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Assessment of Minocycline and Polymyxin B Combination against *Acinetobacter baumannii*

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Antimicrobial resistance among *Acinetobacter baumannii* is increasing worldwide, often necessitating combination therapy. The clinical utility of using minocycline with polymyxin B is not well established. In this study, we investigated the activity of minocycline and polymyxin B against 1 laboratory isolate and 3 clinical isolates of *A. baumannii*. Minocycline susceptibility testing was performed with and without an efflux pump inhibitor, phenylalanine-arginine β-naphthylamide (PAβN). The intracellular minocycline concentration was determined with and without polymyxin B (0.5 μg/ml). Time-kill studies were performed over 24 h using approximately 10^6 CFU/ml of each strain with clinically relevant minocycline concentrations (2 μg/ml and 8 μg/ml), with and without polymyxin B (0.5 μg/ml). The *in vivo* efficacy of the combination was assessed in a neutropenic murine pneumonia model. Infected animals were administered minocycline (50 mg/kg), polymyxin B (10 mg/kg), or both to achieve clinically equivalent exposures in humans. A reduction in the minocycline MIC (≥4×) was observed in the presence of PAβN. The intracellular concentration and *in vitro* bactericidal effect of minocycline were both enhanced by polymyxin B. With 2 minocycline-susceptible strains, the bacterial burden in lung tissue at 24 h was considerably reduced by the combination compared to monotherapy with minocycline or polymyxin B. In addition, the combination prolonged survival of animals infected with a minocycline-susceptible strain. Polymyxin B increased the intracellular concentration of minocycline in bacterial cells and enhanced the bactericidal activity of minocycline, presumably due to efflux pump disruption. The clinical utility of this combination should be further investigated.

Acinetobacter baumannii is a common nosocomial pathogen globally which has been implicated as an etiologic agent in ventilator-associated pneumonia, skin and skin structure infections (traumatic battlefield and other wounds), urinary tract infections, meningitis, and bacteremia (1). Over the past decades, infections due to increasingly resistant strains of *Acinetobacter* have emerged (2, 3). This is particularly alarming because there are very limited therapeutic options for these infections (4, 5). Multidrug-resistant strains of *A. baumannii* have been reported to adversely affect patient outcomes. Studies have found significantly higher rates of hospital mortality in patients infected with multidrug-resistant strains than in patients with susceptible strains (6, 7).

Current treatment strategies for multidrug-resistant *A. baumannii* include combination therapy with tigecycline, minocycline, carbapenems, polymyxins, and even daptomycin (8–11). However, the rationale of using certain agents together in a combination is not well established. In this study, the combination of minocycline and polymyxin B was studied based on a mechanistically plausible approach. Resistance to the tetracycline class in *A. baumannii* is commonly mediated through the upregulation of efflux pumps located within the cell membrane (12). In order to function effectively, the structural units of an efflux pump have to be properly aligned on the cell membrane. It is hypothesized that based on its mechanism of action, polymyxin B would disrupt the cell membrane (13), affect the proper functioning of the efflux pumps, and thereby enhance the activity of minocycline. The purpose of this study was to investigate the utility of using minocycline in combination with polymyxin B against *A. baumannii*.

MATERIALS AND METHODS

Bacterial isolates and *in vitro* susceptibility. Four strains of *A. baumannii* (1 laboratory isolate and 3 clinical isolates) with a broad range of minocycline susceptibility were used (Table 1). All strains belonged to the *Acinetobacter calcoaceticus-A. baumannii* complex based on the API 20 NE system (bioMérieux Vitek, Marcy l'Etoile, France). Identification of *A. baumannii* was confirmed by detection of the OXA51 gene by PCR (14). MICs were determined by broth dilution in triplicate according to the CLSI in the presence and absence of phenylanine-arginine β-naphthylamide (PAβN) (100 μg/ml), an efflux pump inhibitor (15). In order to exclude the inhibitory effect of PAβN, the bacterial burden of each positive control was determined by quantitative culture.

Resistance mechanisms. Colony quantitative PCR (qPCR) was carried out to determine the presence of the *tetA*, *tetB*, *tetM*, and *tet39* genes in *A. baumannii* strains. Colonies of overnight-streaked strains on LB agar plates were chosen, resuspended, heated, and used as PCR templates. The qPCR was run using Sybr green Select master mix (ABI) in an ABI 7000 sequence detection system. The threshold cycle (C_T) values were normalized with the housekeeping gene *recA* of the same strain. The difference (ΔC_T) was used as a logarithmic power (base = 2) to calculate the relative signal of the gene. The transcription levels of the known efflux genes, *adeB*, *adeJ*, and *adeG*, were determined by reverse transcription (RT)-

Received 18 August 2014 Returned for modification 15 November 2014
Accepted 15 February 2015

Accepted manuscript posted online 23 February 2015

Citation Bowers DR, Cao H, Zhou J, Ledesma KR, Sun D, Lomovskaya O, Tam VH. 2015. Assessment of minocycline and polymyxin B combination against *Acinetobacter baumannii*. Antimicrob Agents Chemother 59:2720–2725. doi:10.1128/AAC.04110-14.

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doi:10.1128/AAC.04110-14

TABLE 1 Characteristics of the bacterial isolates used

Isolate	Source	MIC (μg/ml)			Resistance mechanism(s)
		Minocycline	Minocycline + PAβN[a]	Polymyxin B	
AB BAA 747	Laboratory	0.25	0.125	1	Reference *adeB* expression
AB 7283	Clinical	0.5	0.125	1	Moderate overexpression[b] of *adeB*
AB 1261	Clinical	1	0.125	2	Moderate overexpression of *adeB*
AB 7416	Clinical	16	2	1	*tetB*, moderate overexpression of *adeB*

[a] PAβN, Phe-Arg b-naphtylamide (100 μg/ml).
[b] Moderate overexpression, >10× the transcription level by qRT-PCR, compared to that in a wild-type isolate susceptible to minocycline.

qPCR. Cells were grown in cation-adjusted Mueller-Hinton broth (Ca-MHB) and centrifuged, and total RNA was isolated (Ambion RiboPure-Bacteria RNA isolation kit [ABI]). The RT reaction was performed using the TaqMan reverse transcriptase reagent kit (ABI) with a mixture of primers. The results (C_Ts) were normalized with the housekeeping gene *rpoB*.

Time-kill studies. Time-kill studies were performed using approximately 1×10^6 CFU/ml of each strain with clinically relevant minocycline concentrations (2 μg/ml and 8 μg/ml), with and without a subinhibitory polymyxin B concentration (0.5 μg/ml). The time-kill study methods have been described previously (16). Briefly, an overnight culture of the isolate was diluted with prewarmed Ca-MHB and incubated further at 35°C until reaching log-phase growth. The bacterial suspension was further diluted with Ca-MHB according to absorbance at 630 nm. Fifteen milliliters of the suspension was transferred to sterile flasks, containing a specific amount of drug to achieve the desired final concentration, and placed in a shaker bath. Serial samples (0.5 ml) were taken in duplicate at baseline and at 1, 2, 4, 8, 12, and 24 h, centrifuged (10,000 × *g* for 15 min), and reconstituted with sterile normal saline to their original volumes to minimize any drug carryover effect. Bacterial populations were quantified by plating the 10× serial dilutions onto Mueller-Hinton agar plates and incubated at 35°C for 18 to 24 h. The bacterial density from each sample was determined by visual inspection of CFU. The limit of detection for the time-kill studies was 100 CFU/ml. These are based on plating 200 μl of an undiluted sample and visual inspection with ≥20 CFU per plate. Experiments were performed at least in triplicate on different days.

Intracellular concentration. The inoculum was prepared as detailed above. The intracellular minocycline concentration was determined with or without polymyxin B in triplicate. Five milliliters of the bacterial suspension was exposed to minocycline (8 μg/ml) with or without polymyxin B (0.5 μg/ml). After incubation in a water shaker bath at 35°C for 30 min, a sample (500 μl) was taken to determine bacterial density by quantitative culture. The remaining bacterial suspension was centrifuged (3,220 × *g* at 4°C for 10 min). The pellet was washed twice with 50 mM phosphate-buffered saline (PBS) (pH 7.0) and reconstituted in 100 μl water. The samples were transferred to new tubes, and 400 μl acetonitrile with doxycycline (internal standard) was added to extract minocycline from bacterial cells. The tubes were mixed by vortexing for 15 s and centrifuged for 15 min at 18, 000 × *g*, and supernatants were evaporated to dryness under a stream of ambient air. After reconstituting with 1 ml of 50% methanol and centrifuging for 15 min at 18,000 × *g*, the minocycline concentration was determined by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method as detailed below. The final intracellular drug concentration in each sample was normalized by the viable bacterial burden after drug exposure. These experiments were repeated 3 times on different days.

Neutropenic murine pneumonia model. The *in vivo* antibacterial activity of minocycline in combination with polymyxin B was examined using a neutropenic murine pneumonia model. This infection model has been described elsewhere (16, 17). Briefly, transient neutropenia was induced in female Swiss Webster mice weighing between 20 and 25 g (Harlan, Indianapolis, IN). The mice were given food/water *ad libitum*, and were administered two intraperitoneal doses of cyclophosphamide (150 mg/kg at 4 days prior to infection and 100 mg/kg at 1 day prior to infection). The experimental protocol was approved by the University of Houston Institutional Animal Care and Use Committee.

Minocycline pharmacokinetic study. The single-dose pharmacokinetics of minocycline in infected mice were explored. Three mice were sacrificed serially at 0.5, 1, 2, 5, and 8 h after an intraperitoneal injection (50 mg/kg), and blood samples were collected by cardiac puncture. Blood samples were allowed to clot on ice and centrifuged at 10,000 × *g* and 4°C for 15 min. Supernatants (100 μl) were mixed with 100 μl water and 400 μl acetonitrile (with 4 μg/ml doxycycline as an internal standard). Samples were vortexed for 15 min and centrifuged for 15 min at 18,000 × *g*, and supernatants were evaporated to dryness under a stream of ambient air. After reconstituting with 100 μl of 50% methanol and centrifuging for 15 min at 18,000 × *g*, the minocycline concentration was determined by a validated ultraperformance liquid chromatography (UPLC)-UV method, as detailed below. The serum concentrations of minocycline observed at each time point were averaged. A one-compartment model with linear absorption was fit to the average drug serum concentration-time profile. The fit of the model was assessed by the coefficient of determination. Using the best-fit parameters, the area under the concentration-time curve from 0 h to infinity ($AUC_{0-\infty}$) was calculated (dose divided by total clearance). All modeling procedures were performed using the ADAPT II program (18).

Polymyxin B pharmacokinetic study. The single-dose pharmacokinetics of polymyxin B in infected mice were explored in a manner similar to that described above. Three mice were sacrificed serially at 0.5, 1, 2, and 4 h after an intraperitoneal injection (10 mg/kg). To explore the impact of renal function on drug elimination, an additional group of animals were given an intraperitoneal dose of uranyl nitrate (5 mg/kg) on day −2. Renal impairment was confirmed in selected animals on the day of infection by measuring serum creatinine. The polymyxin B concentration in serum was determined by a validated UPLC-MS/MS method (19).

Drug assays. The minocycline concentration in mouse serum was determined by a validated UPLC-UV method. Since a lower concentration was anticipated, a more sensitive LC-MS/MS method was used to determine the minocycline concentration in bacterial cells.

Both the UPLC-UV and LC-MS/MS assays shared the same chromatographic method, which consisted of a Waters Acquity UPLC and a Waters BEH C_{18} column (1.7 μm by 2.1 by 50 mm). Mobile phases A and B were water and acetonitrile, respectively, both containing 0.1% formic acid. The injection volume was 5 μl. The analytes were separated by gradient elution at 45°C. The gradient was as follows: 0 to 0.5 min, 98% A; 0.5 to 0.7 min, 98 to 84% A; 0.7 to 1.2 min, 84 to 76% A; 1.2 to 1.7 min, 76 to 70% A; 1.7 to 2.1 min, 70 to 50% A; 2.1 to 2.5 min, 50 to 5% A; 2.5 to 3.0 min, 5% A; 3.0 to 3.2 min, 5 to 98% A; 3.2 to 5 min, 98% A.

The UV detection was performed on a Waters diode-arrayed detector at 350 nm. The intraday and interday variabilities of the UPLC-UV method were <0.82% and <4.39%, respectively. The linear range of detection was 0.03 to 64 μg/ml. The mass spectrometry analysis was performed on API5500 Qtrap triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with Turbo-Ion-Spray source. The multiple-reaction monitoring (MRM) scan type in positive mode was used. The transitions of *m/z* 458.3 to 441.2 and *m/z*

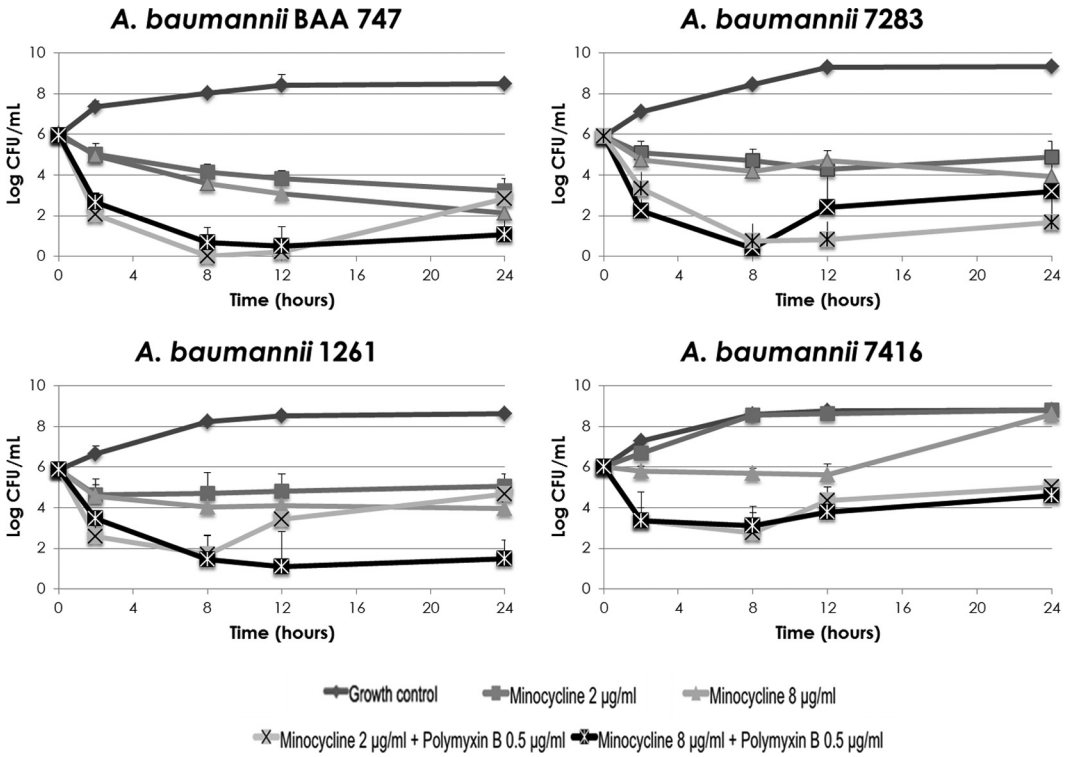

FIG 1 Twenty-four-hour time-kill studies.

445.2 to 154.2 were used for quantifying minocycline and doxycycline, respectively. The intraday variability of the LC-MS/MS system was <7.5%, while the interday variability was <11.4%. The linear range of detection was 0.24 to 500 ng/ml.

***In vivo* antibacterial effect.** The neutropenic mice were anesthetized and inoculated with approximately 1×10^7 bacterial cells under largnygoscopic guidance. The inocula used were guided by pilot inoculum ranging studies to result in >50% mortality of animals at between 48 and 96 h postinfection. Approximately 2 h after infection, the mice were randomly divided into 4 treatment groups: (i) placebo, (ii) minocycline, (iii) polymyxin B, and (iv) a combination of minocycline and polymyxin B. The daily doses of minocycline and polymyxin B used were guided by the pharmacokinetic studies detailed above in order to mimic clinically relevant exposures of these agents. Experiments were performed in duplicate.

The bacterial burden in lung tissue was used to verify the initial inocula at baseline ($n = 2$), at 24 h ($n = 3$), and upon death or up to 96 h ($n = 10$ for each treatment group), as described elsewhere (20). Briefly, lung tissues were aseptically collected and homogenized in 10 ml sterile saline. The samples were centrifuged at 4,000 × *g* and 4°C for 15 min. The pellets were then resuspended in normal saline to 10× their original volumes to minimize any carryover effect from the drugs. The samples were diluted using 10× serial dilutions, quantitatively cultured on Mueller-Hinton agar plates, and then incubated for 24 h. The bacterial burden in lung tissues was calculated via visual inspection of colony growth and compared using Student's *t* test. All animals were observed every 8 h; at each inspection time, any animals that appeared to be moribund were humanely euthanized. Death was recorded as it occurred, at the next inspection time. Any mice remaining at the end of the 96-hour experiment were euthanized by CO_2 asphyxiation. Bacterial burdens observed at the same time frame were compared using Student's *t* test. Survival over time was assessed using Kaplan-Meier survival analysis and the log rank test.

RESULTS

***In vitro* susceptibilities, resistance mechanisms, and time-kill studies.** The *in vitro* susceptibilities of the strains to various agents and the tetracycline resistance mechanisms are shown in Table 1. No effects were attributed to the PAβN concentration used (data not shown). In the presence of PAβN, the MIC of AB7416 was not as low as those of the other strains. It is unclear if this is due to the presence of *tetB* or another, alternative mechanisms(s).

The results of the time-kill studies are shown in Fig. 1. As anticipated, a subinhibitory concentration of polymyxin B was unable to suppress bacterial growth alone (data not shown). However, the addition of polymyxin B suppressed more bacterial growth than either concentration of minocycline. In *A. baumannii* 1261, the high-concentration combination resulted in approximately a 2.5 log decrease compared with growth with minocycline alone after 24 h (means ± standard deviations [SD] were as follows: minocycline at 2 μg/ml, 5.05 ± 0.62 CFU; minocycline at 8 μg/ml, 3.96 ± 0.31 CFU; and minocycline at 8 μg/ml plus polymyxin B at 0.5 μg/ml, 1.48 ± 0.93 CFU). Bacterial regrowth was observed for two strains (AB7283 and AB7416) after 8 h in the combination treatment groups.

Intracellular concentrations. In the presence of polymyxin B, the minocycline intracellular concentration was increased consistently among the bacterial strains. However, the absolute ratio of increase varied widely. Therefore, the intracellular drug concentration was fixed at 1 when minocycline was used alone, and the median values were reported for each isolate, as shown in Fig. 2.

Minocycline pharmacokinetics. The fit of the model was satisfactory; the coefficient of determination (r^2) was 0.99. The elimination half-life, volume of distribution, and clearance of minocycline were 3.2 h, 0.09 liter, and 0.02 liter/h, respectively. The AUC of minocycline was 66 mg · h/liter, which was similar to that reported after an intravenous dose of 200 mg given to humans (67 to 85.8 mg · h/liter) (21).

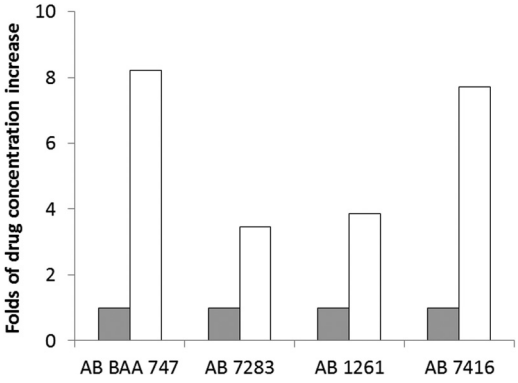

FIG 2 Increased intracellular minocycline concentration with polymyxin B. The value for the minocycline-only group was fixed at 1 (reference) (shaded bars).

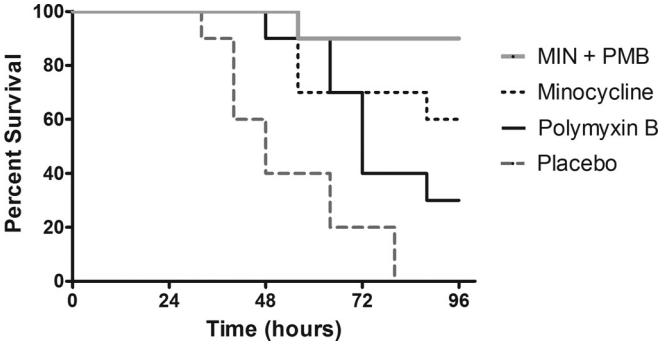

FIG 4 Survival rates of animals infected with AB 1261 ($P < 0.001$ for treatment groups compared to placebo group).

Polymyxin B pharmacokinetics. The fits of the model were satisfactory; the r^2 values were >0.91. The elimination half-life, volume of distribution, and clearance of polymyxin B1 were 2.6 h, 0.01 liter, and 0.00275 liter/h, respectively. The AUC of polymyxin B1 was 68 mg · h/liter, which was similar to the reported range after conventional doses of polymyxin B were given intravenously to humans (22). Despite a >5-fold elevation in baseline serum creatinine, the observed drug exposures were not considerably different. The ratio of AUC_{0-4} in animals with and without renal impairment was 0.91 (data not shown).

***In vivo* antibacterial effect.** The bacterial tissue burdens at 24 h for all 3 clinical strains are shown in Fig. 3. Lung tissue burdens (mean ± SD) at baseline were 7.67 ± 0.15 log CFU/g (for AB 7283), 7.6 ± 0.04 log CFU (for AB 1261) and 8.36 ± 0.03 log CFU/g (for AB 7416). After 24 h, the bacterial burden in lung tissue increased consistently in the placebo group and the group given polymyxin B only. Minocycline monotherapy demonstrated moderate activity in suppressing growth of two susceptible clinical strains (AB 7283 and AB 1261); adding polymyxin B resulted in a further reduction in the bacterial lung burden at 24 h. For AB 7416, no significant differences were observed among the treatment groups (Fig. 3).

Since the most pronounced effect of the combination was found against AB 1261, an additional experiment with treatment of the animals for up to 96 h was performed. Survival of the infected animals over time is shown in Fig. 4. As anticipated, the majority of the animals in the placebo group died by 72 h postinfection. In contrast, 90% of the animals in the combination group survived until the end of the experiment ($P < 0.001$). The relative animal survival among different treatment groups was consistent with the 24-hour bacterial burden in lung tissue. Furthermore, a higher bacterial burden in lung tissue was seen in deceased animals, suggesting that mortality was likely attributable to uncontrolled pneumonia.

DISCUSSION

Infections caused by *A. baumannii* can lead to significant morbidity and mortality. In general, mortality associated with *A. baumannii* infections ranges from 8% to 43% (23, 24). Combination therapy is often used for infections due to multidrug-resistant *A. baumannii*, where limited treatment options exist (25). In a study of *A. baumannii* bacteremia, the authors reported mortality of 30.8% to 58.3%, which was dependent on the antimicrobial combination used (26).

Many antimicrobial combinations have been studied for *A. baumannii* infections (25, 27, 28). In this study, polymyxin B was selected based on its mechanism of action, which would potentially interfere with the mechanism of minocycline resistance. Resistance to the tetracycline class in *Acinetobacter* spp. is commonly mediated through upregulation of efflux pumps located within the cell membrane (12). We hypothesized that polymyxin B could disrupt the cell membrane and the proper functioning of the efflux pumps, thereby enhancing the activity of minocycline. In this study, we demonstrated an increase in bacterial susceptibility to minocycline in the presence an efflux pump inhibitor. Similarly, the addition of polymyxin B was able to increase the intracellular

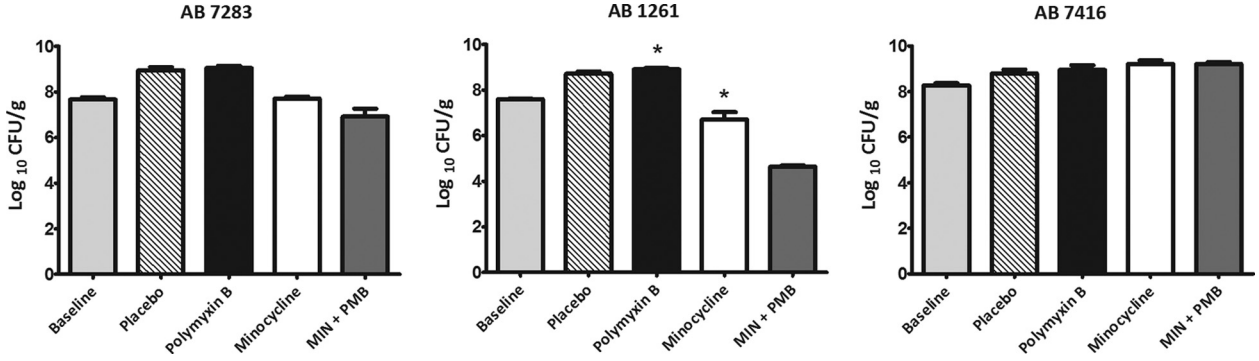

FIG 3 Tissue burdens at 24 h for various clinical strains *, $P < 0.05$ for the combination group compared to either monotherapy group.

concentration of minocycline and enhance the bactericidal activity of minocycline. The increased intracellular concentration of minocycline mediated by polymyxin B was presumably due to disruption of efflux pumps. As polymyxins are known for their ability to increase penetration through the outer membrane, we recognize that the effects seen in this study may also have been due to this mechanism rather than efflux pump disruption. Specifically, for AB7461 and AB747, which vary in their expression of *adeB*, the very high level of minocycline accumulation potential seems to imply that an additional mechanism(s) other than *adeB* could be involved.

There have been several studies to examine *in vitro* combinations of polymyxins (polymyxin B or colistin) with either a tetracycline or glycylcycline (27–29). Zhang and colleagues examined minocycline and polymyxin B for pan-drug-resistant *A. baumannii* (29). The authors found the MIC of each drug was reduced by the combination of minocycline and polymyxin B, providing a supportive framework on which our studies were based. Our study is unique because it is the first *in vivo* study to our knowledge to investigate the combination of minocycline and polymyxin B against *A. baumannii*. In contrast to a previous *in* vitro study using average free serum steady-state concentrations (27), a validated murine pneumonia model was used to enhance the clinical relevance of our findings. Using 2 different study endpoints (i.e., bacterial burden in lung tissue and survival over time), we showed that polymyxin B alone was unsatisfactory in the treatment of pneumonia, even when the bacterial strains were susceptible to polymyxin B. As we have reported previously, despite an apparently adequate systemic exposure, only limited polymyxin B concentrations could be achieved in the epithelial lining fluid (19, 30). Further escalation of the clinical dose is probably not feasible due to concerns about nephrotoxicity (31).

Consequently, our *in vitro* investigations focused on the effect (in any) of low polymyxin B concentrations in enhancing minocycline activity. Regardless of the study design, we were encouraged to see that the *in vitro* antimicrobial effect of minocycline could be augmented even with a subinhibitory concentration of polymyxin B. The mechanism of the synergy was briefly explored, and it was likely due (at least partially) to a higher intracellular minocycline concentration achieved in bacterial cells. Subsequent *in vivo* studies corroborated our *in vitro* findings, attesting to the value of adding polymyxin B to minocycline.

There are several limitations for our study. First, our postulation on efflux pump disruption by polymyxin B was based mostly on circumstantial evidence; we recognize that the increased intracellular concentration of minocycline mediated by polymyxin B could also be due to a nonspecific increase in membrane permeability. Second, survival benefits were demonstrated in only one clinical minocycline-susceptible strain. Pilot studies revealed that the mortality of animals infected with AB 7283 (minocycline susceptible) was 0% when treated with minocycline alone (data not shown); therefore, it was inconceivable that additional benefits could be provided by the combination using our study design. Third, although unlikely to be affected, the pharmacokinetic profiles of minocycline and polymyxin B when both drugs were coadministered were not examined. Finally, with a minocycline-resistant strain (AB 7416), adding polymyxin B did not appear to restore the activity of minocycline adequately. Therefore, the decision to add polymyxin B to minocycline therapy should be based on a careful evaluation of the benefit-to-risk ratio.

In conclusion, polymyxin B could enhance the bactericidal activity of minocycline against *A. baumannii*. The mechanistic framework for synergy and the clinical utility of this combination should be further investigated.

ACKNOWLEDGMENTS

We thank Jie He for technical assistance in assaying polymyxin B concentrations in mouse serum.

Dongxu Sun and Olga Lomovskaya are employees of Rempex Pharmaceuticals. They contributed to the elucidation of resistance mechanisms.

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