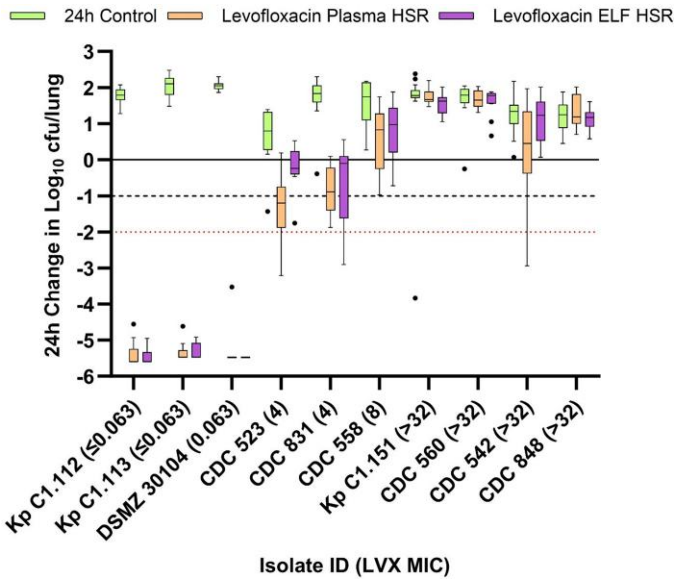

Figure 3. Cfu/lung data following administration of humanized levofloxacin (750 mg every 24 h) plasma and ELF exposures in the COMBINE murine neutropenic pneumonia model against *K. pneumoniae* isolates. Boxes represent IQRs with horizontal lines denoting the medians. Whiskers were determined by Tukey's test and outliers are displayed as individual dots. The solid line denotes stasis; the dashed line denotes 1 log kill; the red dotted line denotes 2 log kill.

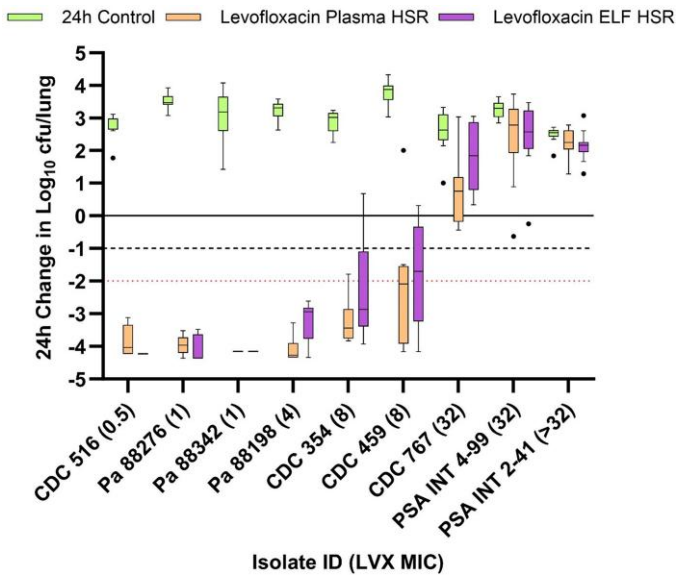

Figure 4. Cfu/lung data following administration of humanized levofloxacin (750 mg every 24 h) plasma and ELF exposures in the COMBINE murine neutropenic pneumonia model against *P. aeruginosa* isolates. Boxes represent IQRs with horizontal lines denoting the medians. Whiskers were determined by Tukey's test and outliers are displayed as individual dots. The solid line denotes stasis; the dashed line denotes 1 log kill; the red dotted line denotes 2 log kill.

These HSRs were subsequently administered to mice infected with various *K. pneumoniae* and *P. aeruginosa* strains, with every isolate tested in duplicate against HSRs of both matrixes. As anticipated, the tigecycline simulated exposures were ineffective in producing cfu/lung reductions against this collection of *K. pneumoniae* isolates. Levofloxacin displayed greater ELF penetration in this murine model relative to humans, necessitating a lower mg/kg dose to simulate human ELF concentration-versus-time profile in relation to the plasma HSR. Both HSRs for levofloxacin achieved multilog kill against all susceptible isolates. The plasma HSR, which overexposes the ELF, led to greater cfu reductions than the ELF HSR in isolates at the resistance breakpoint or a few doubling dilutions greater, underscoring the importance of matching exposures at the site of infection so as to not overpredict activity or efficacy in cases where the penetration is discordant between matrixes.

Of note, these experiments developed humanized serum and ELF exposures of tigecycline commensurate with the FDA-approved dose of 100 mg followed by 50 mg every 12 h. These exposures have demonstrated a lack of clinical efficacy when used to treat Gram-negative bacterial pneumonia, which is consistent with the known PK/PD properties of the drug.[15–17] Therefore, we introduced tigecycline at these exposures as a negative control in the model. Notably, many clinicians utilize an off-label high-dose regimen of 200 mg followed by 100 mg every 12 h, which has been shown to improve clinical outcomes relative to labelled dosing in VAP or hospital-acquired pneumonia.[18,19] This increase in dose better optimizes exposures in regard to PK/PD targets but importantly is not represented in these studies.

As reported previously, when discovered in an earlier phase of model development and validation, it should be noted that the initial bacterial burden between *K. pneumoniae* and *P. aeruginosa* isolates differs.[5] While not ideal, it is necessary to start *K. pneumoniae* at a higher inoculum (≥7 $\log_{10}$ cfu/lung) to better ensure the consistent ability for control animals to achieve ≥1 log of growth, and extending *P. aeruginosa* initial bacterial burden beyond 6 $\log_{10}$ cfu/lung is liable to lead to overt infection-related mortality before 8 h, where drug therapy is unlikely to have any meaningful benefits. This ∼1 log difference in initial bacterial burden between bacterial species could theoretically lead to differences in PD profiling in terms of magnitude of exposure needed, particularly against antibiotic classes that are prone to an inoculum effect, albeit inoculum effects are generally described when assessing multilog differences *in vitro* as opposed to 1 log in this *in vivo* murine pneumonia model.

As the purpose of these experiments was to set quantitative cfu/lung benchmarks in the COMBINE model using the selected isolates that could be replicated by other laboratories, it was imperative that we could replicate our own findings and understand interday variability on an intralaboratory level. Therefore, a strength of this study was the assessment of each HSR against each isolate on two separate study days. These data from separate days were combined and analysed together as one to incorporate the variability between separate investigations and provide a more reasonable benchmark for other investigators to match. This also served as an indirect quality control between each study day, as discordant results between the original and repeated investigation of each isolate and regimen would signal potential error in study methodology. Overtly discordant results were not observed for the efficacy studies of either drug. Interday study variability was generally within 0.5 $\log_{10}$ cfu/lung in either direction, which is a reasonable target for external investigators looking to replicate these findings as the data presented are inclusive of our study-to-study variability.

Similarly, additional efforts were taken to quantify drug exposures during the *in vivo* efficacy assessments after separate PK validation of the HSRs. Relative to original confirmatory PK data, the concentrations obtained in the ELF for tigecycline during the efficacy assessments were very reproducible, albeit serum data were more variable. For levofloxacin, the concentration-versus-time profiles with CDC 831 (*K. pneumoniae*) suggest similar volume of distribution and clearance compared with original PK confirmatory studies, which were also performed in animals infected with a *K. pneumoniae* isolate. However, infection with CDC 767 (*P. aeruginosa*) appeared to lead to a decrease in drug clearance, but again a similar volume of distribution. This difference in clearance may be linked to the mortality observed in animals infected with CDC 767 relative to the two *K. pneumoniae* isolates. As animals become increasingly septic, it would be expected that their renal function declines, leading to decreased drug clearance of renally excreted compounds like levofloxacin. Importantly, drug concentrations over the initial 8–12 h of the model before overt sepsis determine the majority of activity (or lack thereof), so reasonable differences in the exposure of the later portion of the infection model are expected variability.

In this model, both antibiotics tested had ELF penetration discordant from humans. Interestingly, these discordances were in opposing directions, with tigecycline ELF underexposed and

levofloxacin ELF overexposed when serum/plasma human profiles were simulated. While these differences between species may seem trivial, the implications have been proven to be serious when unaccounted for, as demonstrated by ceftobiprole.[4,20] Simply stated, using plasma exposure targets from a murine pneumonia model cannot reliably predict the efficacy or microbiological effect in humans. To enhance clinical translation, murine pneumonia models must quantify ELF exposure. Undoubtedly, PK sampling and analysis of the ELF is more complicated, costly and time-consuming. It also introduces additional variables such as urea concentrations in both plasma and ELF for dilution correction. However, the importance of its characterization cannot be overstated. Another point to consider for clinical translation is the source of human ELF data. Similar to how we conduct ELF PK in infected mice, ideally human PK ought to be generated in patients with infection also. While ELF exposure of levofloxacin was thought to be multitudes higher than in plasma based on healthy volunteer data, it was later demonstrated that the median penetration in infected patients was 0.9 as opposed to 2.4 in healthy volunteers.[6] Developing an ELF HSR based on healthy volunteer data for levofloxacin would have resulted in marked exposure differences and lessened the clinical translation of the model.

The protein-binding characteristics of the tetracyclines are complex and the literature is mixed.[21–23] The class is generally considered concentration-dependent but unlike conventional drug wisdom, does not display inverse proportionality where lower concentrations are bound at a higher percentage and higher concentrations are bound at a lower percentage. Instead, protein binding for this drug class increases with increasing exposure.[24,25] We found similar findings in this model. For protein-binding assessments, *ex vivo* methods are ideal as they maintain a higher level of biological complexity relative to *in vitro* experiments. While murine serum could have been purchased and spiked with tigecycline to determine *in vitro* protein binding, our approach of preparing, infecting and dosing mice consistent with our efficacy studies and sampling directly from the model is likely a more accurate representation of protein binding within the model. This *ex vivo* methodology eliminates variables that need to be accounted for when running *in vitro* assessments such as duration of drug exposure with the matrix, temperature and any additives or pretreatment to the serum.

In summary, this study developed serum (tigecycline), plasma (levofloxacin) and ELF HSRs (both) in the COMBINE murine neutropenic pneumonia model. These regimens were tested in duplicate against a previously characterized challenge set of *K. pneumoniae* and *P. aeruginosa* strains, with efficacy (at least for the ELF HSRs) that could be reasonably anticipated based on MIC. Meropenem, cefiderocol and tobramycin are three additional antibiotics that will undergo similar plasma and ELF HSR development and quantitative cfu/lung assessment in the COMBINE model against the same collection of isolates described previously to provide robust benchmarks using compounds representative of many antibiotic classes for future compound development.

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Transparency declarations

AJF and AMP have none to declare. EMD is an employee of the study funder, CARB-X. DPN is a consultant, speaker bureau member or has received research funding from AbbVie, CARB-X, Cepheid, Innoviva, Merck, Pfizer and Shionogi.

Supplementary data

Figures S1 and S2 and Table S1 are available as Supplementary data at *JAC* Online.

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Development and confirmation of humanized plasma and epithelial lining fluid exposures of meropenem, cefiderocol and tobramycin in a standardized neutropenic murine pneumonia model

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Background: Murine pneumonia models play a fundamental role in the preclinical development of novel compounds seeking an indication for the treatment of pneumonia. It is vital that plasma exposures in these models are not used as a surrogate for exposure in pulmonary epithelial lining fluid (ELF). Herein, human-simulated regimens (HSRs) in both plasma and ELF of meropenem, cefiderocol and tobramycin are described in the standardized COMBINE murine neutropenic pneumonia model.

Materials and methods: HSRs were developed in both plasma and ELF for meropenem and cefiderocol as 2 g q8h 3 h infusions, and tobramycin 7 mg/kg 30 min infusion. Pharmacokinetic studies were performed to confirm plasma and ELF exposures in mice that recapitulated %*f*T > MIC for meropenem and cefiderocol, and *f*Cmax and $fAUC_{0\text{-}24}$ for tobramycin in humans.

Results: Concentration–time profiles and relevant pharmacodynamic exposures for all three compounds were well matched in mice relative to humans. None of the plasma HSRs were able to appropriately humanize the ELF. Thus, modifications of the plasma HSRs were necessary to provide unique HSRs specific to ELF exposure for each compound.

Conclusions: It should not be assumed that lung penetration in mice relative to humans is equivalent. With HSRs confirmed for these three drugs with established clinical use in the treatment of patients with pneumonia, these humanized exposures within the standardized model will allow for back-translation of anticipated efficacy and provide purposeful quantitative benchmarks for cfu/lung assessments for researchers on an international scale.

Background

Preclinical animal infection models continue to play an important role in the development of novel antibacterial compounds.[1] However, many factors can either hinder or improve the clinical translation of the models and therefore the subsequent utility of the derived data. While the murine neutropenic thigh infection model remains the 'gold standard' for initial pharmacokinetic/pharmacodynamic (PK/PD) assessment, it is increasingly important for compounds seeking an indication for pneumonia to undergo assessment in the murine neutropenic pneumonia model.[2] A compound that best exemplifies the relevance of these additional lung investigations is daptomycin, which is inactivated by alveolar surfactant. Unfortunately, this was not discovered until after clinical failure when used to treat severe community-acquired pneumonia during registrational trials.[3]

Akin to the need for accounting for interspecies differences in plasma protein binding in preclinical animal infection models, there is the need to consider interspecies differences in penetration at the site of infection. This is especially true for lung penetration into the epithelial lining fluid (ELF), as the rate and extent of system hysteresis can be significantly mismatched. Therefore, humanizing free plasma exposure in a murine pneumonia model cannot be assumed to achieve clinically translational exposures in the lung, which may be either relatively over- or underexposed.[2]

Further complicating the preclinical landscape of animal infection models is a lack of uniformity between laboratories and research groups.[4,5] To alleviate this concern, the European Innovative Medicines Initiative-funded Collaboration for prevention and treatment of MDR bacterial infections (COMBINE) consortium has defined critical elements of the murine neutropenic pneumonia model in a proposed standardized protocol.[6] The purpose of the studies described here was to use the COMBINE murine neutropenic pneumonia model to develop and confirm human-simulated dosing regimens (HSRs) for three

commonly used antibiotics used to treat critically ill patients with pneumonia: meropenem, cefiderocol and tobramycin. HSRs were created to approximate not only the free plasma exposure, but also the lung ELF, with the hypothesis that different dosing regimens would probably be required to humanize each matrix based on interspecies differences in system hysteresis. Future investigations are planned to use these HSRs against a previously well-defined challenge set of Gram-negative isolates (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) to define quantitative benchmarks for cfu/lung in the standardized model that could be replicated and utilized by research laboratories internationally.[7]

Materials and methods

Antimicrobial agents

Commercial vials were acquired as follows: meropenem 1 g (PremierProRx, Lot #0004E21), cefiderocol 1 g (Shionogi, Lot #0021) and tobramycin 80 mg/2 mL (Mylan, Lot #7608713). Vials were reconstituted as necessary per the manufacturers' instruction and further diluted with normal saline to achieve concentrations required to deliver weight-based dosing to the mice.

Bacterial isolates

K. pneumoniae CDC 851 was sourced from the CDC and FDA Antibiotic Resistance Isolate Bank (Atlanta, GA, USA) and stored locally frozen at −80°C in skim milk. Before pharmacokinetic studies, CDC 851 was subcultured twice on Trypticase soy agar with 5% sheep blood (Becton Dickinson and Co., Sparks, MD, USA) and incubated at 37°C for ∼16 h. Bacterial colonies from the overnight culture plate were suspended in NS to a McFarland target of ∼1.25 to produce the final inoculum. CDC 851 (meropenem MIC ≤0.12 mg/L, tobramycin MIC 16 mg/L, cefiderocol MIC undetermined) has the following known resistance mechanisms: EMRD, fosA5, KDEA, oqxA, oqxA, oqxB19, oqxB19 and SHV-11.[8]

Ethics

Animals were maintained and utilized in accordance with National Research Council recommendations. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Hartford Hospital (Assurance #A3185-01).

Laboratory animals and the neutropenic pneumonia model

The model followed the COMBINE protocol with laboratory-specific detailed methods as follows.[6] Specific pathogen free CD-1, female mice 6–8 weeks old were acquired from Charles River Laboratories, Inc. (Raleigh, NC, USA). All animals were allowed to acclimatize for 72 h before any study procedures and were housed as groups of six at controlled room temperature in HEPA-filtered cages (Innovive, San Diego, CA, USA). Study rooms were maintained with diurnal cycles (12 h light/12 h dark) and food and water were provided *ad libitum*.

Neutropenia was achieved through 0.2 mL intraperitoneal (IP) administrations of cyclophosphamide 150 mg/kg on day minus 4 and an additional 100 mg/kg on day minus 1. A predictable degree of renal impairment was produced using 5 mg/kg of uranyl nitrate (Electron Microscopy Sciences, Hatfield, PA, USA) dissolved in sterile water administered as a 0.2 mL IP injection on day minus 3. Bacterial colonies from the overnight culture plate were suspended in normal saline to a McFarland target of 1.25 to produce the final inoculum. Mice were anaesthetised using inhaled isoflurane, manually restrained upright and infected with 50 µL of bacterial suspension via the nares. Each bacterial inoculation suspension was used within 30 minutes of initial preparation.

Pharmacokinetic studies

Ex vivo tobramycin plasma protein binding studies

Escalating single doses of tobramycin (2.5, 5 and 10 mg/kg) were administered subcutaneously to determine tobramycin plasma protein binding. Triplicate pooled plasma and ultrafiltrate samples were collected at 1 h (5 mice per replicate, 15 mice per dose) and stored at −80°C until concentration determination. Whole blood was collected in K_2EDTA tubes and then subsequently centrifuged at 4°C at 3000**g** for 10 minutes. Plasma was separated (total plasma) and 900 µL was added to an ultrafiltration device (Centrifree®, Merck Millipore Ltd, Ireland) and centrifuged using a fixed rotor at 10°C at 2000**g** for an additional 45 minutes to obtain protein free ultrafiltrate. The triplicate free and total tobramycin concentrations were averaged and then free fractions were calculated using the equation: free fraction = $\text{concentration}_{\text{ultrafiltrate}}/\text{concentration}_{\text{total plasma}}$.

Human-simulated exposure pharmacokinetic studies

The purpose of these studies was to establish HSRs in both plasma and ELF in the COMBINE murine neutropenic pneumonia model equivalent to clinical doses of meropenem (2 g every 8 h as 3 h infusion),[9] cefiderocol (2 g every 8 h as 3 h infusion)[10] and tobramycin (7 mg/kg as 0.5 h infusion)[11–13] based on unbound (free) exposures. Human plasma protein binding values of 2%, 58% and 10%, respectively, were used to determine unbound human exposures.[14–16] While the human plasma concentration–time profile for tobramycin is well described in the literature and made for clear exposure targets around which to develop a plasma HSR in the model, the available literature on the human ELF concentration–time profile was sparser and provided only point-to-point penetration estimates. Because overall AUC exposures between plasma and ELF are a better characterization of penetration, we fit a linear regression (R^2 = 0.986) to single point penetration ratios at 0.5, 2, 4 and 8 h.[12] Using the equation $C_{ELF} = C_{plasma} \times (0.1223 \times \text{time} + 0.1567)$, a physiologically plausible human ELF exposure versus time profile was generated that yielded a AUC_{ELF}/AUC_{plasma} penetration ratio of 0.69, which is similar to the 0.64 reported in a recent population PK analysis of tobramycin human ELF.[17]

At predefined timepoints (shown in Figures 1–3), groups of six mice had blood sampled via retro-orbital bleeding (two time points, under anaesthesia with isoflurane 2%–3% v/v in 100% oxygen via inhalation) and/or cardiac puncture (one terminal time point). Proparacaine hydrochloride ophthalmic solution 0.5% was applied to the eye (1–2 drops) after blood sampling via retro-orbital bleeding. The volume of blood collected was 0.15 mL per sample via retro-orbital bleeding with subsequent fluid replacement using 0.2 mL normal saline given IP. Mice were euthanized by CO_2 exposure before cardiac puncture. Following blood collection by cardiac puncture, but before cervical dislocation, bronchoalveolar lavage (BAL) fluid was collected from the mice at the same time points. A catheter was inserted into the trachea of the mice, and lungs were lavaged with four separate aliquots of 0.4 mL of normal saline. The BAL fluid was withdrawn immediately after injection and pooled for each animal. Pooled BAL was centrifuged for 10 min at 4°C and the supernatant was collected for analysis.

Total plasma concentrations were corrected to free using murine protein binding percentages determined presently for tobramycin, and previously determined values for meropenem (8%) and cefiderocol (31.6%).[18,19] Drug concentrations in ELF were estimated by correcting the drug concentration in BAL fluid for the dilution with normal saline during lung lavage using the following formula: $\text{compound}_{ELF} = \text{compound}_{BAL} \times (\text{urea}_{plasma}/\text{urea}_{BAL})$, where compound_{BAL} is the measured drug concentration in the BAL fluid sample and urea_{plasma} and urea_{BAL} are the concentrations of urea in paired plasma and BAL fluid

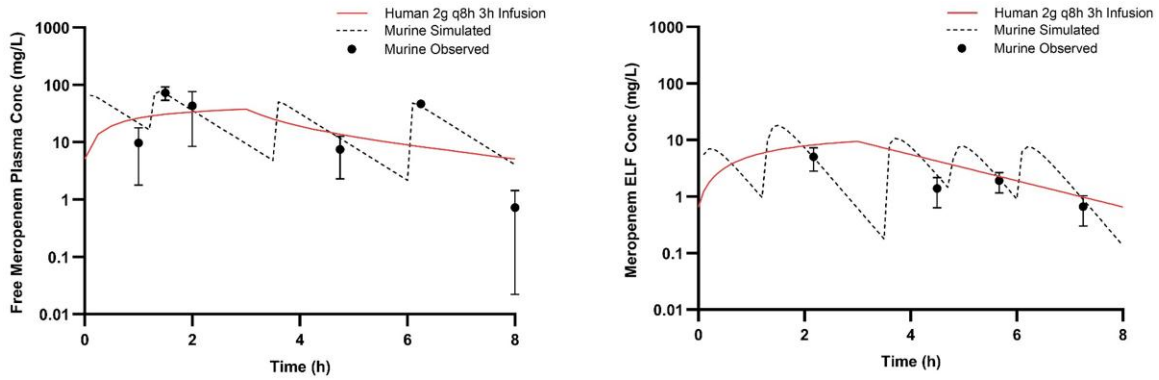

Figure 1. Humanized plasma and pulmonary ELF meropenem exposures after administration of respective HSRs in the COMBINE murine neutropenic pneumonia model. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

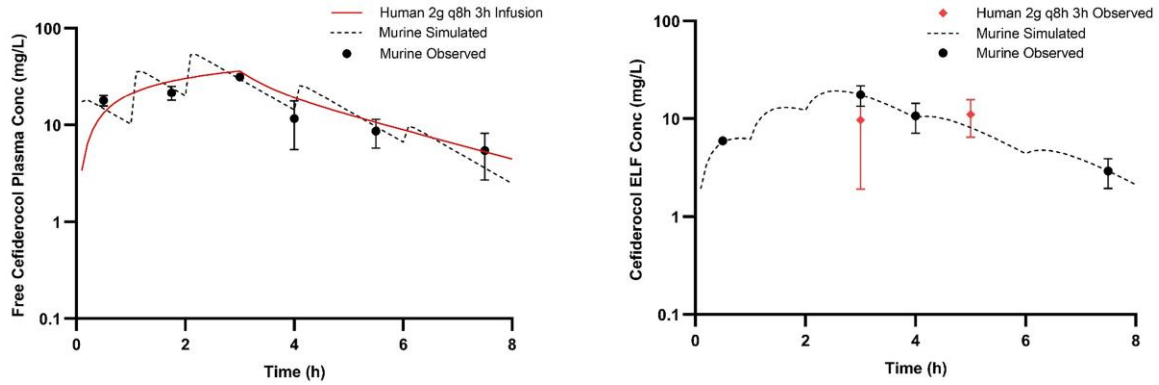

Figure 2. Humanized plasma and pulmonary ELF cefiderocol exposures after administration of respective HSRs in the COMBINE murine neutropenic pneumonia model. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

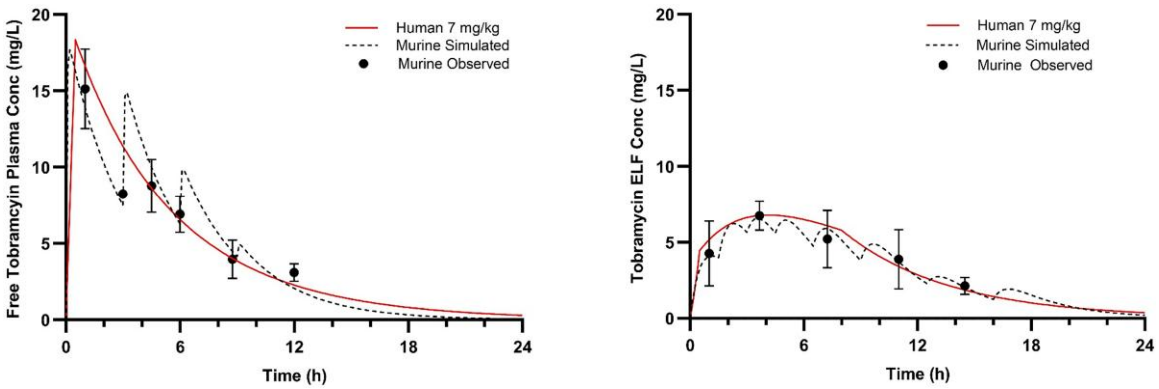

Figure 3. Humanized plasma and pulmonary ELF tobramycin exposures after administration of respective HSRs in the COMBINE murine neutropenic pneumonia model. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

samples from each mouse, respectively. Owing to the lack of albumin in BAL fluid, total ELF concentrations were considered unbound.

Statistical outliers for each respective analyte were removed by the interquartile range method. Previously reported meropenem, cefiderocol and plazomicin (an aminoglycoside similar to tobramycin) plasma HSRs developed in alternative murine models were used as a baseline.[19,20] Mathematical modifications were made to the baseline regimens as necessary to achieve translational exposures in the COMBINE model and repeat confirmatory PK studies were undertaken for both plasma and ELF. A pharmacokinetic model was fit to the plasma and ELF concentrations of each compound and the best-fit estimate parameters were determined by nonlinear least-square techniques (WinNonlin, version 8.3, Pharsight

Corp., Mountain View, CA, USA). Compartment model selection was based on visual inspection of the fit and the Akaike information criterion. The parameter estimates for each respective drug and matrix were used to calculate $fAUC_{0\text{-}24}$, fC_{max} and %$fT>MIC$ (meropenem and cefiderocol only) at each doubling dilution.

The sample size calculation was performed using nQuery Advisor based on the following: (i) the mean %CV of the PK parameter for typical antibiotics is usually <30%, and (ii) a two-sided 95% confidence interval with 80% probability will have an interval that extends no more than 1 SD from the observed mean. As a result, the sample size of six mice per time point is sufficient for the assessment of drug disposition.

Analytical procedures

All compounds were analysed using a Waters Acquity UPLC H-Class system with tandem TQ-XS mass spectrometer (LC-MS/MS) equipped with an Acquity UPLC BEH C18 (1.7 μm, 2.1×50 mm) column maintained at 40°C and a sample manager cooled to 5°C unless otherwise stated. All reagents were obtained from commercial sources and used without further purification. Assay protocols were validated in accordance with Food and Drug Administration bioanalytical method validation guidelines.[21] All samples above the limit of quantification were diluted with blank matrix before sample preparation. Mean interday coefficients of variance (CV) for each assay method are shown in Table 1.

Urea concentrations in BAL and murine K_2EDTA plasma were determined using standards in saline (5–500 μg/mL) and [^{13}C]-urea as the internal standard. The internal standard was diluted with 3:4 acetonitrile:water to yield a 950 ng/mL solution of [^{13}C]-urea for protein precipitation. Compounds were monitored using an ESI probe in positive acquisition mode. The quantitative mass transition for urea was 61.0→43.8. The quantitative mass transition for [^{13}C]-urea was 62.0→45.0. For the preparation of all standards and samples, 630 μL of protein precipitation solution was added to a well plate containing 30 μL of standard or sample. Nominal concentrations of the low quality control (LQC), medium quality control (MQC) and high-quality control (HQC) for urea in saline were 7.5, 75 and 400 μg/mL, respectively.

Concentrations of meropenem in murine K_2EDTA plasma (0.01–100 μg/mL) and saline (0.01–100 μg/mL) were determined using meropenem-d_6 as the internal standard. The internal standard was diluted with 9:1 acetonitrile:water to yield a 50 ng/mL solution of meropenem-d_6 for protein precipitation. Compounds were monitored using an ESI probe in positive acquisition mode. The quantitative and qualitative mass transitions for meropenem were 384.2→141.2 and 384.2→320.17, respectively. The quantitative mass transition for meropenem-d_6 was 390.2→147.0. For the preparation of plasma standards and samples, 250 μL of protein precipitation solution was added to a microcentrifuge tube containing 50 μL of standard or sample. The suspension was vortexed to mix and centrifuged at 15 000**g** for 2 minutes. For analysis, 50 μL of supernatant transferred to a 96-well plate and diluted with 200 μL of water. For the preparation of saline standards and BAL samples, 125 μL of protein precipitation solution was added to a well plate containing 25 μL of standard or sample. This mixture was then diluted with 600 μL of water. Nominal concentrations of the LQC, MQC and HQC for meropenem in both matrices were 0.025, 1 and 50 μg/mL, respectively.

Concentrations of cefiderocol in murine K_2EDTA plasma (0.1–100 μg/mL) and saline (0.025–100 μg/mL) were determined using cefiderocol-d_8 as the internal standard. The internal standard was diluted with 9:1 acetonitrile:water to yield a 100 ng/mL solution of cefiderocol-d_6 for protein precipitation. Compounds were monitored using an ESI probe in positive acquisition mode. The quantitative and qualitative mass transitions for cefiderocol were 752.2→285.0 and 752.2→214.1, respectively. The quantitative mass transition for cefiderocol-d_8 was 760.3→293.0. Cefiderocol standards and samples were prepared in the same manner as described for meropenem in plasma and saline previously. Nominal concentrations of the LQC, MQC and HQC for cefiderocol in both matrices were 0.15, 1 and 50 μg/mL, respectively.

Concentrations of tobramycin in murine K_2EDTA plasma (0.5–100 μg/mL) and saline (25–10 000 ng/mL) were determined using tobramycin-d_5 as the internal standard and a Kinetex F5 (2.6 um, 150×2.1 mm) column maintained at 40°C. Compounds were monitored using an ESI probe in positive acquisition mode. The quantitative and qualitative mass transitions for tobramycin were 468.2→163.4 and 468.2→324.0, respectively. The quantitative mass transition for tobramycin-d_5 was 479.5→331.0. For the preparation of plasma standards and samples, the internal standard was diluted with 9:1 acetonitrile:water to yield a 500 ng/mL solution of tobramycin-d5 for protein precipitation. To a microcentrifuge tube containing 50 μL of standard or sample, was added 250 μL of protein precipitation solution. The suspension was vortexed to mix and centrifuged at 15 000**g** for 2 minutes. For analysis, 50 μL of supernatant transferred to a 96-well plate and diluted with 200 μL of water containing 3% (v/v) formic acid in water. Nominal concentrations of the LQC, MQC and HQC for tobramycin in plasma were 0.75, 7.5 and 75 μg/mL, respectively. For the preparation of saline standards and BAL samples, the internal standard was diluted with water to yield a 500 ng/mL solution of tobramycin-d_5. To a well plate containing 25 μL of standard or sample was added 25 μL of tobramycin-d_5 solution and 200 μL of water containing 2.5% (v/v) trichloroacetic acid. Nominal concentrations of the LQC, MQC and HQC for tobramycin in saline were 75, 500 and 7500 ng/mL, respectively.

Table 1. Mean interday CVs for each assay method

Analyte	Matrix	LQC (%)	MQC (%)	HQC (%)
Urea	Saline	9.9	9.6	11.5
Meropenem	Saline	12.4	3.5	4.2
	Plasma	9.2	3.5	5.8
Cefiderocol	Saline	2.7	3.5	3.5
	Plasma	10.8	4.1	1.6
Tobramycin	Saline	10.0	9.8	6.4
	Plasma	5.2	5.7	5.7

Results

Pharmacokinetic studies

Ex vivo tobramycin serum protein binding studies

Mean percentages of protein binding (±standard deviation) were 27.1% (2.9%), 20.5% (9.3%) and 19.8% (1.5%) at the 1-h timepoint after receiving doses of 2.5, 5 and 10 mg/kg, respectively. These plasma protein binding percentages were considered exposure-independent across the studied dose range, and thus the average observed value across the three doses of 22.4% (77.6% unbound) was applied uniformly to correct for free tobramycin plasma concentrations. Protein binding in humans, while often considered clinically negligible, has been shown to be ~10%.[16]

Human-simulated exposure pharmacokinetic studies

The observed meropenem, cefiderocol and tobramycin plasma and ELF concentrations were satisfactorily described using one-compartment pharmacokinetic models with first order elimination. The best-fit parameters for each drug in each matrix are

Table 2. Best-fit plasma pharmacokinetic parameters in the COMBINE murine neutropenic pneumonia model

Drug	V_{plasma} (L/kg)	K_a (1/h)	K_{el} (1/h)
Meropenem	0.04	1.34	27.13
Cefiderocol	0.19	21.71	0.74
Tobramycin	0.37	23.68	0.31

V_{plasma}, volume of distribution in central compartment; K_a, absorption rate constant into central compartment; K_{el}, elimination rate constant from central compartment.

Table 3. Best-fit ELF pharmacokinetic parameters in the COMBINE murine neutropenic pneumonia model

Drug	V_{ELF} (L/kg)	$K_{a\text{-}ELF}$ (1/h)	$K_{el\text{-}ELF}$ (1/h)
Meropenem	1.60	7.00	2.50
Cefiderocol	0.33	1.94	0.72
Tobramycin	0.79	1.74	0.35

V_{ELF}, volume of distribution in ELF compartment; $K_{a\text{-}ELF}$, absorption rate constant into ELF compartment; $K_{el\text{-}ELF}$, elimination rate constant from ELF compartment.

displayed in Tables 2 and 3. Importantly, the pharmacokinetic parameters coupled with interspecies differences in protein binding did not allow for utilization of the same murine dosing regimen to appropriately simulate humanized exposures in both matrices for any of the three drugs investigated. Therefore, separate dosing regimens were required for each individual matrix of each compound. The murine dosing regimens administered to achieve humanized exposures of each of the three compounds in both matrices are listed in Table 4. Owing to the increased lung penetration observed in the murine model for both meropenem and cefiderocol compared to humans, humanizing the ELF required a 35%–40% reduction in the total drug delivered relative to the plasma HSR. By contrast, due to lower ELF penetration of tobramycin in mice, a 35% increase in total drug delivered was required to approximate the ELF exposure in man. The murine plasma and ELF concentration–time profiles after administration of respective plasma and ELF HSRs for each compound are presented in Figures 1–3. Using the interquartile range method, 10/108 (9.3%) plasma samples and 7/78 (9.0%) BAL samples were excluded from final pharmacokinetic analyses.

The %*f*T > MIC at relevant doubling dilutions achieved in the model with separate HSRs to characterize the plasma and ELF of meropenem and cefiderocol are appropriately matched to average exposures in humans receiving 2 g every 8 h as 3 h infusions as shown in Table 5. For tobramycin, the fC_{max} and $fAUC_{0\text{-}24}$ achieved with both the plasma and ELF HSRs were well matched to humans receiving 7 mg/kg. The human plasma fC_{max} and $fAUC_{0\text{-}24}$ compared with murine plasma were 18.3 versus 17.7 mg/L and 103 versus 103 mg h/L, while the human to murine ELF were 6.8 versus 6.6 mg/L and 79 versus 76 mg h/L, respectively.

Discussion

Characterization of lung ELF is critical for the clinical translation of preclinical animal pneumonia models. Utilizing the standardized COMBINE protocol for the murine neutropenic pneumonia model, we developed and confirmed HSRs in both plasma and ELF for meropenem, cefiderocol and tobramycin: three antibiotics commonly used to treat critically ill patients with pneumonia. Different dosing regimens were required for each matrix, resulting in a total of six unique HSRs across the three compounds, with the general understanding that HSRs based on ELF exposures provide enhanced translation to the clinic relative to plasma HSRs when interspecies differences in target site penetration exist. It should be noted that all these regimens were developed in the model with mice infected with a singular strain. Alternative isolates may have differing levels of virulence and propensity to cause septic shock, which could result in some alteration in the renal clearance of these compounds. However, as has been demonstrated previously, these changes can generally be considered negligible and fall well within the expected variability seen in humans.[22]

While human plasma concentration–time profiles are often well characterized, ELF profiles, especially in infected patients, are often inadequately defined. Existing literature for meropenem ELF pharmacokinetics in patients with ventilator-associated pneumonia provided sufficient %*f*T > MIC exposure targets to allow for the development of an ELF HSR.[9] On the other hand, cefiderocol ELF exposures in mechanically ventilated patients with pneumonia using the clinical dose of 2 g every 8 h as a 3 h infusion have been sparsely characterized with two sampling timepoints at 3 h (end of infusion) and 5 h.[10] Based on these limited data, it could be reasonably concluded that cefiderocol ELF exposures are likely to remain above the susceptibility breakpoint of 4 mg/L for a sufficient percentage of the dosing interval to predict efficacy, which was recapitulated with the cefiderocol ELF HSR. Similarly, because aminoglycoside activity is described by C_{max}/MIC and *f*AUC/MIC, we needed to estimate these values in tobramycin human ELF through applying a regression based on single point-to-point penetration estimates from plasma over time to predict an ELF concentration–time curve. While this extrapolation was performed with one external pharmacokinetic dataset, the AUC_{ELF}/AUC_{plasma} penetration ratio of 0.69 was consistent with the result of 0.64 reported in a recent population PK study.[17] It is imperative that future PK studies undertaken for novel compounds seeking an indication for pneumonia characterize lung exposures throughout the entire dosing interval. Otherwise, preclinical models seeking to simulate humanized exposures will continually rely on methods that are imprecise and based on many assumptions.

Our development of humanized exposure profiles for both plasma and ELF in the well-defined COMBINE murine neutropenic pneumonia model provides a translational link between preclinical studies, *in vitro* potency and the clinical efficacy of meropenem, cefiderocol and tobramycin for the treatment of Gram-negative pneumonia. Furthermore, the application of these defined HSRs in the COMBINE model will allow for the establishment of quantitative bacterial density benchmarks to better delineate the potential clinical utility of novel therapies relative to accepted standard of care treatments.[22]

Table 4. Dosing regimens administered to achieve humanized exposures in plasma and ELF

Human regimen	Matrix	Murine regimen (dorsal subcutaneous injections in 0.1 mL)
Meropenem 2 g every 8 h as a 3 h infusion	Plasma	65 mg/kg at 0 h, 65 mg/kg at 1.25 h, 45 mg/kg at 3.5 h, 45 mg/kg at 6 h repeated every 8 h
	ELF	20 mg/kg at 0 h, 50 mg/kg at 1.25 h, 30 mg/kg at 3.5 h, 20 mg/kg at 4.75 h, 15 mg/kg at 6 h repeated every 8 h
Cefiderocol 2 g every 8 h as a 3 h infusion	Plasma	5 mg/kg at 0 h, 7.5 mg/kg at 1 h, 10 mg/kg at 2 h, 3.5 mg/kg at 4 h, 1 mg/kg at 6 h repeated every 8 h
	ELF	3.75 mg/kg at 0 h, 5 mg/kg at 1 h, 6.25 mg/kg at 2 h, 1.75 mg/kg at 4 h, 1 mg/kg at 6 h repeated every 8 h
Tobramycin 7 mg/kg as a 30 min infusion	Plasma	9 mg/kg at 0 h, 4 mg/kg at 3 h, 2 mg/kg at 6 h, 0.5 mg/kg at 9 h
	ELF	4.8 mg/kg at 0 h, 3.6 mg/kg at 1.5 h, 2.4 mg/kg at 3 h, 2.4 mg/kg at 4.5 h, 2.7 mg/kg at 6.5 h, 2.4 mg/kg at 9 h, 1.2 mg/kg at 12.5 h, 1.2 mg/kg at 16 h

Table 5. Comparison of %*f*T > MIC values achieved with meropenem and cefiderocol in both plasma and ELF at each MIC in humans and in mice receiving respective humanized regimens

			%*f*T > MIC for a MIC (μg/mL) of:							
Drug	Matrix	Species	1 (%)	2 (%)	4 (%)	8 (%)	16 (%)	32 (%)	64 (%)	128 (%)
Meropenem	Plasma	Mouse[a]	100	100	94	75	55	29	5	0
		Human[b]	100	100	100	78	50	19	0	0
	ELF	Mouse[c]	78	62	43	14	3	0	0	0
		Human[b]	90	71	50	16	0	0	0	0
Cefiderocol	Plasma	Mouse[a]	100	100	91	76	50	16	0	0
		Human[b]	100	99	98	76	48	11	0	0
	ELF	Mouse[c]	100	100	85	50	14	0	0	0
		Human[b]	Minimally characterized, see Figure 2							

[a]Average exposures achieved using the plasma HSRs listed in Table 4.
[b]Average exposures achieved with 2 g every 8 h as a 3 h infusion.
[c]Average exposures achieved using the ELF HSRs listed in Table 4.

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Transparency declarations

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Quantitative performance of humanized plasma and epithelial lining fluid exposures of meropenem, cefiderocol and tobramycin against a challenge set of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in a standardized neutropenic murine pneumonia model

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Background: The COMBINE murine neutropenic pneumonia model looks to standardize an important element of preclinical development and provide interlaboratory uniformity. Herein we provide quantitative bacterial density in lung benchmark efficacy data of humanized exposures of meropenem, cefiderocol and tobramycin in plasma and epithelial lining fluid (ELF) against a collection of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

Methods: In accordance with the COMBINE protocol, human-simulated regimens (HSRs) based on both plasma and ELF exposures of meropenem, cefiderocol (both as 2 g q8h as 3 h infusions) and tobramycin 7 mg/kg as 30 min infusions were tested against *K. pneumoniae* and *P. aeruginosa* isolates. The 24 h change in cfu/lung for each HSR was calculated. Each isolate was tested in duplicate against both the plasma and ELF HSRs on separate experiment days.

Results: Meropenem HSRs demonstrated >1 $\log_{10}$ kill against all *P. aeruginosa* isolates with MICs of ≤16 mg/L, but only against *K. pneumoniae* isolates with MICs of ≤2 mg/L as isolates with MICs of >2 mg/L generally harboured carbapenemases. Cefiderocol HSRs uniformly achieved >1 $\log_{10}$ kill against both species at MICs of ≤8 mg/L, with net growth and extensive variability in *P. aeruginosa* isolates with MICs of 16 mg/L. All tobramycin-susceptible isolates demonstrated >1 $\log_{10}$ kill, while non-susceptible isolates did not. Differences in cfu/lung magnitude between the plasma and ELF HSRs were most pronounced around the clinical breakpoints.

Conclusions: In the COMBINE pneumonia model, administration of plasma and ELF HSRs of meropenem, cefiderocol and tobramycin demonstrated 24 h cfu/lung within reason of expectation given known PK/PD properties and existing clinical breakpoints.

Introduction

Preclinical pharmacodynamic (PD) infection models play an integral role in the development of novel anti-infective compounds.[1] However, the absence of standardization within these models between laboratories is a complicating factor in clinical translation and benchmarking.[2] Establishing some level of model consistency is paramount to levelling the preclinical playing field and providing a means to better ensure reproducible findings.[3,4] To that end, the Collaboration for prevention and treatment of MDR bacterial infections (COMBINE) project has developed a standardized protocol for the murine neutropenic pneumonia model.[5] Prior efforts have established a diverse phenotypic and genotypic challenge set of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolates suitable for use in this standardized model.[6] The isolates have reproducibly demonstrated stable MIC values via broth microdilution (BMD), consistent recovery after inoculation and establishment of sufficient growth over the duration of study.

Traditional preclinical pharmacokinetic/PD (PK/PD) dose ranging and fractionation studies are important in informing clinical dose selection in humans. However, these studies ultimately tend to describe single traditional PK/PD indices (i.e. AUC/MIC,

C_{max}/MIC, or $\%T_{>MIC}$) without appreciating the collinearity or co-dependence between all of them on predicting efficacy. As such, simulating the human profile of compounds allows for the additional clinical translation that antimicrobial efficacy is sustained at clinically relevant exposures.[7] Another important attribute to the clinical translation of preclinical animal infection models is the careful consideration of exposures at the site of infection; in the case of the pneumonia model this is the pulmonary epithelial lining fluid (ELF). It has been well described that interspecies differences in target site penetration, including ELF, commonly exist and therefore considering only plasma concentrations may lead to relative overexposure or underexposure at the infection site in mice.[8] To this end, we endeavoured to develop within the COMBINE murine neutropenic pneumonia model human-simulated regimens (HSRs) separately in both plasma and ELF for five antibiotics representative of different antibiotic classes. With HSRs pharmacokinetically confirmed, they were tested against the *K. pneumoniae* and *P. aeruginosa* challenge set to provide robust quantitative benchmarks using compounds representative of many antibiotic classes for future compound development. The cfu/lung benchmarks for HSRs of tigecycline and levofloxacin have been previously reported.[9] Herein, we provide the cfu/lung benchmarks for HSRs of the remaining compounds: meropenem, cefiderocol and tobramycin. The development and confirmation of the HSRs in the model have been previously described.[10]

Materials and methods

Antimicrobial agents

Commercial vials were acquired as follows: meropenem 1 g (PremierProRx, Lot #0004E21), cefiderocol 1 g (Shionogi, Lot #0021), tobramycin 80 mg/2 mL (Mylan, Lot #7608713). Vials were reconstituted as necessary per manufacturer's instructions and further diluted with normal saline to achieve concentrations required to deliver weight-based dosing to the mice.

Bacterial isolates

K. pneumoniae and *P. aeruginosa* isolates were procured from the isolate repositories at the Center for Anti-Infective Research and Development (CAIRD) (Hartford, CT, USA), the CDC and FDA Antibiotic Resistance Isolate Bank (CDC Bank) (Atlanta, GA, USA), the Paul Ehrlich Institute (PEI) (Berlin, Germany) and the Leibniz Institute (DSMZ) (Brunswick, Germany). Prior to experimentation, each isolate was subcultured twice on Trypticase soy agar with 5% sheep blood (Becton Dickinson and Co., Sparks, MD, USA) and incubated at 37°C for approximately 16 h. All isolates will be made readily available for investigators through the DSMZ.

Laboratory animals and the neutropenic pneumonia model

The COMBINE protocol was adhered to with laboratory-specific methods as follows.[5] Specific pathogen-free CD-1, 6–8 week-old female mice were obtained from Charles River Laboratories, Inc. (Raleigh, NC, USA). All animals were housed as groups of six at controlled room temperature in HEPA-filtered cages (Innovive, San Diego, CA, USA). Animals underwent an acclimatization period of 72 h prior to any study procedures. Food and water was provided *ad libitum* and diurnal cycles (12 h light/12 h dark) were maintained within study rooms.

Neutropenia was achieved through intraperitoneal administrations of cyclophosphamide 150 mg/kg on Day −4 and an additional 100 mg/kg on Day −1. A predictable degree of renal impairment was produced using 5 mg/kg of uranyl nitrate administered intraperitoneally on Day −3. The final inoculum was produced from suspension of bacterial colonies from the overnight culture plate in normal saline. Mice were anaesthetized using inhaled isoflurane, manually restrained upright, and infected with 50 µL of bacterial suspension via the nares to target starting bacterial lung burdens of 10^6 for *P. aeruginosa* and 10^7 for *K. pneumoniae* isolates, as previously described.[6] Each bacterial inoculation suspension was utilized within 30 min of initial preparation.

In vivo efficacy studies

Controls were sacrificed 2 h following inoculation and just prior to antibiotic initiation (0 h controls) and 24 h later. All groups contained six mice except for 24 h controls, which contained 4–6 mice. Meropenem, cefiderocol or tobramycin plasma and pulmonary ELF HSRs were administered over 24 h (or equal volume of normal saline in controls), then animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Lungs from individual animals were aseptically harvested and homogenized in normal saline. Homogenized tissue was then serially diluted 10-fold and 50 µL was plated for cfu/lung quantification with a lower limit of quantification of 1×10^2 cfu/lung. The change in cfu/lung count over 24 h for each treatment was calculated relative to the initial bacterial burden. Each isolate was tested in duplicate against both the plasma and ELF HSRs on separate experiment days to incorporate interday model variability. The raw data from individual experiments were combined and analysed as a whole. Among isolates that were tested against multiples of the three drugs, each drug was tested separately on independent study days.

Meropenem plasma and ELF HSRs were tested against 10 *K. pneumoniae* and 9 *P. aeruginosa* isolates with meropenem MICs previously determined by BMD ranging from ≤0.063 to >64, and 1 to >64 mg/L, respectively. Cefiderocol plasma and ELF HSRs were assessed against 10 *K. pneumoniae* isolates (cefiderocol MIC range: 0.125 to >32 mg/L) and 10 *P. aeruginosa* isolates (cefiderocol MIC range: 0.063–16 mg/L). Tobramycin HSRs were evaluated against six *K. pneumoniae* isolates (tobramycin MIC range: 0.25–32 mg/L) and six *P. aeruginosa* isolates (tobramycin MIC range: 0.05 to >64 mg/L). The phenotypic and known genotypic profiles of these isolates are shown in Table 1 and have been reported previously.[6] Additionally, a *K. pneumoniae* isolate (CDC 831) served as an internal QC that was tested against previously described plasma and ELF HSRs of levofloxacin on each study day.[9] To demonstrate consistency with prior model experience, QC acceptance criteria for the model were based on the following: 24 h control growth of ≥1 $\log_{10}$ cfu/lung relative to the 0 h control; greater magnitude of bacterial reduction with the levofloxacin plasma HSR relative to the ELF HSR; and achievement of net killing with the plasma HSR.[9] In instances where any of the above criteria were not met, all data from the study run were discarded and experiments were subsequently repeated.

Sample size for *in vivo* efficacy studies was calculated as follows: (i) for typical antimicrobial agents, optimal dosing regimens usually produce approximately 2–3 $\log_{10}$ decrease in bacterial density with 40% coefficient of variation (CV); and (ii) in order to have an observed mean that deviates from the true mean by no more than 1 SD using a two-sided 95% CI with 80% probability, six datapoints are required.

Although the plasma and ELF HSRs for the respective drugs were determined and confirmed previously, additional efforts to characterize exposures concurrently with the *in vivo* efficacy runs were undertaken utilizing methodology used in the historic confirmations to ensure stability of the regimens in the model over time and among animals infected with different isolates.[10] In brief, at predefined timepoints, groups of mice receiving respective HSRs of meropenem, cefiderocol or tobramycin had blood sampled via retro-orbital bleeding (two timepoints, under anaesthesia with isoflurane 2%–3% v/v in 100% oxygen via inhalation) and/

Table 1. Phenotypic and known genotypic profiles of *K. pneumoniae* and *P. aeruginosa* isolates utilized during *in vivo* efficacy studies

			MIC (mg/L)			
	Isolate origin	Isolate ID	MEM	FDC	TOB	Known genotypic information
K. pneumoniae	CDC Bank	106	>64	8	>64	aac(3)-IId, aac(6′)-Ib, aadA1, aadA2, aph(3″)-Ib, aph(3′)-VI, aph(6)-Id, armA, ARR-2, ble-MBL, catB4, cmlA5, CTX-M-15, dfrA1, dfrA12, EMRD, ere(A), fosA5, KDEA, mph(E), msr(E), NDM-1, oqxA, oqxB20, OXA-1, OXA-9, qacEdelta1, sat2, SHV-28, sul1, sul2, TEM-1A
	CDC Bank	129	16	2	32	aac(6′)-Ib, aadA2, aph(3′)-Ia, catA1, dfrA12, KPC-3, mph(A), OmpK35, oqxA, oqxB, sul1, TEM-1A
	CDC Bank	160	8	0.125	0.25	fosA, oqxA, oqxB, OXA-48, SHV-11
	CDC Bank	504	8	0.25	8	aac(6′)-Ib-cr, catB4, CTX-M-15, OXA-48(c), SHV-OSBL(b), tet(A), tet(R)
	CDC Bank	523	64	2	8	aadA1, aph(3′)-Ia, dfrA1, KPC-2, sul1
	CDC Bank	542	2	>32	>64	aac(6′)-Ib, aadA2, catA1, dfrA12, EMRD, KDEA, mph(A), oqxA, oqxB, SHV-12, sul1
	CDC Bank	548	16	1	0.25	aph(3″)-Ib, aph(6)-Id, CTX-M-15, dfrA14, EMRD, fosA, KDEA, KPC-3, oqxA, oqxB20, QnrS1, SHV-28, sul2, TEM-1
	CDC Bank	555	>64	4	>64	aac(6′)-Ib-G, aadA1, aph(3′)-Ia, ARR-2, ble-MBL, catB, CTX-M-15, dfrA14, EMRD, KDEA, mph(A), NDM-5, oqxA, oqxB25, OXA-232, OXA-9, QnrS1, rmtF1, SHV-12, sul1, TEM-1A
	CDC Bank	560	>64	16	>64	aac(3)-IId, aac(6′)-Ib-AKT, aadA1, armA, ARR-2, ble-MBL, catA1, catB4, cmlA5, CMY-4, CTX-M-15, dfrA1, EMRD, ere(A), fosA, KDEA, mph(E), msr(E), NDM-1, oqxA, oqxB20, OXA-1, OXA-9, sat2, SHV-100, sul1, TEM-1A
	CDC Bank	831	≤0.063	0.5	8	aac(3)-IIa, aph(3′)-Ib, aph(6)-Id, catB4, CTX-M-15, dfrA14, OXA-1, QnrB1, SHV-187, sul2, TEM-1B, tet(A), tet(R)
	CDC Bank	848	64	4	>64	aac(3)-IIa, aac(6′)-Ib-cr, catB4, CTX-M-15, dfrA1, Omp35, OmpK35, oqxA, oqxB, OXA-1, OXA-48, QnrS1, SHV-11, sul1, TEM-1B, tet(A), tet(R)
	PEI	Kp C1.112	≤0.063	0.25	0.5	Unknown
	PEI	Kp C1.113	≤0.063	0.25	0.25	Unknown
	PEI	Kp C1.147	32	2	>64	Unknown
	PEI	Kp C1.151	>64	2	32	KPC
	DSMZ	30104	0.125	0.25	0.125	Unknown
P. aeruginosa	CDC Bank	459	16	0.063	2	OXA-50
	CDC Bank	767	>64	8	>64	GES-20
	CDC Bank	771	>64	1	>64	GES-19, GES-20
	PEI	Pa 88198	2	0.25	0.5	Unknown
	PEI	Pa 88276	1	0.5	1	Unknown
	PEI	Pa 88356	32	4	2	Unknown
	CAIRD	PSA INT 2-41	16	16	>64	aac(6′)-Ib, aac(6′)-Ib-cr, aadA6, aph(3′)-IIb, CTX-M-2, OXA-488, PDC-35, catB7, fosA, qacE, sul1
	CAIRD	PSA INT 4-99	8	8	>64	aac(6′)-Ib-cr, aadA6, aph(3′)-IIb, OXA-14, OXA-488, PDC-35, catB7, cml, cmlA1, fosA, qacE, sul1
	CAIRD	PSA INT 5-19	>64	0.125	>64	OXA-488, PAO (PDC-35), GES-5
	CAIRD	PSA INT 5-35	16	0.5	0.5	aph(3′)-IIb, OXA-488, PDC-471, catB7, fosA
	CAIRD	PSA INT 12-28	8	1	16	aac(6′)-Ib, aadA6, aph(3′′)-Ib, aph(3′)-IIb, aph(3′)-VIb, aph(3′)-XV, aph(6)-Id, GES-1, OXA-488, PDC-35, PER-1, catB7, fosA, qacE, sul1, tet(G)
	CAIRD	PSA US 4-27	64	16	>64	OXA-17/142, OXA-395, PAO (PDC-44), VIM-2

LVX, levofloxacin; MEM, meropenem; FDC, cefiderocol; TOB, tobramycin.

or cardiac puncture (one terminal timepoint). Proparacaine hydrochloride ophthalmic solution 0.5% was applied to the eye (1–2 drops) after blood sampling via retro-orbital bleeding. The volume of blood collected was 0.15 mL per sample via retro-orbital bleeding with subsequent fluid replacement using 0.2 mL normal saline given intraperitoneally. Mice were euthanized by CO_2 exposure prior to cardiac puncture. Following blood collection by cardiac puncture, but prior to cervical dislocation, bronchoalveolar lavage (BAL) fluid was collected from the mice at the same timepoints. A catheter was inserted into the trachea of the mice, and lungs were lavaged with four separate aliquots of 0.4 mL of normal saline. The BAL fluid was withdrawn immediately after injection and pooled for each animal. Pooled BAL was centrifuged for 10 min at 4°C and the supernatant was collected for analysis. Mice receiving plasma-based HSRs did not undergo BAL, and mice receiving ELF-based HSRs did not undergo retro-orbital bleeding.

Ethics

Animals were maintained and utilized in accordance with National Research Council recommendations. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Hartford Hospital (Assurance #A3185-01).

Results

In vivo efficacy studies

Levofloxacin quality control

The QC isolate CDC 831 was tested against levofloxacin plasma and ELF HSRs during 27 separate *in vivo* efficacy experiments. The quantitative cfu/lung values from each study are presented in Figure S1 (available as Supplementary data at *JAC* Online). The data from four experiments were discarded because the QC failed to meet the prespecified criteria for acceptance as follows: two failed to reach 24 h control growth of ≥1 $\log_{10}$ cfu/lung relative to the 0 h control; one failed to achieve greater magnitude of bacterial reduction with the levofloxacin plasma HSR relative to the ELF HSR; and one failed to achieve net bacterial reduction with the plasma HSR.

Meropenem

Across all tested *K. pneumoniae* isolates, the mean ± SD $\log_{10}$ cfu/lung starting bacterial burden and 24 h net growth were 7.30 ± 0.16 and 1.77 ± 0.23, respectively. For *P. aeruginosa* isolates, they were 5.95 ± 0.31 and 3.05 ± 0.70 $\log_{10}$ cfu/lung, respectively. The 24 h change in $\log_{10}$ cfu/lung after receiving saline control, meropenem plasma HSR and meropenem ELF HSR for each isolate is presented in Figure 1.

Among *K. pneumoniae* isolates there was clear differentiation of activity between the three isolates with MICs of ≤2 mg/L (the CLSI intermediate breakpoint), and the seven isolates with MICs of >4 mg/L (the CLSI resistant breakpoint).[11] Resistant isolates all experienced net growth on both the meropenem plasma and ELF HSR, often similar to saline control, while intermediate and susceptible isolates experienced multilog$_{10}$ kill regardless of meropenem HSR. Conversely, both meropenem HSRs achieved ≥1 $\log_{10}$ kill in *P. aeruginosa* isolates with MICs of ≤16 mg/L. Both *P. aeruginosa* isolates with MICs of >64 mg/L demonstrated multilog$_{10}$ growth upon administration of the meropenem ELF HSR, but PSA INT 5-19 showed 1 $\log_{10}$ kill against the plasma HSR, whereas PSA 1866 grew similarly to the growth control.

Meropenem exposures quantified concurrently with *in vivo* efficacy studies were concordant with the original regimen confirmation studies in both plasma and ELF matrices. These data are presented graphically in Figure S2.

Cefiderocol

Among the 10 *K. pneumoniae* isolates tested against the cefiderocol HSRs, the mean ± SD $\log_{10}$ cfu/lung starting bacterial burden and 24 h net growth were 7.27 ± 0.12 and 1.66 ± 0.24,

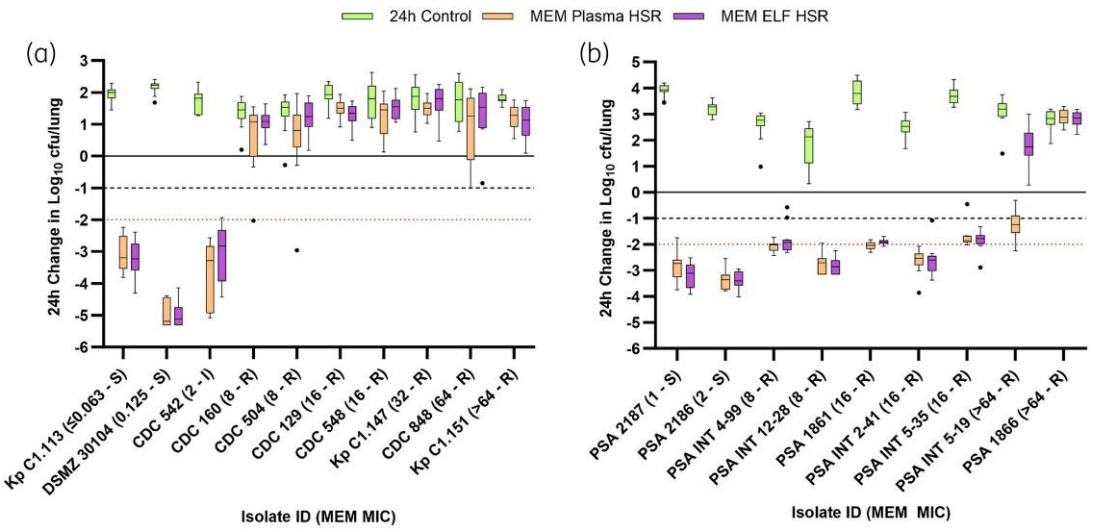

Figure 1. Quantitative cfu/lung data following administration of humanized meropenem (2 g every 8 h as 3 h infusion) plasma and ELF exposures in the COMBINE murine neutropenic pneumonia model against (a) *Klebsiella pneumoniae* and (b) *Pseudomonas aeruginosa* isolates. Boxes represent IQR, with horizontal line denoting the median. Whiskers determined by Tukey's test and outliers displayed as individual dots. Solid line denotes stasis. Dashed line denotes 1 $\log_{10}$ kill. Red dotted line denotes 2 $\log_{10}$ kill. S/I/R, susceptible/intermediate/resistant according to CLSI M100 Thirty-Fifth Edition.

respectively. For *P. aeruginosa* isolates they were 5.74 ± 0.25 and 3.58 ± 0.69 $\log_{10}$ cfu/lung, respectively. Individual isolate 24 h change in $\log_{10}$ cfu/lung after administration of saline control, cefiderocol plasma HSR and cefiderocol ELF HSR is shown in Figure 2.

The efficacy of the cefiderocol HSRs aligned well with the phenotypic profiles and existing CLSI breakpoints (S: ≤ 4 mg/L; I: 8 mg/L; R ≥ 16 mg/L) across both species.[11] All susceptible and intermediate isolates achieved ≥1 $\log_{10}$ kill with minimal differentiation in the magnitude of killing between the plasma and ELF HSRs. *K. pneumoniae* isolate CDC 560 (cefiderocol MIC = 16 mg/L) demonstrated 1 $\log_{10}$ kill with both cefiderocol HSRs. Differences in cfu reduction between the matrix HSRs were most pronounced among *P. aeruginosa* isolates with MICs of 16 mg/L (PSA INT 2-41 and PSA US 4-27) at the resistance breakpoint, although both resulted in net mean growth with considerable intermouse variability. *K. pneumoniae* isolate CDC 542 (cefiderocol MIC > 32 mg/L) grew similarly to untreated control.

Cefiderocol concentration-versus-time profiles from PK sampling taken during *in vivo* efficacy studies are shown in Figure S3. The ELF profile was well aligned with historic HSR determination, as was the plasma profile over the first 3 h, but there was some variance from historic determinations at the later observed timepoints.

Tobramycin

K. pneumoniae and *P. aeruginosa* isolates examined in the COMBINE murine neutropenic pneumonia model against humanized plasma and ELF exposures of tobramycin displayed similar mean ± SD $\log_{10}$ cfu/lung starting bacterial burden and 24 h net growth to those tested against meropenem and cefiderocol—7.33 ± 0.29 and 1.71 ± 0.27 $\log_{10}$ cfu/lung, respectively, for *K. pneumoniae*, and 6.23 ± 0.12 and 3.00 ± 0.74 for *P. aeruginosa*. Figure 3 contains individual isolate data for untreated controls and tobramycin plasma and ELF HSRs.

Tobramycin HSR efficacy in the model was harmonious with clinical breakpoints for both *K. pneumonia* (S: ≤2 mg/L; I: 4 mg/L; R ≥ 8 mg/L) and *P. aeruginosa* (S: ≤1 mg/L; I: 2 mg/L; R ≥ 4 mg/L) isolates.[11] Susceptible isolates demonstrated multilog$_{10}$ killing over 24 h after administration of the HSR associated with either matrix. The *P. aeruginosa* isolate at the intermediate breakpoint (CDC 459—tobramycin MIC = 2 mg/L) showed extensive variability but net growth. All resistant isolates failed to achieve ≥1 $\log_{10}$ of kill with either tobramycin HSR, with *P. aeruginosa* isolates growing similarly to untreated controls, and *K. pneumoniae* isolates demonstrating net growth but numerically lower cfu/lung values at 24 h compared with saline control.

Tobramycin plasma exposures were quantified in mice receiving the plasma HSR on *in vivo* efficacy study days (Figure S4). Observed concentrations reasonably recapitulated the historic plasma HSR regimen confirmation. Similarly, BAL samples were collected in mice receiving the ELF HSR, but inconsistencies in the analytical outputs for these samples precluded their reporting as reanalysis of individual samples were unable to reproduce concentrations within 20% of the original, and sample volume was depleted.

Discussion

These data describe the quantitative cfu/lung performance of humanized plasma and ELF exposures of meropenem, cefiderocol and tobramycin against a challenge set of *K. pneumoniae* and *P. aeruginosa* in the COMBINE murine neutropenic pneumonia model. This manuscript is the last in a series of four manuscripts to: establish a diverse pheno-genotypic challenge set of *K. pneumoniae* and *P. aeruginosa* suitable for use in the COMBINE murine

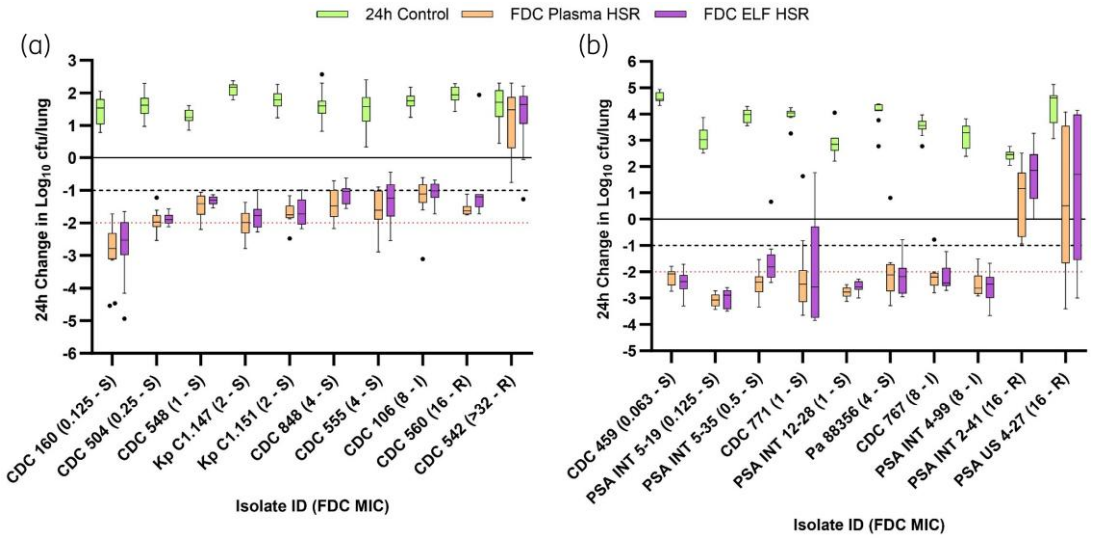

Figure 2. Quantitative cfu/lung data following administration of humanized cefiderocol (2 g every 8 h as 3 h infusion) plasma and ELF exposures in the COMBINE murine neutropenic pneumonia model against (a) *Klebsiella pneumoniae* and (b) *Pseudomonas aeruginosa* isolates. Boxes represent IQR, with horizontal line denoting the median. Whiskers determined by Tukey's test and outliers displayed as individual dots. Solid line denotes stasis. Dashed line denotes 1 $\log_{10}$ kill. Red dotted line denotes 2 $\log_{10}$ kill. S/I/R, susceptible/intermediate/resistant according to CLSI M100 Thirty-Fifth Edition.

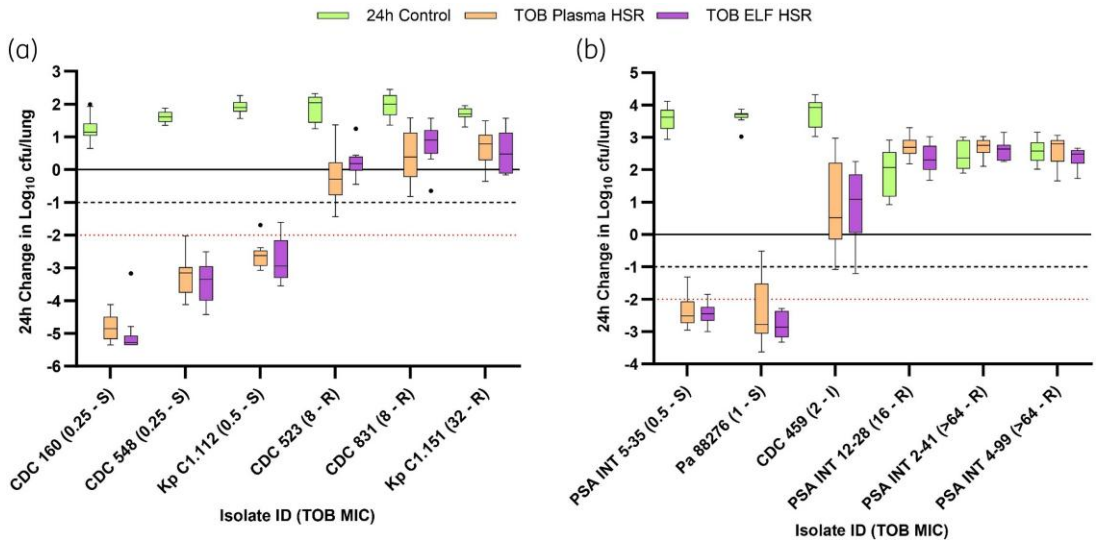

Figure 3. Quantitative cfu/lung data following administration of humanized tobramycin (7 mg/kg as 30 min infusion) plasma and ELF exposures in the COMBINE murine neutropenic pneumonia model against (a) *Klebsiella pneumoniae* and (b) *Pseudomonas aeruginosa* isolates. Boxes represent IQR, with horizontal line denoting the median. Whiskers determined by Tukey's test and outliers displayed as individual dots. Solid line denotes stasis. Dashed line denotes 1 $\log_{10}$ kill. Red dotted line denotes 2 $\log_{10}$ kill. S/I/R, susceptible/intermediate/resistant according to CLSI M100 Thirty-Fifth Edition.

neutropenic pneumonia model; develop and confirm HSRs in both plasma and ELF matrices for tigecycline, levofloxacin, meropenem, cefiderocol and tobramycin; and define quantitative cfu/lung benchmarks against this isolate challenge set after administration of each matrix HSR for the respective compounds.[6,9,10]

The standardization of multiple facets of preclinical animal infection models has the potential to provide benefit and clarity to the development space. The COMBINE murine neutropenic pneumonia model protocol defines specifications for many of the most essential model elements while still allowing enough flexibility for existing laboratories to maintain many of their existing workflows and techniques. Therefore, a shift towards general acceptance of this protocol should be minimally disruptive to research laboratories, especially given that bacterial isolates with defined phenotypic and genotypic profiles that reproducibly induce infection under the stipulated constructs of the COMBINE protocol have already been defined.[6] Importantly, isolates other than those sourced from the CDC Bank will be made readily available to investigators through the DSMZ. While these efforts are a significant first step towards global standardization of preclinical animal infection models, it should be noted that they incorporate only two bacterial species and only one specific infection model. The preclinical infectious disease research community could additionally benefit from similar analyses with *Acinetobacter baumannii* in this model, and the consensus on standardized protocols for other infection models like the murine neutropenic thigh infection model.

Meropenem, cefiderocol and tobramycin have well-defined PK/PD targets based on plasma exposures.[12–14] The purpose of our current study was to establish quantitative bacterial density benchmarks after exposures to humanized plasma and ELF profiles and not to re-define PK/PD targets for these compounds. However, existing clinical breakpoints heavily weigh PK/PD targets in the context of PTA at a given dose, which allows for clinical back-translation of these HSRs in terms of expected efficacy. In general, there was strong agreement between clinical breakpoints and efficacy of humanized exposures across all three tested antibiotics, which represents a validation of established PK/PD targets and the translational relevance of the COMBINE murine neutropenic lung infection model. Perhaps the starkest contrast was the different activity of the meropenem HSRs between *K. pneumoniae* and *P. aeruginosa*. *K. pneumoniae* isolates with meropenem MICs of 8 and 16 mg/L uniformly demonstrated net growth on meropenem HSRs, whereas *P. aeruginosa* isolates with the same meropenem phenotype were able to achieve >1 $\log_{10}$ of kill. Based on free plasma exposures (on which PK/PD targets are often derived), it would not be unreasonable to expect this activity in *P. aeruginosa* at MICs up to 16 mg/L based on the PK/PD target achievement of >40% $fT_{>16\ mg/L}$ with an optimized meropenem dose of 2 g q8h as a 3 h extended infusion. However, recent efforts have shown that phenotype alone may be insufficient to predict antibiotic efficacy, especially for β-lactams when carbapenemases are present.[15,16] Underlying mechanisms of meropenem resistance between *K. pneumoniae* and *P. aeruginosa* are often quite different, with Enterobacterales being driven by carbapenemases and *P. aeruginosa* mainly through porin and efflux pump regulation.[17] Indeed, all tested *K. pneumoniae* with meropenem MICs of 8 or 16 mg/L harboured a carbapenemase (either KPC or OXA-48), whereas no carbapenemases were present in the *P. aeruginosa* isolates with the same meropenem MICs. This alone may be sufficient to explain the differentiation in activity between the two bacterial species when administered equivalent humanized exposures of meropenem. Nonetheless, as previously reported, it should be noted that the starting bacterial burden of *K. pneumoniae* isolates were uniformly targeted 1 $\log_{10}$ higher relative to *P. aeruginosa* isolates in the COMBINE model in order to achieve viability.[6] This

difference could plausibly influence antibiotic efficacy but was not noted with either cefiderocol or tobramycin.

Throughout this four-part series we have belaboured the point of quantifying drug exposures at the target site of infection in preclinical animal models to improve bench-to-bedside translation because interspecies differences in penetration are common. As described in the development and confirmation of the HSRs for meropenem, cefiderocol and tobramycin, these three drugs were no exception.[10] As the magnitude of penetration varied by ~30%–50% between mouse and man, it would not be expected for the efficacy of the plasma and ELF HSRs to be different for every isolate. In fact, only isolates on the slippery slope of PK/PD attainment at or near the breakpoint are likely to be impacted. Differences in penetration of this magnitude would be unlikely to sway target exposure attainment for isolates that are either exquisitely susceptible or highly resistant. Indeed, with few exceptions this is what our dataset demonstrated.

Modern PK/PD preclinical animal model assessments generally determine PK in infected animals up front and then assume that these exposures are consistent with those attained during *in vivo* efficacy studies, without actual reconfirmation of exposures. In general, this is a very reasonable assumption as the reconfirmation of exposures concurrently with *in vivo* efficacy studies requires a notable level of effort and resources. Although identical exposures are not expected to be achieved *in vivo* over a large series of experiments as we have conducted herein due to the intra-individuality of PK in the mice, observed variability during treatment is expected to encompass that which has been originally defined in the HSR regimens, as well as the increases seen in human PK studies of infected patients. Indeed, as shown in the Supplementary data, we demonstrated remarkable PK consistency between the original HSRs and the profiles obtained during the *in vivo* efficacy studies for meropenem in both matrixes, and cefiderocol in the ELF. Reasonably expected variation from the original HSRs was observed for both tobramycin and cefiderocol plasma. Having demonstrated variability within our own laboratory, it is highly encouraged that confirmatory PK studies be undertaken prior to future use of our mg/kg dosing schemes as differences in the drug products (i.e. pharmaceutical versus analytical grade), formulations and manufacturers, as well as mice vendors may result in considerably different drug exposures in each of the biological matrixes.

An additional strength of these investigations was the inclusion of an internal QC with every *in vivo* efficacy assessment. Similar to the reconfirmation of drug exposures, this practice is not commonplace for PK/PD animal infection models for many of the same reasons. However, it should be acknowledged that these models are highly complex biological systems, with many moving parts and both internal and external variables. While all experiments are well intentioned, it may not be possible to identify when one of the variables, whether it be previously understood or currently unrecognized, has been changed or manipulated. To this end, the addition of a QC can help demonstrate model stability and reassure that any unknowing changes in study variables are of limited impact. Of the 27 experiments undertaken, there were 4 instances (15%) in which the QC demonstrated unexpectedly discordant results. Inclusion of this QC isolate in each investigational run revealed a high level (85%) of model reproducibility over the course of months of repetitive studies, while also serving to identify experimental inconsistencies that might have otherwise been missed when using different isolates from run to run. Although not typically done for conventional PD profiling studies, inclusion of the QC isolate confirmed the robustness of the experimental model, as well as the suitability and applicability of the defined bacterial density benchmarks determined for the drug–bug combination studies.

In summary, we have (i) established a diverse phenotypic and genotypic challenge set of *K. pneumoniae* and *P. aeruginosa* suitable for use in the COMBINE murine neutropenic pneumonia model, (ii) developed and confirmed HSRs in both plasma and ELF biological matrixes for tigecycline, levofloxacin, meropenem, cefiderocol and tobramycin and (iii) now have set quantitative cfu/lung benchmarks using both plasma and ELF HSRs with all five compounds against the isolate challenge set. This dataset and the use of the COMBINE protocol serves to further minimize experimental inconsistency and improve the interpretability of endpoint data such as quantitative bacterial density among different laboratories. Moreover, this collective series of studies[6,9,10] supported by CARB-X has broad utility for the development of novel therapies (i.e. small molecules, phage, lysins) because these investigations provide translational PK and PD benchmarks for an array of antibacterials previously demonstrated to be effective in the treatment of infection in man.

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Transparency declarations

A.J.F. and A.M.P. have none to declare. E.M.D. is an employee of the study funder, CARB-X. D.P.N. is a consultant, speaker bureau member or has

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Supplementary data

Figures S1 to S4 are available as Supplementary data at *JAC* Online.

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