Original article

Efficacy of EBL-1003 (apramycin) against *Acinetobacter baumannii* lung infections in mice

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ABSTRACT

Objectives: Novel therapeutics are urgently required for the treatment of carbapenem-resistant *Acinetobacter baumannii* (CRAB) causing critical infections with high mortality. Here we assessed the therapeutic potential of the clinical-stage drug candidate EBL-1003 (crystalline free base of apramycin) in the treatment of CRAB lung infections.
Methods: The genotypic and phenotypic susceptibility of CRAB clinical isolates to aminoglycosides and colistin was assessed by database mining and broth microdilution. The therapeutic potential was assessed by target attainment simulations on the basis of time—kill kinetics, a murine lung infection model, comparative pharmacokinetic analysis in plasma, epithelial lining fluid (ELF) and lung tissue, and pharmacokinetic/pharmacodynamic (PKPD) modelling.
Results: Resistance gene annotations of 5451 CRAB genomes deposited in the National Database of Antibiotic Resistant Organisms (NDARO) suggested >99.9% of genotypic susceptibility to apramycin. Low susceptibility to standard-of-care aminoglycosides and high susceptibility to EBL-1003 were confirmed by antimicrobial susceptibility testing of 100 *A. baumannii* isolates. Time—kill experiments and a mouse lung infection model with the extremely drug-resistant CRAB strain AR Bank #0282 resulted in rapid 4-log CFU reduction both *in vitro* and *in vivo*. A single dose of 125 mg/kg EBL-1003 in CRAB-infected mice resulted in an AUC of 339 h × μg/mL in plasma and 299 h × μg/mL in ELF, suggesting a lung penetration of 88%. PKPD simulations suggested a previously predicted dose of 30 mg/kg in patients (creatinine clearance (CLCr) = 80 mL/min) to result in >99% probability of −2 log target attainment for MICs up to 16 μg/mL.
Conclusions: This study provides proof of concept for the efficacy of EBL-1003 in the treatment of CRAB lung infections. Broad *in vitro* coverage, rapid killing, potent *in vivo* efficacy, and a high probability of target attainment render EBL-1003 a strong therapeutic candidate for a priority pathogen for which treatment options are very limited. **Katja Becker, Clin Microbiol Infect 2021;27:1315**
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Introduction

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) strains are characterized by nosocomial transmission, high-level drug resistance, and high mortality. CRAB has consistently been ranked at the top of both the World Health Organization's (WHO's) list of

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critical priority pathogens and the US Centers for Disease Control and Prevention's (CDC's) most urgent health threats [1,2]. CRAB strains are primarily of concern in ventilator-associated bacterial pneumonia (VABP) and bloodstream infections (BSIs) in critical ill patients, and treatment options often rely on combination therapy that includes a last-resort drug, often colistin [3,4]. Drug resistance is prevalent among clinical *A. baumannii* isolates, and the proportion of CRAB strains has reached a concerning level of up to 90% in some parts of the world [5].

Aminoglycosides used to play an important role in treatment of CRAB, representing a common constituent in drug combination therapy. However, the use of standard-of-care aminoglycosides such as amikacin is frequently compromised by increasing levels of drug resistance [6]. Various mechanisms confer resistance to aminoglycoside in *A. baumannii*, including 16S ribosomal RNA methyltransferases (RMTases) or aminoglycoside-modifying enzymes (AMEs) such as *N*-acetyltransferases (AACs), *O*-phosphotransferases (APHs), and *O*-nucleotidyltransferases (ANTs) [7,8]. RMTase-mediated pan-aminoglycoside resistance has been observed with particularly high prevalence in CRAB and other carbapenem-resistant Gram-negative isolates, stressing the serious threat of antimicrobial resistance and the pressing need for new treatment options [9,10].

EBL-1003, a crystalline free base of apramycin, is a candidate drug currently in clinical development with support from the Innovative Medicines Initiative's European Gram-Negative Antibacterial Engine (IMI ENABLE) [11]. Based on its unique chemical structure, comprising an unusual bicyclic octose moiety, apramycin evades almost all clinically relevant AMEs and is also unaffected by RMTase-mediated pan-aminoglycoside resistance [12]. Modification by the *N*-acetyltransferase AAC(3)-IV remains the sole clinically relevant resistance mechanism for apramycin [12,13]. Several research groups have independently reported outstanding *in vitro* activity of apramycin against highly drug-resistant clinical isolates of *A. baumannii* [12,14,15].

Based on these encouraging reports on apramycin activity, we sought proof of concept for the therapeutic efficacy of EBL-1003 in pulmonary infections, and insights into lung penetration and therefore the probability of target attainment (PTA) in respiratory CRAB infection.

Methods

We analysed the resistance gene annotations of CRAB isolates deposited in the National Database of Antibiotic Resistant Organisms (NDARO) and subsequently assessed the activity of EBL-1003 against a panel of clinical *A. baumannii* isolates. The efficacy of EBL-1003 against CRAB was further investigated in a murine lung infection model. We used matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) to visualize drug distribution in the lung. Lastly, we studied the pharmacokinetics and pharmacodynamics (PKPD) to assess the PTA in epithelial lining fluid (ELF). Full details of all methods are provided in the web-only Supplementary Material.

Results

Genotypic analysis of CRAB clinical isolates

First, we wanted to understand the prevalence of aminoglycoside resistance in a large panel of CRAB isolates. Towards this end we analysed the resistance gene annotations of 5707 clinical *A. baumannii* isolates deposited in the National Database of Antibiotic Resistant Organisms (NDARO) and classified 5451 isolates (95.5%) as carbapenem-resistant (CRAB) based on carbapenemase

annotations in those genomes; 4305 (78.0%) of these CRAB genomes contained at least one aminoglycoside resistance gene. The most prevalent aminoglycoside resistance genes were *aph(3′)-I*, annotating to 2275 (41.7%) CRAB genomes, followed closely by the RMTase *armA* (2164; 39.7%) and the AMEs *aac(6′)-I* (1507; 27.7%), *aph(3′)-VI* (1366; 25.1%), and *aac(3)-I* (1002; 18.4%) (Fig. 1A); *aac(3)-IV* was the least prevalent aminoglycoside resistance gene and annotated to only one (0.02%) of the 5451 CRAB genomes. The only colistin resistance gene in the CRAB isolates was *mcr-4* found in two genome annotations (0.04%).

From the annotation of aminoglycoside resistance genes, we inferred an aminoglycoside resistome for each isolate that we translated into genotypic resistance to individual aminoglycosides, and the lack of any resistance gene into genotypic susceptibility (Fig. 1B, Supplementary Material Table S1). This allowed us to compare the genotypic susceptibility to apramycin in deposited NDARO isolates with that to other aminoglycosides and colistin. Genotypic susceptibility of CRAB isolates to apramycin and to colistin was >99.9%. The genotypic susceptibility to standard-of-care aminoglycosides was considerably lower than for apramycin or colistin.

Antimicrobial susceptibility of CRAB clinical isolates

Next, we determined the *in vitro* activity of EBL-1003 in comparison to standard-of-care drugs in a panel of 100 geographically and phenotypically diverse *A. baumannii* isolates, of which 86 were identified as CRAB (meropenem MIC ≥4 μg/mL). In the overall panel, 99 *A. baumannii* isolates were susceptible to EBL-1003 (applying an apramycin epidemiological cut-off value (ECOFF) of 16 μg/mL [12]), and the MIC_{90} for EBL-1003 was 8 μg/mL. The MIC_{90} of amikacin, tobramycin, gentamicin and plazomicin was >128 μg/mL. Of the 86 CRAB isolates, 85 were apramycin susceptible and 79 showed susceptibility to colistin (MIC <2 μg/mL) while all other tested drugs failed to provide sufficient coverage of the CRAB isolates in the panel (Fig. 2). All of the 68 *A. baumannii* isolates with a pan-aminoglycoside-resistant phenotype had an apramycin MIC of ≤16 μg/mL. Notably, 63 *A. baumannii* isolates and 57 CRAB isolates (66.3%) were of confirmed respiratory origin.

The *in vitro* activity of EBL-1003 against CRAB led us to further explore the bactericidal activity in time–kill experiments. For subsequent analyses we selected the XDR strain *A. baumannii* AR Bank #0282, which is characterized by OXA-23- and OXA-66-mediated carbapenem resistance [16]. The MIC of EBL-1003 was determined as 4 μg/mL for this strain (Supplementary Material Table S2). Concentrations as low as 3 μg/mL (0.75-fold MIC) resulted in CFU reductions, and concentrations of ≥16 μg/mL (≥4-fold MIC; ¼ of C_{max} observed in mice after a single administration of 20 mg/kg) irrevocably suppressed regrowth of persister phenotypes (Supplementary Material Fig. S1).

Lung efficacy dose fractionation studies, lung PK, and MALDI-MS imaging

We then tested the efficacy of EBL-1003 in a murine *A. baumannii* lung infection model with the strain AR Bank #0282. All mice survived the predefined study period of 26 h post infection. A subcutaneous dose of 5 mg/kg EBL-1003 at q6h (total daily dose of 20 mg/kg) resulted in a >2-log_{10} (>99%) reduction in CFU counts in lung tissue at 24 h when compared with the start of treatment. Doses of >5 mg/kg at q6h resulted in a >4-log_{10} (>99.99%) reduction in bacterial burden (Fig. 3A, Supplementary Material Fig. S2).

The pharmacokinetics and lung penetration of EBL-1003 were assessed in the same lung infection model as the efficacy. For a single dose of 20 mg/kg, the C_{max} in plasma was 67 μg/mL at
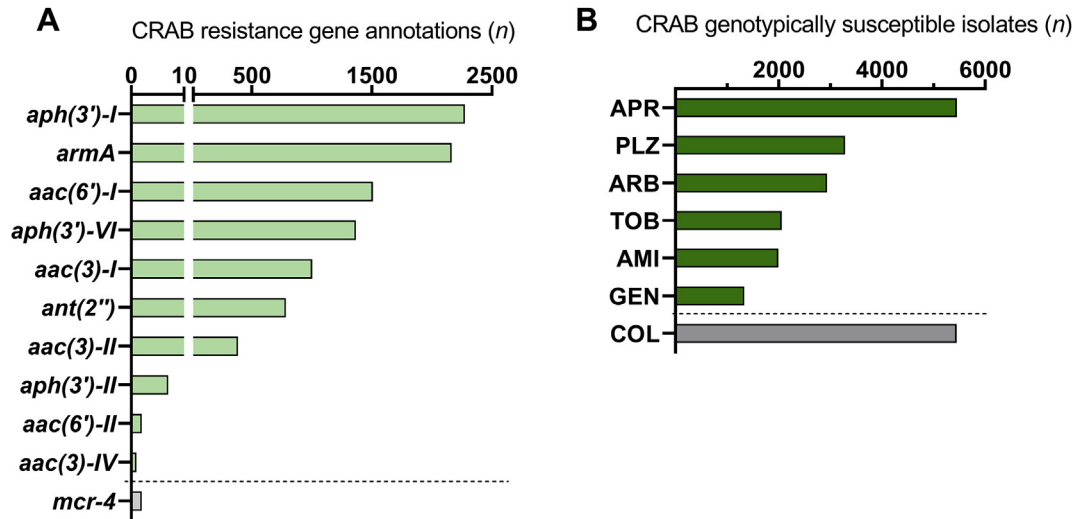
Fig. 1. Prevalence of aminoglycoside- and colistin-resistance genes and inferred genotypic susceptibility of 5451 carbapenem-resistant *Acinetobacter baumannii* (CRAB) isolates. (A) Aminoglycoside resistance gene annotations for 5451 CRAB genomes deposited in the National Database of Antibiotic Resistant Organisms (NDARO) as of 1st September 2020. (B) The resulting genotypic susceptibility of CRAB isolates to apramycin (APR) in comparison to plazomicin (PLZ), arbekacin (ARB), tobramycin (TOB), amikacin (AMI), gentamicin (GEN), and colistin (COL).
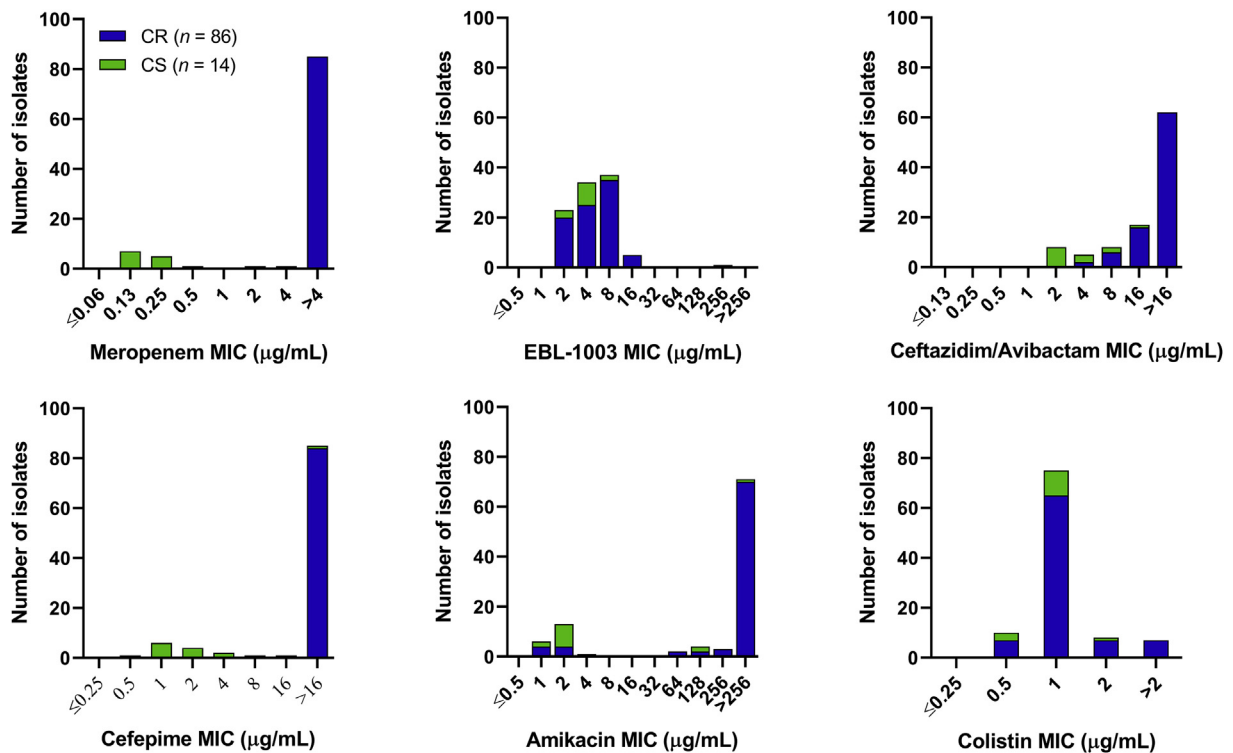

Fig. 2. Minimal inhibitory concentration (MIC) distributions for 100 clinical *Acinetobacter baumannii* isolates: carbapenem-resistant (CRAB) $n = 86$; carbapenem-susceptible *A. baumannii* (CSAB) $n = 14$.

$T_{max} = 0.167$ h. Overall, the C_{max} of EBL-1003 in the ELF was lower than in plasma, but the AUC in ELF resembled the AUC in plasma (Fig. 3B,C). Hence, EBL-1003 penetrated into the lung efficiently and reached an $AUC_{0-24,ELF}/AUC_{0-24,plasma}$ ratio of 0.88 at a dose of 125 mg/kg. The AUC_{plasma} of 339 h × µg/mL for this dose in mice roughly corresponds to the expected therapeutic AUC in humans (Fig. 3B). The human dose predicted to achieve a similar AUC_{plasma} exposure level is approximately 30 mg/kg [17].

Tissue distribution of EBL-1003 in the lungs was further studied by MALDI-MSI after subcutaneous administration in healthy mice. We were thus able to visualize the penetration of EBL-1003 into the lung tissue. The MS images indicated that EBL-1003 was distributed evenly across the entire lung, and confirmed peak tissue concentration at about one hour post administration. Drug clearance over time in lung tissue was were in agreement with decreasing drug concentration in ELF (Fig. 3D).
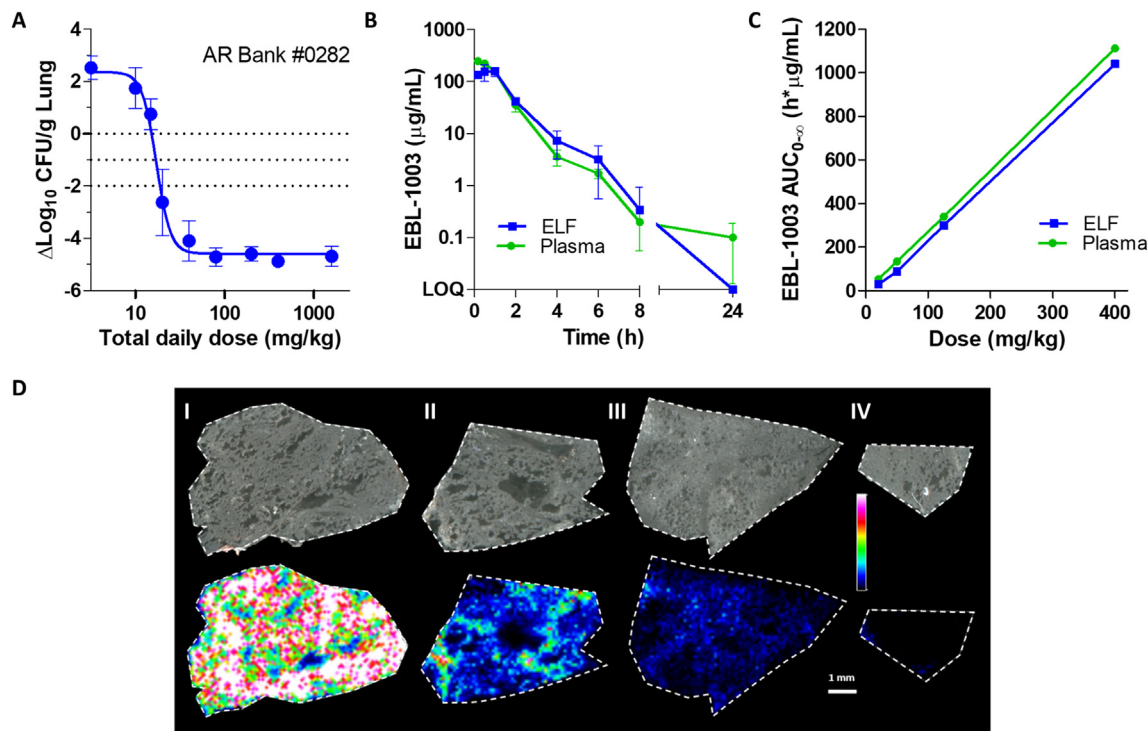
Fig. 3. Mouse lung efficacy and pharmacokinetics of EBL-1003 (apramycin). (A) Efficacy against XDR carbapenem-resistant *Acinetobacter baumannii* (CRAB) strain AR Bank #0282 in a murine lung infection model. Doses were administered q6h and the cumulative daily dose is depicted. Composite results of two dose-fractionation studies are shown with colony-forming unit (CFU) reductions calculated against the study-specific baseline (mean ± SD). (B) Concentration of EBL-1003 in the epithelial lining fluid (ELF) and plasma of CRAB-infected mice after a single subcutaneous dose of 125 mg/kg (mean ± SD; LOQ = 0.01). This dose resulted in an $AUC_{0-24,plasma}$ of 339 h × μg/mL and an $AUC_{0-24,ELF}$ of 299 h × μg/mL (lung penetration ratio = 0.88). (C) The $AUC_{0-\infty}$ in ELF and plasma after single subcutaneous doses of 20, 50, 125 and 400 mg/kg. (D) Distribution of EBL-1003 in lung tissue after a single subcutaneous dose of 400 mg/kg in healthy mice. Tissue sections were analysed by matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) quantifying EBL-1003 relative to kanamycin as internal standard. Ion distribution images of m/z 578.24 (potassiated EBL-1003) at (I) 1 h, (II) 2 h, (III) 4 h post administration, and (IV) in control tissue. Ion intensities are shown using a rainbow scale with maximum intensity scaled to 3%. Scale bar 1 mm; lateral resolution 100 μm.

PKPD and probability of target attainment (PTA) analysis

Next, we integrated plasma and ELF data with the results of the efficacy study (pharmacodynamic, PD) to study which PK/PD index reveals the best correlation. PK/PD indices based on plasma and ELF concentrations had similar correlations. The two indices $fAUC_{ELF}/MIC$ and $fC_{max,ELF}/MIC$ showed good correlation with R^2 values of 0.82 and 0.87, respectively, whereas fT/MIC showed lower R^2 values of 0.59–0.66. The AUC and the C_{max} were therefore much better predictors of CFU reduction in lung than the time over MIC. $fAUC_{ELF}/MIC$ target values for stasis, 1-$\log_{10}$ and 2-$\log_{10}$ CFU reduction in lung tissue at 24 h compared to start of treatment were 6.31, 7.26 and 8.34 (Fig. 4A,B).

Lastly, we simulated the PTA in humans based on a predicted human dose of 30 mg/kg, a clearance rate of 120 and 80 mL/min, and the assumption of 88% lung penetration. For all effect targets in adult healthy males with a clearance of 120 mL/min, the PTA was >99.9% for MICs ≤8 μg/mL. For an MIC of 16 μg/mL, the PTA remained >95% for stasis and 1-$\log_{10}$ CFU reduction but dropped to 90.1% for 2-$\log_{10}$ CFU reduction. For a hypothetical MIC of ≥32 μg/mL, the PTA dropped below 50% for all effect targets. In adult males with reduced renal function (creatinine clearance (CLCr) 80 mL/min), higher PTA was predicted due to a higher drug exposure by the same dose. In these patients, the PTA was >99% for all effect targets up to an MIC of 16 μg/mL. The PTA dropped below 85% for all effect targets for an MIC ≥32 μg/mL (Fig. 4 C,D). PTA results were only slightly affected for dose variations between 20 and 35 mg/kg (Supplementary Material Fig. S3).

Discussion

The findings in this study provide the first proof of concept for lung penetration and the efficacy of apramycin in lung infections in mice. Results were assessed in a PKPD evaluation that supports target attainment in ELF for the predicted therapeutic dose of EBL-1003 in humans. The study further confirms and extends on previous reports of the superior *in vitro* activity of apramycin against *A. baumannii* clinical isolates by analysing the relevant resistance gene annotations in an unprecedented high number of 5451 CRAB genomes.

In CRAB genomes deposited in NDARO, we screened for gene annotations and inferred a resistance phenotype based on this genotypic information which allowed us to assess a large number of isolates. However, in this screening, unspecific resistance mediated by drug efflux and potential gradual effects on susceptibility (intermediate versus resistant) could not be considered, and thus genotypic assessment might not in all cases fully reflect the phenotype. Based on AMEs and RMTases as the major aminoglycoside resistance mechanisms in CRAB, we inferred a very high genotypic susceptibility from the low prevalence of apramycin and colistin resistance genes in CRAB. Colistin is a last-resort drug often used in therapeutic regimens for CRAB infection, but is only used in patients with limited treatment options because of the very high nephrotoxicity of this drug [3,18,19]. Our database analysis suggested high susceptibility to apramycin, rendering EBL-1003 an alternative candidate drug with a coverage of CRAB isolates that compares well with that of colistin. Our data also provide a
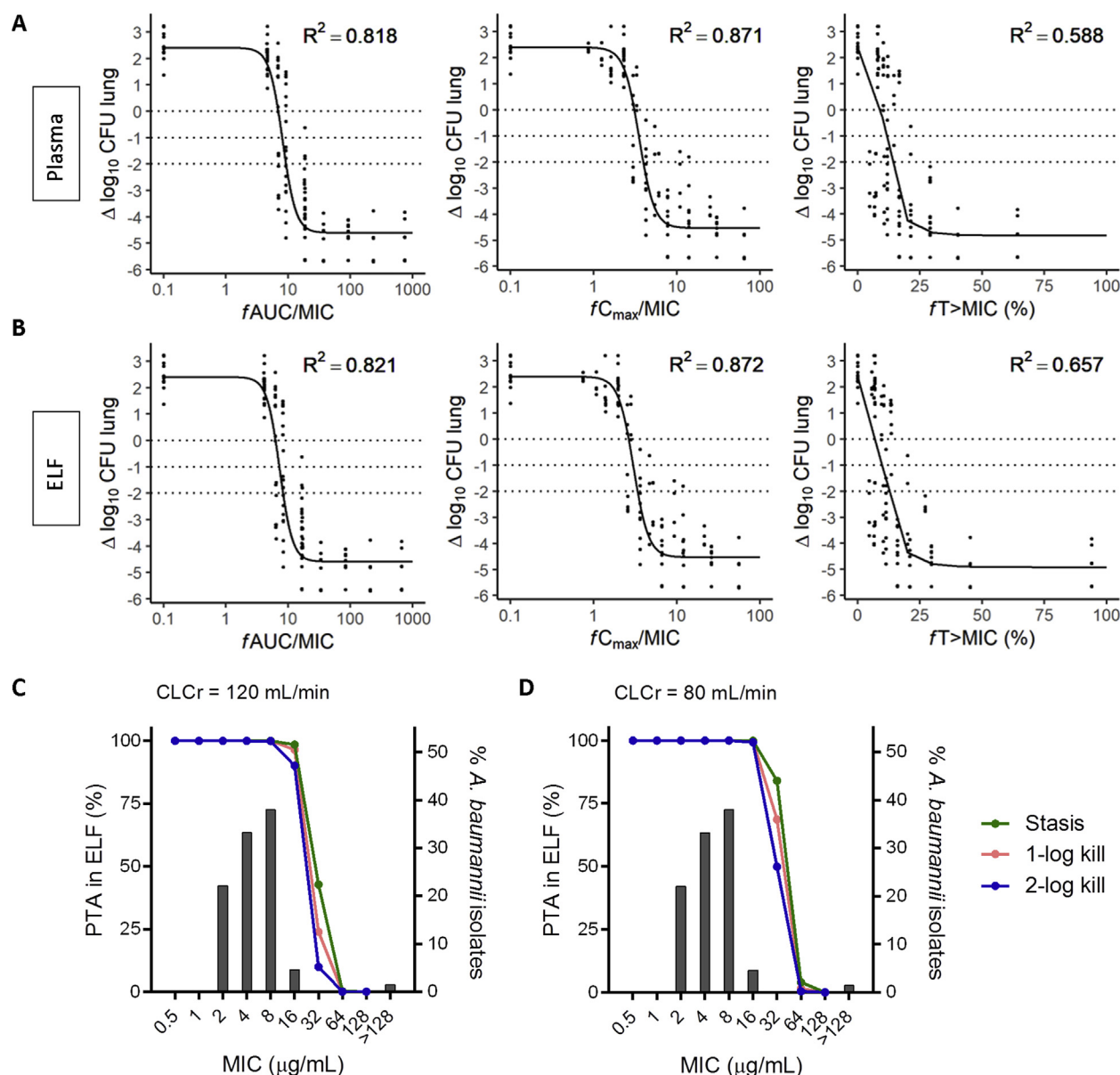
Fig. 4. Pharmacokinetic/pharmacodynamic (PK/PD) analysis of EBL-1003 (apramycin) in plasma and epithelial lining fluid (ELF). (A) Plasma and (B) ELF PK/PD index correlation for *Acinetobacter baumannii* strain AR Bank #0282 with an MIC of 4 μg/mL. For the fAUC/MIC and fC$_{max}$/MIC graphs, PK/PD index value for the drug-free vehicle (which should be 0) was set to 0.1 to enable semi-logarithmic plotting. (C) Probability of target attainment (PTA) in the lung for stasis, 1- and 2-log$_{10}$ kill based on 1000 simulations of EBL-1003 administration at a dose of 30 mg/kg as 30-minute intravenous infusion to an adult healthy male, creatinine clearance (CLCr) = 120 mL/min, and (D) to an adult male with reduced renal function, CLCr = 80 mL/min.

rationale for limited clinical utility of other aminoglycoside antibiotics in CRAB infections, providing further support for the specific differentiation of EBL-1003 within its own drug class.

Both EBL-1003 and colistin also showed very good *in vitro* coverage of 100 clinical *A. baumannii* isolates, the majority of which were CRAB and/or pan-aminoglycoside-resistant. Time−kill experiments confirmed rapid multi-log bactericidal activity of EBL-1003 against the XDR *A. baumannii* isolate AR Bank #0282. Phenotypic assessments in the present and in previous studies are in agreement with the genotypic resistance profiling of the CRAB genomes deposited in the NDARO, indicating that EBL-1003 and colistin are the only two effective antibiotics in the panel tested against CRAB. Although a relatively small number of isolates was assessed *in vitro* in comparison to the genotypic analysis, the results are generally in agreement with previous studies on the

susceptibility of *A. baumannii* and other Gram-negatives to apramycin [12,15,20,21]. Genetic information was unavailable for the isolates tested in this study, but it is reasonable to assume that the single clinical isolate resistant to EBL-1003 carries the *aac(3)-IV* resistance gene, which has been described as the only apramycin resistance mechanism of clinical relevance [12,13]. Notably, a significant proportion of the clinical isolates included in our *in vitro* assays were of respiratory origin, and respiratory CRAB infections have been reported as one of the largest hospital burdens, with colistin often left as the only effective treatment option currently available on the market [18,22].

The *in vivo* efficacy of apramycin has previously been demonstrated by James Kirby and colleagues in a murine thigh infection model [23]. To our best knowledge, this was the first and thus far the only proof of concept for the remarkable efficacy of apramycin

against *A. baumannii*. The outstanding *in vitro* activity of EBL-1003 in our murine *A. baumannii* lung infection model supports the notion of apramycin as a potent alternative to colistin in the treatment of respiratory CRAB infections, and provides proof of concept for the efficacy in lung infections as well. As was expected, based on the *in vitro* time−kill analysis, apramycin led to highly potent killing of *A. baumannii* AR Bank #0282 with low fAUC/MIC targets in both ELF and plasma.

The excellent efficacy of EBL-1003 in the lungs of infected mice indicated sufficient penetration into lung tissue and ELF. Indeed, our pharmacokinetic studies revealed a high AUC in ELF resembling the AUC in plasma, suggesting a lung penetration of 88% at a dose predicted to result in human therapeutic plasma exposure. MS imaging suggested even tissue distribution of the candidate drug. The human AUC lung penetration ratio of aminoglycosides has been reported to range from a low 13% for plazomicin to a high 77% for gentamicin [24−26]. High lung penetration and efficacy of EBL-1003 in mice warrants further clinical assessment of EBL-1003 to demonstrate sufficient drug penetration into human ELF after parenteral administration in humans.

In our model, the EBL-1003 exposure in plasma and ELF (fAUC$_{plasma}$ and fAUC$_{ELF}$, respectively) achieved by doses corresponding to the predicted therapeutic dose for systemic *Escherichia coli* infections in humans (30 mg/kg) were considerably higher than the predicted target values for *A. baumannii* [17]. PK/PD predictions suggested >99.9% PTA for MIC ≤8 μg/mL in the lungs of healthy individuals and even higher PTA in patients with a reduced (80 mL/min) creatinine clearance, representative of typical patients in the target patient population [27,28]. In the absence of a model for the human PK of EBL-1003, human concentration time profiles were simulated from a model of human PK of gentamicin. While being an assumption, this hypothesis is backed up by the chemical similarity of the two molecules and the similarity of their PK in various animal species [17].

The present study emphasizes the great potential of EBL-1003 in the treatment of *A. baumannii* infections based on its unprecedented coverage of multidrug-, carbapenem- and aminoglycoside-resistant isolates, its efficient lung penetration, and its promising PTA. Intrinsically lower toxicity of EBL-1003 has been proposed to provide for an improved safety margin when compared to colistin or other aminoglycosides antibiotics [29,30].

The WHO has not only prioritized CRAB infections as the most critical medical need in antibacterial therapy, as indicated above. It has also monitored the clinical development activities in the field to find a critical gap between the prioritized pathogens and the current clinical pipeline [11]. The current pipeline is dominated by β-lactam/β-lactamase inhibitor combinations, which have in part been successful in addressing critical infections with carbapenem-resistant *Enterobacterales* (CRE), but have demonstrated little benefit in treating *P. aeruginosa* and *A. baumannii*. Of the 52 antibiotics listed in the last WHO clinical pipeline report in 2019, EBL-1003 is one of only five candidate drugs that may hold promise for efficacy against CRAB infections. Further evaluation in patients will be required to underscore the clinical utility and lasting success of new antibiotics in the treatment of CRAB infections.

Author contributions

KB and SNH analysed the NDARO data base. KB, KH, SC, DH and SNH coordinated the *in vitro* microbiology work. SC performed time−kill experiments. KB and SNH coordinated the *in vivo* microbiology including plasma and ELF pharmacokinetics. AN, RS, BP and PEA contributed to the MS imaging of lung tissue. VAC and LEF performed PK analysis, PKPD evaluation, and PTA simulations. LEF and SNH conceptualized the study. KB, VAC, ECB, LEF and SNH wrote the manuscript. All authors reviewed and approved the manuscript.

Transparency declaration

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.12.004.

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