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Resistance Emergence Mechanism and Mechanism of Resistance Suppression by Tobramycin for Cefepime for *Pseudomonas aeruginosa*

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The panoply of resistance mechanisms in *Pseudomonas aeruginosa* makes resistance suppression difficult. Defining optimal regimens is critical. Cefepime is a cephalosporin whose 3′ side chain provides some stability against AmpC β-lactamases. We examined the activity of cefepime against *P. aeruginosa* wild-type strain PAO1 and its isogenic AmpC stably derepressed mutant in our hollow-fiber infection model. Dose-ranging studies demonstrated complete failure with resistance emergence (both isolates). Inoculum range studies demonstrated ultimate failure for all inocula. Lower inocula failed last (10 days to 2 weeks). Addition of a β-lactamase inhibitor suppressed resistance even with the stably derepressed isolate. Tobramycin combination studies demonstrated resistance suppression in both the wild-type and the stably derepressed isolates. Quantitating the RNA message by quantitative PCR demonstrated that tobramycin decreased the message relative to that in cefepime-alone experiments. Western blotting with AmpC-specific antibody for *P. aeruginosa* demonstrated decreased expression. We concluded that suppression of β-lactamase expression by tobramycin (a protein synthesis inhibitor) was at least part of the mechanism behind resistance suppression. Monte Carlo simulation demonstrated that a regimen of 2 g of cefepime every 8 h plus 7 mg/kg of body weight of tobramycin daily would provide robust resistance suppression for *Pseudomonas* isolates with cefepime MIC values up to 8 mg/liter and tobramycin MIC values up to 1 mg/liter. For *P. aeruginosa* resistance suppression, combination therapy is critical.

Treatment of serious infections with *Pseudomonas aeruginosa* remains one of the greatest tests for a clinician. This organism has a large variety of resistance mechanisms available to it, and these may act in combination, rendering even our most potent agents useless.

While the Infectious Diseases Society of America has identified the lack of new agents as a national emergency (4), the wait for the advent of such agents clinically will be significant. It is obvious that it is in our best interest to explore approaches that will help suppress emergence of resistance and preserve both current and future agents for use in the treatment of serious *P. aeruginosa* infections.

Cefepime is regarded as a "4th-generation" cephalosporin with potent activity against *Pseudomonas aeruginosa*. Ceftazidime preceded cefepime as an excellent antipseudomonal cephalosporin. Resistance emergence with ceftazidime was often due to stable derepression of the AmpC β-lactamase (Sabath-Abraham type Id, or now called *P*seudomonas-derived cephalosporinase [PDC]) carried by this organism. Cefepime was designed with a change in its 3′ side chain. This altered side chain was shown to affect the physiological affinity of the drug for the β-lactamase (16). It was this alteration which generated the potent activity profile of cefepime against *Pseudomonas aeruginosa*, as noted in MIC activity (25).

Antibiotic susceptibility testing using broth macrodilution Clinical and Laboratory Standards Institute (CLSI) methods (7) indicates that the bacterial inoculum should be 10^5 to 10^6 CFU/ml. If microdilution methods are employed, the total population burden is 1/10 this range. Such a population burden will highly likely be less than the inverse of the mutational frequency to resistance. Our intent was to test cefepime in our *in vitro* hollow-fiber infection model (HFIM) to ascertain the impact of greater population burdens on the activity of the drug against *P. aeruginosa*. We chose to examine the *P. aeruginosa* PAO1 wild-type (WT) isolate and its isogenic mutant with a stably derepressed AmpC β-lactamase. Given our experience with meropenem (17), we also chose to examine combination chemotherapy with cefepime plus tobramycin for these isolates.

MATERIALS AND METHODS

Many of the methods employed in these experiments were the same as those that we previously published (17).

Microorganisms. *Pseudomonas aeruginosa* strain PAO1 and its isogenic AmpC-overexpressed mutant were the kind gift of Karen Bush and Marie Queenan. We used CLSI broth macrodilution methods to determine MIC values for both cefepime and tobramycin (7). The mutational frequency to resistance was estimated by plating 5 ml of an overnight growth of the WT *P. aeruginosa* PAO1 strain and its isogenic AmpC-overexpressed mutant onto agar containing 3× baseline MIC of either cefepime or tobramycin. The concentration of microbes in the bacterial suspension was determined by quantitative cultures. The ratio of the number of resistant clones to the total population provided the estimate of the frequency of resistant isolates (22, 23). This was done on at least three occasions. At least three colonies were randomly picked from each resistance plate and tested for the change in MIC from baseline.

Hollow-fiber infection model. The HFIM was first described by Blaser et al. (2) as a pharmacodynamic system for bacteria. It was employed by Bilello et al. (1) for HIV pharmacodynamic studies. Schematic dia-

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grams of the system with a description of its use were presented previously in our study of *Mycobacterium tuberculosis* (14). Here, cefepime and/or tobramycin was directly injected into the central reservoir on the specified administration schedule for each agent, depending on the experiment. For combinations of cefepime plus tobramycin, a half-life of 2.25 h was simulated for both drugs.

Hollow-fiber studies. The inoculum was prepared by growing 3 medium-sized overnight-grown colonies of *P. aeruginosa* in cation-adjusted Mueller-Hinton broth (Ca-MHB) at 35°C. Hollow-fiber systems were maintained at 35°C in a humidified incubator. Approximately 15 ml of bacterial culture in late-log-phase growth (1.5×10^8 CFU/ml) was infused into each cartridge, one for each nominal dose. Exposures were to simulate steady-state human pharmacokinetics of unbound drugs. Cefepime was assumed to be 20% protein bound, and tobramycin was assumed to be completely unbound. Experimentally attained cefepime and tobramycin concentration exposures were determined by quantifying drug concentrations using validated liquid chromatography-tandem mass spectrometry (LC/MS/MS) methods in samples taken from the central bioreactor loop at 10 to 18 time points over 48 h for monotherapy and for both agents in combination regimens over 48 h (except for one experiment, where samples were taken later). At 0 (baseline), 0.2, 1, 2, 3, 4, 6, 8, 10, 13, and 14 days of the experiment, samples of the bacterial cultures were obtained from the cartridges, washed, and resuspended in normal saline in order to minimize drug carryover effect. Samples were serially diluted and were quantitatively cultured onto drug-free Mueller-Hinton II agar plates to enumerate the total bacterial population. A portion of the bacterial suspensions was also cultured onto agar that was supplemented with either cefepime at 3× or 5× baseline MIC or tobramycin at 3× baseline MIC for each isolate in order to assess the effect of each regimen on the less susceptible bacterial populations. Plates were incubated at 35°C for 24 h (total population) and 72 h (resistant subpopulations) before the results were read. Cefepime and tobramycin MIC values were determined on a subset of the colonies that grew on drug-supplemented agar to confirm the emergence of resistance.

Types of studies. (i) WT isolate. The first study was dose ranging and consisted of a no-treatment control and 6 active regimens with dosing ranging from 250 mg every 8 h (q8h) to 3,000 mg q8h.

The second study examined different starting inocula. Starting inocula were 5.0 $\log_{10}$(CFU/ml), 6.5 $\log_{10}$(CFU/ml), and 8.0 $\log_{10}$(CFU/ml). Cefepime regimens included 6 g as a continuous infusion daily, 2 g q8h, and 6 g q8h.

The third study examined cefepime alone, tobramycin alone, and both drugs in combination. There was a no-treatment control, 3 cefepime-alone arms (563, 1,500, and 3,000 mg q8h), 3 tobramycin-alone arms (3, 7, and 10 mg/kg of body weight daily), and all possible combination regimens (9 arms).

(ii) Stably derepressed AmpC isogenic mutant. The experiments with the wild-type strain described above suggested strongly that regimen failures were due to stable derepression of the AmpC enzyme. We then employed the isogenic stably derepressed PAO1 isolate to further examine this question. In the first study, there was a no-treatment control plus 8 active regimens. Seven of the regimens were cefepime alone administered every 8 h at simulated doses of 1 g, 2 g, 3 g, 4 g, 6 g, and 8 g plus a continuous-infusion evaluation of 8 g daily. The last active regimen was 1 g cefepime q8h, but with 4 mg/liter of the broad-spectrum β-lactamase inhibitor NXL104 (which inhibits AmpC) always present.

Because of the success of the combination regimen for the wild-type organism, we examined the combination of cefepime and tobramycin for the stably derepressed mutant of PAO1. There was a no-treatment control plus 3 active arms: cefepime alone at 1 g and 2 g q8h and cefepime at 563 mg q8h in combination with 3 mg/kg of tobramycin daily.

Pharmacokinetic methods. Concentration-time profiles were analyzed employing maximum likelihood estimation with the Identification module of the ADAPT II package of programs (8). A 1-compartment open model with zero-order input and first-order elimination was employed, as computer-controlled infusion pumps drove the profile.

We estimated the time that the free drug concentration is greater than the MIC by integrating the following differential equation, which was a system output [$Y(2)$].

$$\begin{aligned} &\text{If } ([X/V_1] \text{ GE MIC}) \text{ Then} \\ &dX(3)/dt = 1.0 \\ &\text{Else} \\ &dX(3)/dt = 0.0 \\ &\text{End if} \end{aligned} \tag{1}$$

where X is the amount of drug in the central compartment, V_1 is the volume of the central compartment, MIC is the MIC of the appropriate drug for the pathogen being studied, and GE is greater than or equal to.

We calculated the area under the concentration-time curve from time zero to time τ ($AUC_{0-\tau}$) by integration. The differential equation was written as

$$dX(2)/dt = X/V_1 \tag{2}$$

where the definitions are the same as those presented above and the system output was $Y(3) = X(2)$. This integrates $AUC_{0-\tau}$.

We employed these methods to calculate the time that the free drug concentration is greater than the MIC for cefepime and the free-drug AUC/MIC ratio for tobramycin. We then employed the Simulation module of the ADAPT II package to perform a 9,999-subject Monte Carlo simulation to determine the frequency with which specific doses of cefepime and tobramycin achieved the above-described goals. As we were interested in pulmonary infections, we used information from two papers (3, 6) to identify the fractional penetration of cefepime and tobramycin into epithelial lining fluid (ELF) in patients with pulmonary infections. For cefepime, Boselli et al. (3) administered the drug by continuous infusion and demonstrated 100% penetration. As the profile of drug in ELF either is similar to that in the plasma or is slightly flatter due to system hysteresis, we employed the time that the free drug concentration is greater than the MIC noted in the HFIM as the target in the Monte Carlo simulation. For tobramycin, the study of Carcas et al. (6) allowed us to directly analyze the data provided using a population pharmacokinetic modeling approach, as we have done previously (10, 19). We used the point estimate of ELF penetration to correct the plasma profile of tobramycin in our Monte Carlo simulation for the target attainment analysis.

LC/MS/MS methods for cefepime and tobramycin. Mueller-Hinton II broth pharmacokinetic simulation samples were diluted with high-pressure liquid chromatography (HPLC)-grade water (0.050 ml sample into 1.00 ml water) and were analyzed concurrently by LC/MS/MS for tobramycin and cefepime concentrations. The LC/MS/MS system comprised a Shimadzu Prominence HPLC system and an Applied Biosystems/MDS Sciex API5000 LC/MS/MS apparatus.

For gentamicin, chromatographic separation was performed by use of a modification of a method by Dussault et al. (12) using a Thermo Scientific Hypersil Gold C_{18} column (particle size, 5 μm; 150 by 4.6 mm) and a gradient employing mobile phases consisting of water with 5% methanol and 0.5% trifluoroacetic acid and methanol at a flow rate of 0.8 ml/min. Methanol is added postcolumn at 0.5 ml/min to enhance sensitivity.

Tobramycin and cefepime concentrations were obtained using LC/MS/MS monitoring of the MS/MS transitions m/z 468 → m/z 163 and m/z 481 → m/z 396, respectively. Analysis run time was 7.0 min. The assay was linear over the ranges of 0.050 to 50.0 μg/ml ($r^2 > 0.996$) for tobramycin and 1.0 to 150 μg/ml ($r^2 > 0.994$) for cefepime. The interday coefficients of variation (CVs) for the tobramycin quality control samples analyzed in replicates of three at three concentrations on each analysis day (0.100, 1.00, and 10.0 μg/ml) ranged from to 2.93 to 6.30%, with accuracies (% REC [recovery]) ranging from 102% to 103%. The interday CVs for the cefepime quality control samples analyzed in replicates of three at three

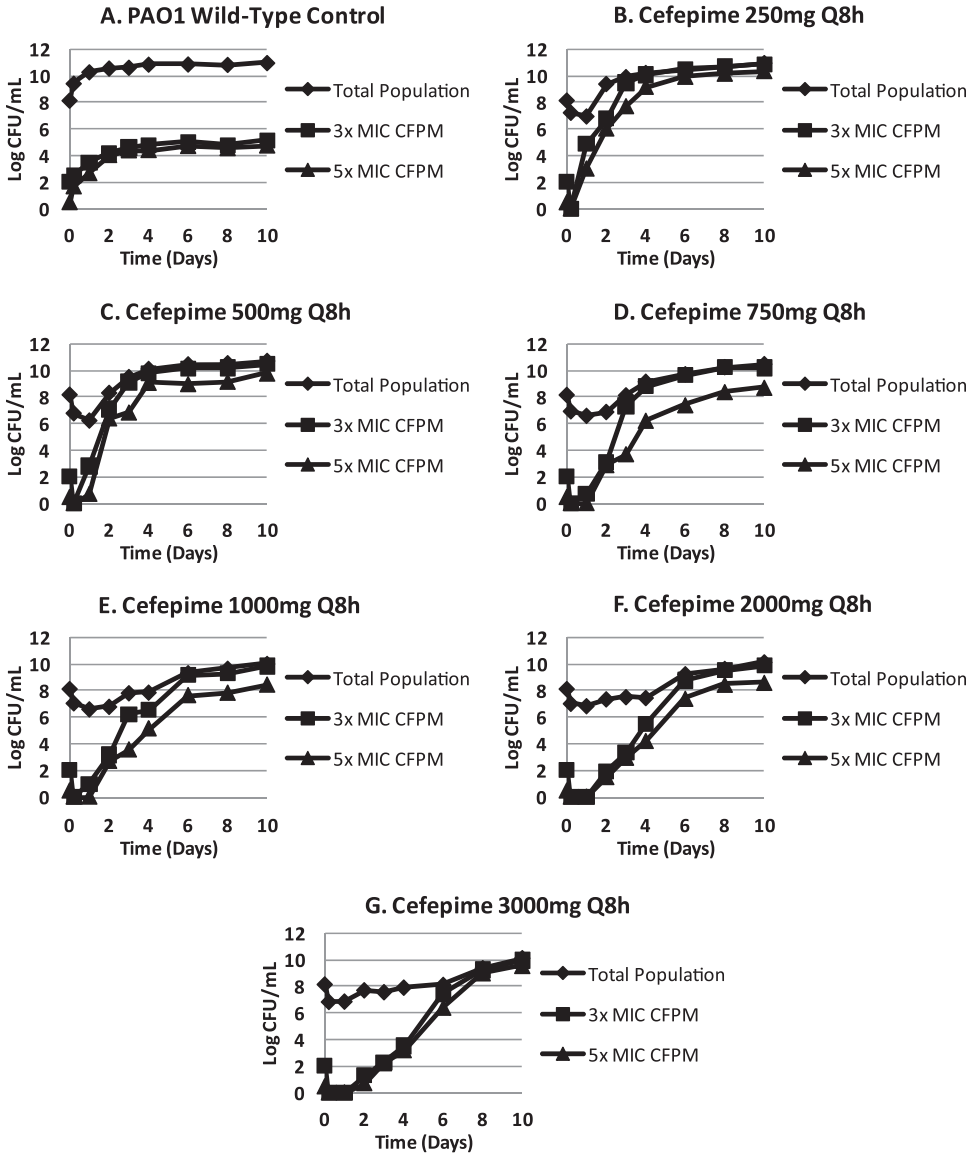

FIG 1 Dose-ranging experiment of cefepime (CFPM) against WT *Pseudomonas aeruginosa* PAO1. Total results and results for cefepime-resistant populations are displayed.

concentrations on each analysis day (5.0, 20.0, and 100 μg/ml) ranged from to 4.57 to 7.05%, with accuracies (% REC [recovery]) ranging from 98.9% to 106%.

Quantitative PCR (qPCR) methods for quantitative assessment of AmpC β-lactamase. RNA was isolated using an RNeasy kit (Qiagen, Inc.) and treated with DNase on the column according to the manufacturer's instructions. A second course of DNase treatment was performed on eluted samples with Turbo DNA-free reagent (Ambion, Inc.). After total RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Inc.), first-strand cDNA was synthesized from 1 μg total RNA using random hexamers (Invitrogen) and a two-step reverse transcription-PCR kit (USB). Real-time PCR was performed with Maxima SYBR green (Fermentas, Inc.) utilizing an ABI 7900HT real-time PCR detection system to quantify levels of gene expression compared to that of the reference gene, *rpsL* (21). The primers used for *ampC* were renamed ON-7 (5′-AGATTCCCCTGCCTGTGC-3′) and ON-8 (5′-GGCGGTGAAGGTCTTGCT-3′) for this study and have been described previously (17). The primers for *rpsL* were ON-19 (5′-GTACATCGGTGGTGAAGGTC-3′) and ON-20 (5′-ACCCTGCTTACGGTCTTTG-3′) and were designed with the aid of the Primer3 program (20).

Western blot analysis. To generate specific antibodies to detect expression of the AmpC of *P. aeruginosa*, the PDC-3 β-lactamase was purified from the *P. aeruginosa* 18SH strain. Briefly, *P. aeruginosa* 18SH was grown overnight in 500-ml cultures in SOB (Super Optimal Broth medium). The PDC-3 enzyme was purified by preparative isoelectric focusing (pIEF) and fast-performance liquid chromatography with a Sephadex Hi Load 16/60 column and a HiTrap high-performance sulfopropyl strong cation exchanger (Pharmacia, Uppsala, Sweden). The protein was quantified by bovine serum albumin assay, and purity was assessed by 5% stacking, 12% resolving SDS-PAGE. Finally, the atomic mass was verified by mass spectrometry.

Anti-PDC-3 polyclonal rabbit antibodies were produced by New England Peptide (Gardner, MA) from 3.0 mg of PDC-3 protein. The antibodies were isolated from rabbit sera using a Hi-Trap protein G column (GE Healthcare). Briefly, rabbit serum was diluted in 20 mM NaH_2PO_4 (pH 7.0) and bound to the column. Antibodies were eluted with 0.1 M glycine-

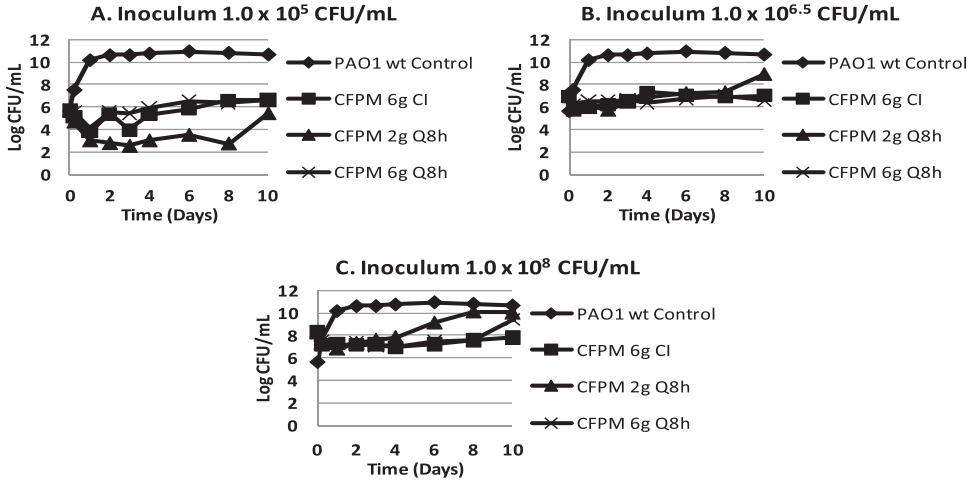

FIG 2 Impact of initial bacterial inoculum on activity of cefepime against WT *Pseudomonas aeruginosa* PAO1. CI, continuous infusion.

HCl (pH 2.7) and added to 1 M Tris-HCl (pH 9.0) to yield a neutralized storage buffer. The concentration was measured by spectrophotometric determination at 280 nm, and the samples were aliquoted and frozen at −20°C for long-term storage.

Purified β-lactamases from the laboratory of R.A.B. were used to screen for specificity; specifically, 500 ng each of class A β-lactamases SHV-1 and KPC-2, class B β-lactamase CcrA, class C β-lactamases CMY-2, P99, and ADC, and class D β-lactamase OXA-1 was tested. All these control proteins were purified from *Escherichia coli* cells containing *bla* genes encoded on expression vectors.

In addition to the purified β-lactamases, we tested the specificity of the antibody by preparing crude cell lysates of a panel of *P. aeruginosa* isolates by growing each strain to an optical density at 600 nm of 0.8 and lysing the cells by 10 min incubation at 100°C in SDS loading dye buffer. These *P. aeruginosa* strains included PAO1, 18SH, MK1184, and 143724R and clinical isolates UL140 and DB322. Crude lysates were also prepared for the ATCC strains *Klebsiella pneumoniae* ATCC 70063 and *P. aeruginosa* ATCC 27853 and a clinical strain of *E. coli* which produces SHV, TEM, and CTX-M β-lactamases.

Cell lysates were prepared by bringing the cell pellet up to a volume of 500 μl in Solulyse reagent (Genlantis, San Diego, CA) with Halt protease inhibitor cocktail, EDTA free (Thermo Fisher, Rockford, IL). After lysis, protein samples were concentrated by ultrafiltration using Micron centrifugal filter devices (Millipore, Billerica, MA). Protein was quantified spectrophotometrically using a Coomassie Bradford protein assay kit (Pierce, Rockford, IL). Appropriate sample volumes were boiled for 10 min in Laemmli sample buffer and loaded to a 12% SDS-polyacrylamide gel. The gel was run at 116 V for 90 min, followed by transfer of the separated proteins to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) by wet electroblotting overnight at 4°C. The blot was blocked for 90 min at room temperature with 5% dry, nonfat milk in Tris-buffered saline–Tween 80 (TBS-T). Anti-PDC-3 (anti-AmpC) rabbit polyclonal primary antibody was used at a concentration of 100 ng/ml prepared in TBS-T with 5% milk. Primary

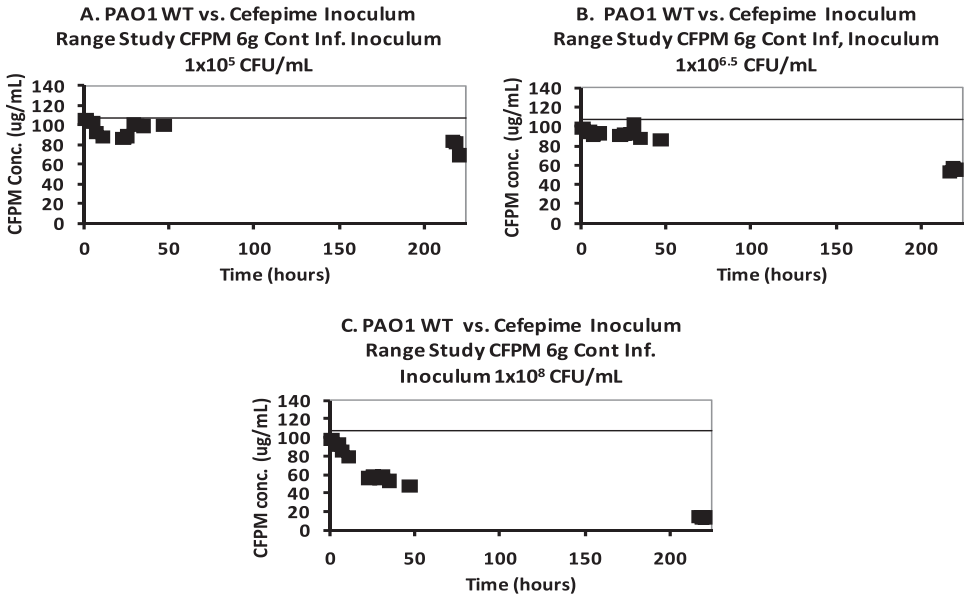

FIG 3 Concentration-time profile of cefepime administered as a continuous infusion (Cont Inf.) with three different initial inocula of WT *Pseudomonas aeruginosa* PAO1.

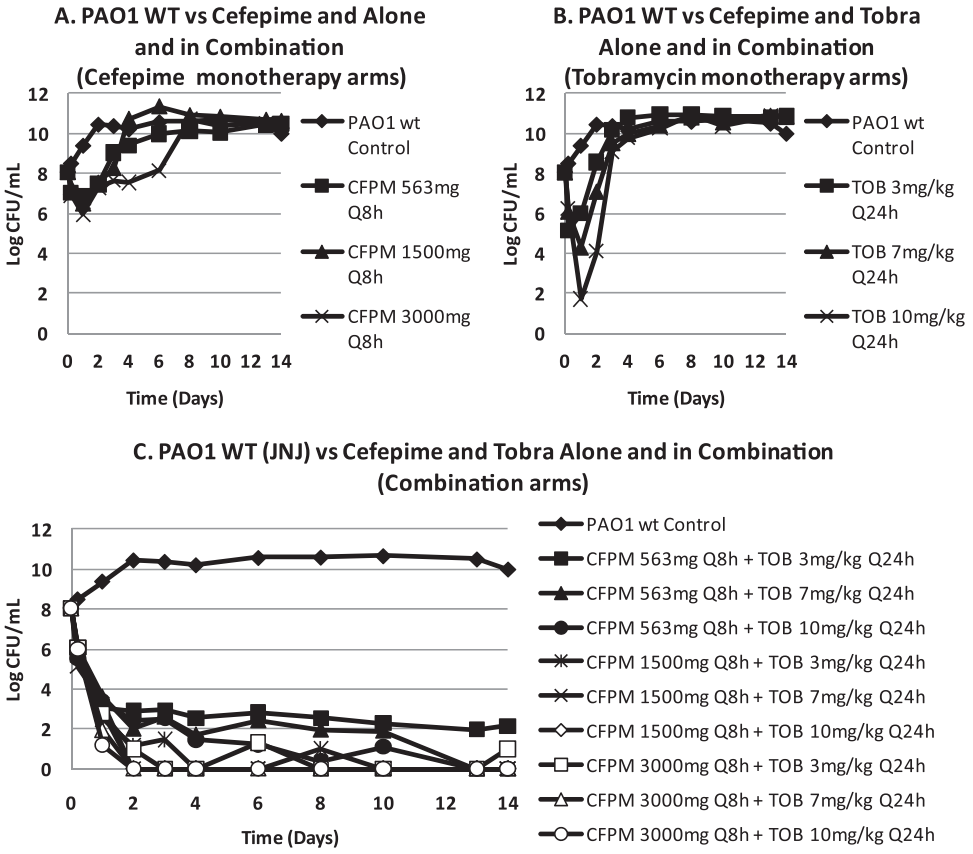

FIG 4 Dose-ranging study of cefepime (A) and tobramycin (Tobra, TOB) (B) alone and in combination against WT *P. aeruginosa* PAO1.

probing was done at room temperature for 80 min, followed by 30 min of washing by frequent changes of TBS-T. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, MA) was diluted 1:20,000 in TBS-T with 5% milk and used to probe for 60 min at room temperature. Washes with TBS-T were repeated for 30 more minutes, before the blot was exposed to enhanced chemiluminescence chemicals (Abcam, Cambridge, MA) and then used to expose film for development.

Examination of drug interaction between cefepime and tobramycin. We examined the stably derepressed mutant organism of PAO1 which overexpresses the AmpC β-lactamase. We examined a 6-by-6 checkerboard broth macrodilution (1 ml of medium) tube evaluation in which cefepime concentrations were 0, 1.0, 2.0, 8.0, 16.0, and 32.0 mg/liter and tobramycin concentrations were 0, 0.25, 0.5, 1.0, 4.0, and 8.0 mg/liter. The baseline number of organisms was 1.15×10^5 CFU/ml. The low inoculum was to minimize the probability of preexisting mutants, so that we could directly examine drug interaction for cell kill and avoid confounding by amplification of resistant subpopulations, which often results in a mosaic surface. Data analysis was as previously defined (9) and employed a Greco universal response surface approach.

RESULTS

MIC values (broth macrodilution method). The cefepime MIC values for the PAO1 WT isolate were 1.0 mg/liter on 5 occasions and 0.5 mg/liter once. For the stably derepressed AmpC mutant, the cefepime MIC rose to 8 mg/liter on 4 occasions and was 16 mg/liter once. The tobramycin MIC was 1.0 mg/liter for both strains.

Mutational frequency to resistance. For the WT isolate, the mutational frequencies to resistance were $1/7.861 \times 10^7$ at 3× MIC and $1/2.069 \times 10^8$ at 5× MIC. On another occasion, the frequency was $1/1.360 \times 10^6$ at 3× MIC. For the isogenic stably derepressed mutant, the mutational frequency to resistance was $1/4.375 \times 10^7$. For tobramycin, this value was $1/1.975 \times 10^7$ at 3× MIC.

Results of therapy with cefepime with or without tobramycin for the wild-type isolate. (i) Initial dose-ranging experiment. The total colony counts and resistant colony counts for 3× MIC and 5× MIC for all regimens are displayed in Fig. 1. All active treatments demonstrate early resistance emergence on both 3 ×MIC and 5× MIC plates, even with simulated monotherapy regimens of 3 g of cefepime q8h (Fig. 1G). Isolates from the drug-containing plates had MIC values of 8 to 32 mg/liter. When cefepime concentrations were measured, the nominal values were achieved during the first dosing interval with excellent accuracy and precision, but all later intervals showed that the cefepime concentrations achieved were biased low, suggesting hydrolysis by β-lactamase (data not shown).

(ii) Inoculum range study. We examined the impact of the initial inoculum on the activity of cefepime. There were 3 active regimens (2 g q8h, 6 g/24 h as a continuous infusion, and 6 g q8h) and a no-treatment control. Initial inocula evaluated were 1×10^5, 3.16×10^6, and 1×10^8. All regimens ultimately failed. However, the low-inoculum regimens tended to fail last, owing to a longer time to amplification of resistant mutants. It should be recognized that the system volume was 15 ml, indicating that the population burden was increased by 15-fold over the initial organ-

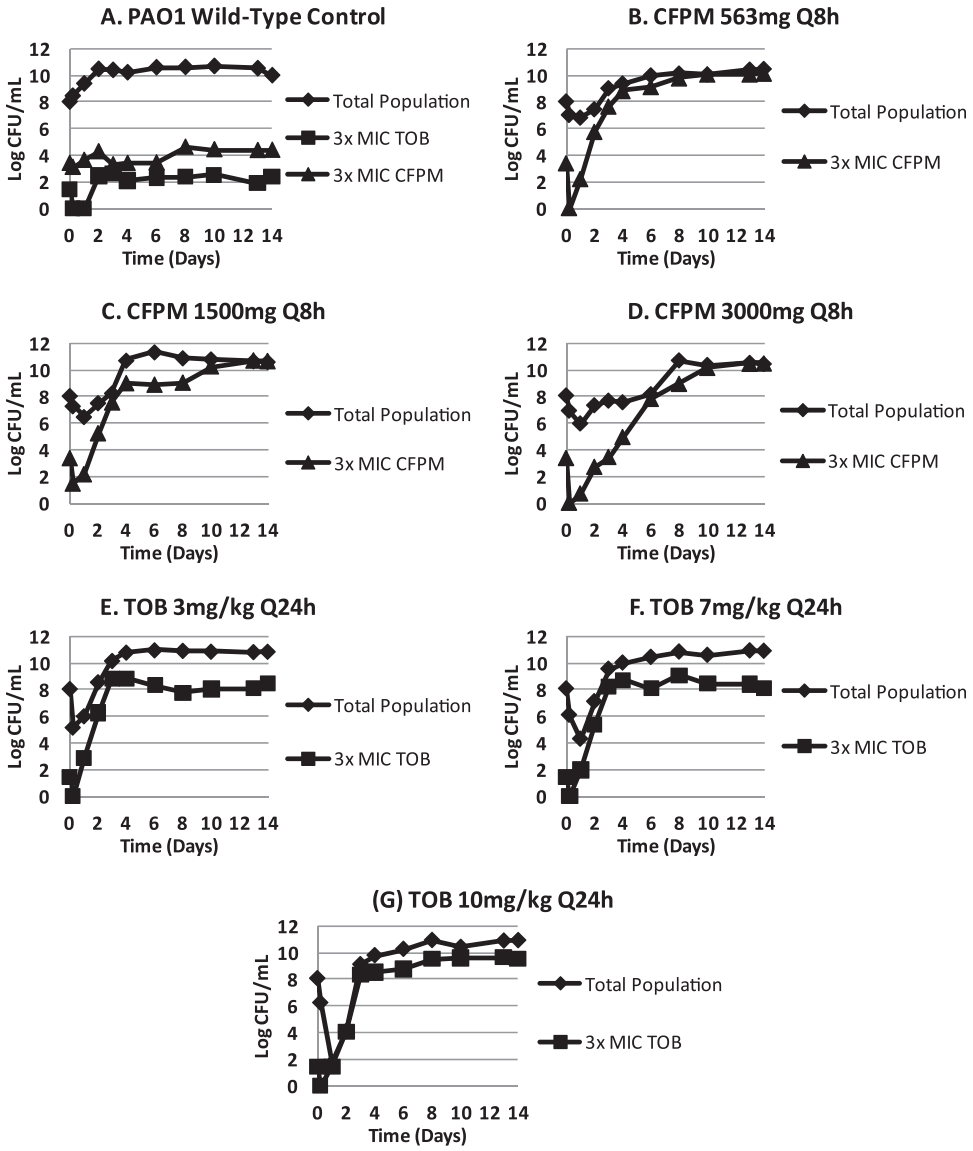

FIG 5 Effects of cefepime and tobramycin regimens alone and in combination on amplification of resistant subpopulations of WT *P. aeruginosa* PAO1.

ism concentration. The results are displayed in Fig. 2. The cefepime concentrations are displayed in Fig. 3 for the continuous-infusion arms. The others were also biased low. For the continuous-infusion arms, we checked cefepime concentrations out to hour 240. As the initial inoculum increased, the drug concentrations achieved were reduced, and this reduction occurred earlier. Again, it would appear that β-lactamase-mediated drug hydrolysis is responsible for regimen failure. All active arms failed with resistance emergence (data not shown). Of interest, at the lowest inoculum, all regimens failed late, after day 10. It is likely that the low number of resistant mutants expected at baseline required more time for amplification.

(iii) Impact of combination therapy with tobramycin. We examined the combination of cefepime and tobramycin. The doses of cefepime were 563 mg, 1,500 mg, and 3,000 mg, all administered every 8 h. All these (except the lowest-dose cefepime regimen) had previously been shown to fail as monotherapy (see above). For tobramycin dosing, we evaluated 3, 7, and 10 mg/kg administered daily. All single-agent-therapy arms failed. All failures were due to resistance emergence. All combination-therapy arms succeeded, with no resistance amplification during the 14-day study period. The total population responses are displayed in Fig. 4, and the amplification of resistance is displayed in Fig. 5 for the monotherapy arms. Again, no resistance was seen in the combination therapy arms. Organisms taken from the drug-containing plates had stable MIC values of 8 to 16 mg/liter for cefepime and 8 mg/liter for tobramycin.

Results of therapy with cefepime with or without tobramycin for the isogenic AmpC stably derepressed isolate. (i) Impact of β-lactamase inhibition. We employed NXL104 as a probe to examine the hypothesis that β-lactamase hydrolysis was responsible for the failure of cefepime monotherapy. We employed the isogenic stably derepressed mutant of PAO1, as this would test the efficacy of NXL104, an agent with potent inhibitory activity

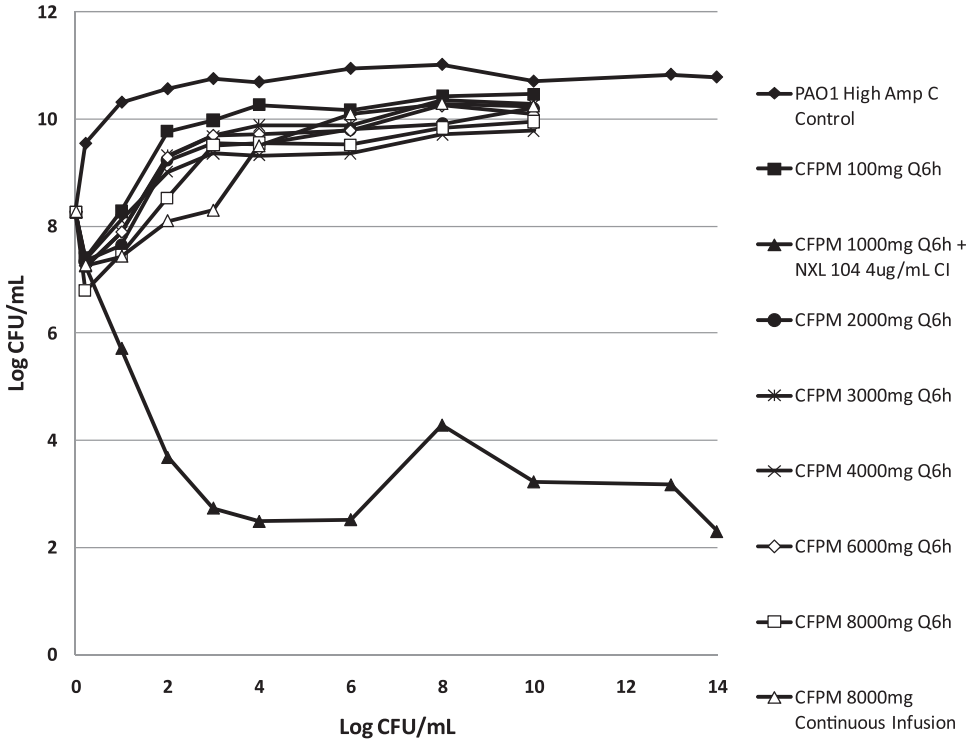

FIG 6 Dose ranging of cefepime against an isogenic isolate of *P. aeruginosa* PAO1 stably derepressed for high expression of AmpC. One arm (▲) demonstrates the effect of adding the β-lactamase inhibitor NXL104.

against AmpC β-lactamases All active regimens with cefepime alone failed rapidly (Fig. 6) due to resistance emergence (data not shown). The regimen in which NXL104 was introduced at a continuous concentration of 4 mg/liter was successful for the full 14 days of the experiment, with no resistance emergence. Importantly, we examined the concentration-time profiles for all regimens. These are displayed in Fig. 7. The impact of β-lactamase hydrolysis is apparent, with none of the monotherapy regimens achieving the nominal concentration-time profile. In contrast, inhibition of the AmpC β-lactamase allows excellent attainment of the nominal concentration-time profile of cefepime. It should be noted that the difficulty in attaining the desired profile is time dependent, with the first dose being quite close to nominal but with increasing discordance with time. It is also important to note that the continuous-infusion regimen demonstrates the greatest discordance from nominal. We speculate that the process of enzyme-mediated hydrolysis is rate limiting and that as intracellular concentrations approach and exceed the K_m of cefepime for the AmpC of *P. aeruginosa*, the rate of hydrolysis decreases and approaches V_{max}. The much higher serum peaks associated with the intermittent administration likely transiently saturate the enzyme (exceed k_{cat}). Continuous infusion never achieves saturation (V_{max}) at the doses simulated and therefore presents a profile that is the most discordant from the nominal profile. Isolates from the resistance plates changed from having a baseline MIC of 8 mg/liter for cefepime to one of 128 to 256 mg/liter.

(ii) Combination therapy with cefepime plus tobramycin. Given the results that we had seen with the WT isolate and combination therapy with cefepime and tobramycin (see above), we decided to test only a low dose of cefepime and a low dose of tobramycin in combination. There was a no-treatment control and two cefepime-alone arms that had previously been shown to fail with monotherapy which were positive therapy controls. The combination regimen was 563 mg q8h of cefepime plus 3 mg/kg of tobramycin. As before, both cefepime-only arms failed and the combination regimen succeeded, with no amplification of a resistant subpopulation. This is presented in Fig. 8. Figure 9 shows the emergence of resistant isolates for the monotherapy arms (none was seen in the combination therapy). As previously, organisms taken from the drug-containing plates had increased cefepime MIC values.

Quantification of AmpC β-lactamase amounts by qPCR and Western blot analysis. Given the data presented above, we had a hypothesis that failure of therapy was due to elaboration of AmpC β-lactamase. Further, we hypothesized that the administration of the aminoglycoside reduced expression of the enzyme, which was the mechanism of improved cell kill and resistance suppression. The amounts of message for AmpC enzyme are presented in Table 1. Values are taken early (hour 5) and normalized to the value for housekeeping gene *rpsL* (21). The fold differences in Table 1 are normalized to the values at time zero.

The Western blot indicates that message trends translate to amounts of protein expressed. Samples were taken at 2 h of exposure and 5 h of exposure. Exposures produced the same AUC values at the indicated time points as the plasma concentration-time profiles would for each of the drugs alone and in combination. Aminoglycoside exposure shuts off expression of β-lactamase. Concentrations of cefepime that kill *P. aeruginosa* are still enough to increase expression. The combination reduces expression to that seen with the control

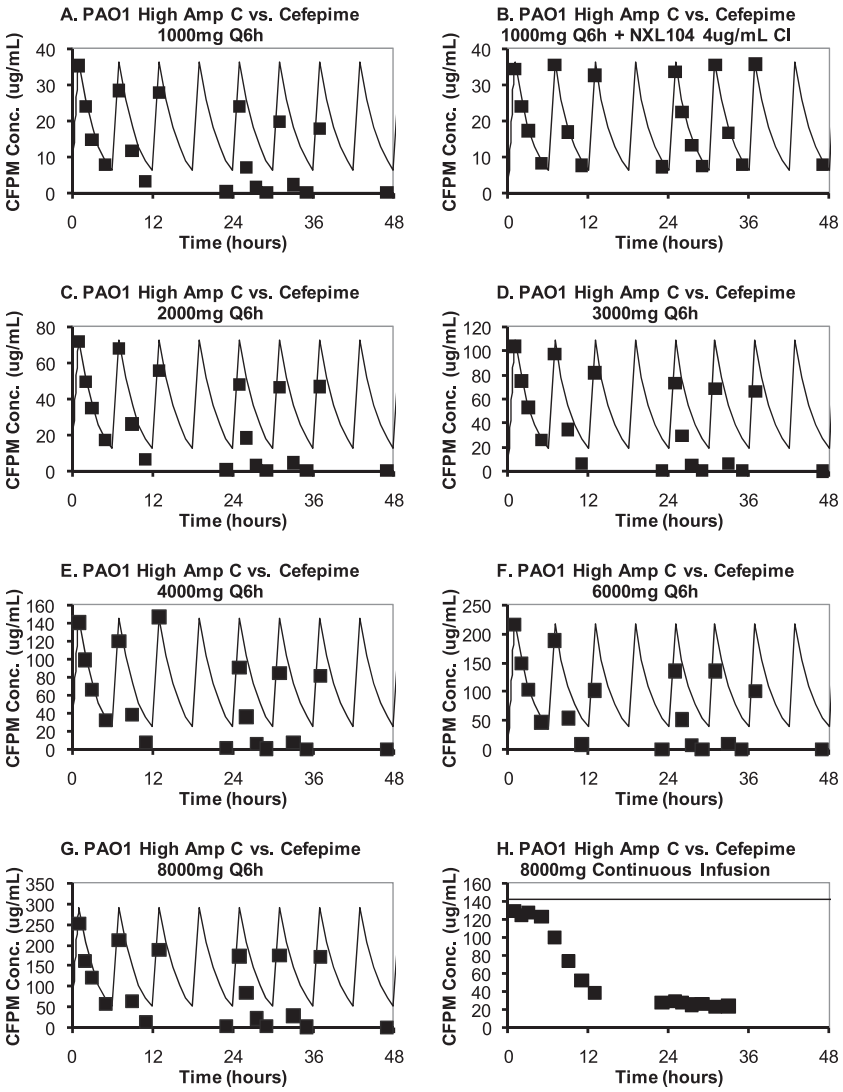

FIG 7 Impact of β-lactamase expression on the ability to attain desired cefepime concentrations over time and impact of addition of the β-lactamase inhibitor NXL104.

(Fig. 10). The antibody prepared for these studies readily detects *P. aeruginosa* AmpC with a sensitivity of 50 ng (unpublished data).

Analysis of drug interaction between cefepime and tobramycin. The 50% inhibitory concentrations for cefepime and tobramycin were 2.65 and 18.0 mg/liter, respectively. Hill's constants were 2.33 and 1.59, respectively. The interaction parameter (α) was 0.07 and had a standard deviation that caused the 95% confidence interval to cross zero, indicating an additive interaction for cell kill. It should be noted that interaction for resistance suppression is quite different and may be synergistic. We have recently demonstrated that drug interaction can be synergistic for resistance suppression while even being antagonistic for cell kill (11).

Analysis of success of combination cefepime plus tobramycin therapy. We noted above that relatively low doses of both cefepime and tobramycin in combination suppressed the emergence of resistant mutants when cefepime alone was unable to do so, even at doses considerably higher than those approved for use in humans by the FDA. This was true for both the wild-type isolate and the isogenic mutant stably derepressed for high AmpC expression (Fig. 4 and 8). The MIC values for the mutant were 8 mg/liter for cefepime and 1 mg/liter for tobramycin. We used these to calculate the exposure targets in this successful experiment. For cefepime, the dose of 563 mg every 8 h produced a profile that was above the MIC value of 8 mg/liter for 24.7% of the dosing interval in the hollow-fiber system. For tobramycin, the AUC in the successful experiment with 3 mg/kg of tobramycin daily was 58.06 mg · h/liter. With the tobramycin MIC value of 1.0 mg/liter, we get the same value for the AUC/MIC ratio.

Because Boselli et al. (3) demonstrated a 100% penetration of cefepime into ELF in intensive care unit patients and because ELF penetration is associated with system hysteresis, which tends to flatten the concentration-time profile, we used the target derived in the hollow-fiber system without modification, as this is conservative. We used the cefepime pharmacokinetic values of Tam et al. (24) for the Monte Carlo simulation. We used the creatinine clearance distribution from a clinical trial (26), as the study of Tam et al. (24) made the drug clearance proportional to creatinine clearance plus an intercept. We chose to evaluate the Monte Carlo

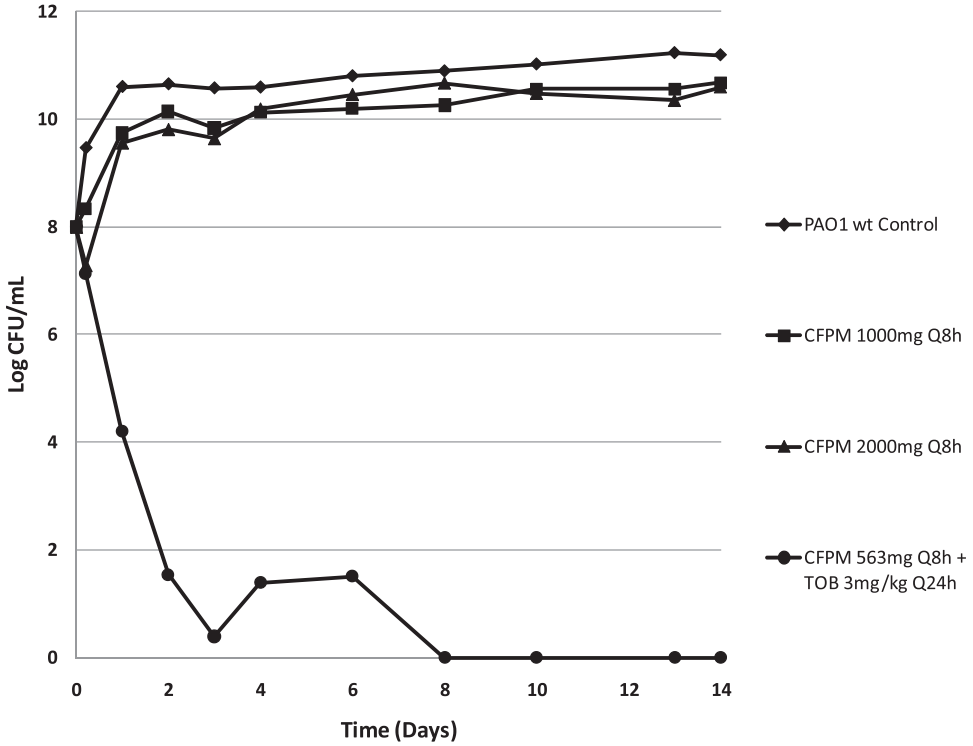

FIG 8 Effect of addition of tobramycin to cefepime at a low dose of both drugs relative to effect of higher doses of cefepime alone against *P. aeruginosa* PAO1 stably derepressed for high expression of AmpC.

simulation only at an MIC value of 8 mg/liter. At this value, for a dose of 2 g of cefepime every 8 h, the target attainment for the percentage of the time that the free drug concentration is greater than the MIC of 24.7% of the dosing interval was 99.5%.

For tobramycin, we used the population pharmacokinetic model of Inciardi and Batra (15). When we analyzed the penetration of tobramycin into the ELF from the data of Carcas et al. (6), the penetration was 44% if the mean parameter vector was employed and 54% if the median parameter vector was employed, and if one simply took the mean values at the times provided and calculated the plasma and ELF AUC values, their ratio was 50%. Given the general concordance of the approaches, we chose to use the 50% value. For the Monte Carlo simulation, we employed a dose of 7 mg/kg of tobramycin.

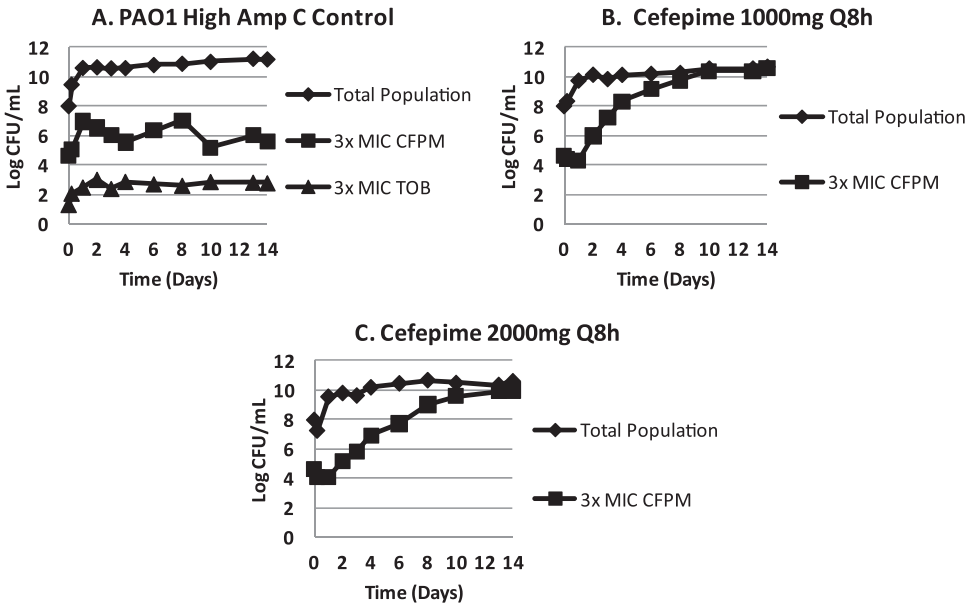

FIG 9 Resistant subpopulation amplification for cefepime monotherapy against *P. aeruginosa* PAO1 stably derepressed for high expression of AmpC.

TABLE 1 Amount of AmpC β-lactamase determined by quantitative PCR in cultures of *Pseudomonas aeruginosa* PAO1 AmpC-overexpressed isogenic mutant in presence of cefepime alone or in combination with tobramycin at hour 5 of drug exposure

Regimen	Fold increase in AmpC RNA expression (95% confidence interval)
Growth control	22.0 (5.1–95.0)
Cefepime, 2,000 mg	259.0 (125.0–5,540.0)
Tobramycin, 7 mg/kg	0.7 (0.3–1.7)
Cefepime + tobramycin	13.9 (7.5–25.5)

TABLE 2 Target attainment for suppression of emergence of resistance for cefepime plus tobramycin[a]

Tobramycin MIC (mg/liter)	Target attainment (% of 9,999 simulated subjects)
0.25	100
0.5	100
1.0	70
2.0	<1
4.0	0

[a] Cefepime and tobramycin were administered at doses of 2 g every 8 h and 7 mg/kg/day, respectively. The rates of cefepime's target attainment for the 2-g-every-8-h dose are 100% at an MIC of 4 mg/liter and 99.5% at an MIC of 8 mg/liter.

The target attainment estimates for tobramycin in the ELF for MIC values of 0.25 mg/liter through 4 mg/liter are 100% to 0%. These are displayed in Table 2. Both agents must attain the target. However, as is displayed above, cefepime has a very high target attainment exceeding 99% even at an MIC of 8 mg/liter. Consequently, the target attainments listed in Table 2 for tobramycin can be assumed to have the cefepime attainment rate included.

DISCUSSION

Little is known about how to optimize chemotherapy for *Pseudomonas aeruginosa*. Generally, chemotherapy fails for one of three reasons. The first is that the patient comes to appropriate medical care too late in the process and that by the time that active chemotherapy is administered, the physiological deterioration is irreversible. The second is that drug therapy is appropriate, in that the MIC is in the susceptible range but the dose and/or schedule is such that inadequate cell kill is obtained and the patient's clinical status does not improve. The third is that while there is adequate bacterial cell kill early on, the therapy produces exposures on the upswing of the inverted U plot (22) and there is ultimate resistance emergence during therapy. The latter outcome occurs surprisingly often, particularly with monotherapy against *P. aeruginosa*. Both ciprofloxacin and imipenem have demonstrated monotherapy resistance rates in nosocomial pneumonia of between 40 and 50% for imipenem and between 33 and 70% for ciprofloxacin (5, 13, 18) (the latter very high monotherapy resistance rate was due in the main to inadequate dosing early in ciprofloxacin's clinical utilization).

Consequently, cefepime was seen to be an important addition to the physician's therapeutic armamentarium, with a very impressive scientific data set with regard to enzyme stability being available, particularly for AmpC-type β-lactamases (16). Little regarding the optimization of cefepime dosing for use against *P. aeruginosa* has been done. In this set of experiments, we examined a WT strain, *P. aeruginosa* PAO1, and its isogenic stably derepressed mutant. As we wanted to evaluate the drug under circumstances that mimicked its use in nosocomial pneumonia, we chose to employ a high inoculum (circa 10^8 CFU/ml) for evaluation of the agent, and we also chose to simulate drug exposures higher than those for which cefepime is currently licensed by the FDA.

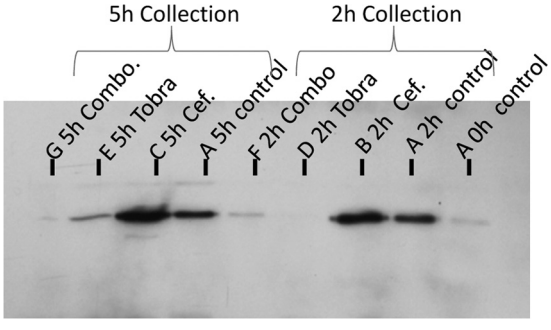

FIG 10 Western blotting for the amount of expressed AmpC β-lactamase in control cultures as well as cultures exposed to cefepime, tobramycin, and the combination of cefepime plus tobramycin over time.

In the original evaluation, simulated doses of 250 mg q8h through 3 g q8h were administered. To our surprise, all regimens failed with emergence of resistance (Fig. 1), and organisms taken from drug plates had stable increases in the MIC to cefepime from a baseline value of 1 mg/liter to 8 mg/liter. This caused us to examine the impact of the starting inoculum on the outcome. In this evaluation, exposures up to 6 g q8h were evaluated. Again, all therapeutic regimens failed with emergence of resistance (Fig. 2), but the 10^5-CFU/ml inoculum regimens fared best and were the last to fail with resistance emergence.

We also noted that after the first dose, concentrations of cefepime were biased low relative to the nominal values. Indeed, the continuous-infusion arms for all three inocula are displayed in Fig. 3 and show inoculum-dependent and time-dependent decrements in concentration relative to the nominal values. This strongly suggests that the failure of therapy is due to elaboration of the AmpC β-lactamase and that the resulting mutants are stably derepressed relative to baseline, which is also consistent with the change in the MIC value from 1 mg/liter to 8 mg/liter.

Given our previous experience with the combination of meropenem plus levofloxacin (17), we felt that it was important to examine combination chemotherapy. In this instance, we examined cefepime alone, tobramycin alone, and both agents in combination. Doses of cefepime of 563 mg q8h through 3 g q8h were examined along with daily doses of 3, 7, and 10 mg/kg of tobramycin. All possible combinations of these regimens were evaluated. In Fig. 4 and 5, it is clear that all monotherapy arms for both drugs failed (Fig. 4 for cell kill) with resistance emergence (Fig. 5). In contrast, all combination therapy arms suppressed resistance, although it should be noted that the regimen with the two lowest doses (563 mg q8h of cefepime and 3 mg/kg daily of tobramycin) generated the smallest log bacterial cell kill (circa 6 log units from baseline) over 14 days. However, it is also true that no resistance emergence was seen. We speculate that these organisms may have been of the nonreplicating persister (NRP) phenotype. As with our previous experience, the combination therapy was able to completely shut off resistance.

Normally, there would be an analysis for synergy performed by bacterial cell kill versus synergy for resistance suppression, as was done previously (17). In this instance, however, resistance occurs in all monotherapy regimens and occurs relatively early in the course of therapy, confounding attempts at examining the issue of synergy for bacterial cell kill.

Given all the previous data, we were even more convinced that the stable derepression of the AmpC β-lactamase was the driver behind the failure of therapy. In the next experiment, we tested this hypothesis directly by employing the stably derepressed isogenic mutant (stably derepressed for AmpC expression) and examining cefepime alone at very high doses (1 g, 2 g, 3 g, 4 g, 6 g, and 8 g q6h as well as 8 g daily by continuous infusion) and also added an arm with the lowest cefepime dose (1 g q6h), but with the β-lactamase inhibitor NXL104 present in the broth at a continuous concentration of 4 mg/liter (a concentration without an independent microbiological effect). In Fig. 6, we see that only the arm with NXL104 coadministered succeeded. Much the same as was seen earlier (Fig. 3), we see in Fig. 7 that there is considerable hydrolysis of cefepime, so that the observed concentrations are always biased below the desired (nominal) concentrations of the drug. This bias is seen after the first dose. Importantly, this is seen most with the continuous-infusion regimen, strongly suggesting a Michaelis-Menten event (the peaks likely saturate the β-lactamase, while with the continuous-infusion regimen hydrolysis remains in a range where it is efficient). Again, this strongly suggests that the enzyme is at the heart of the failures. When it is taken out of the equation by the β-lactamase inhibitor, cefepime succeeds, even at a relatively modest dose.

We also wanted to evaluate combination therapy, as before, but with the stably derepressed isogenic isolate. Given our prior experience, we evaluated only a low combination therapy dose of cefepime plus tobramycin (563 mg q8h plus 3 mg/kg daily of tobramycin) against two regimens that had already demonstrated failure in monotherapy (1 g or 2 g q8h of cefepime). In Fig. 8, we see clear regimen failure for both monotherapy regimens and success for the low-dose combination regimen. In Fig. 9, we see the resistance emergence for the two monotherapy regimens.

Finally, we hypothesized that the aminoglycoside worked for resistance suppression at a low dose, in the main, because, as a protein synthesis inhibitor, it would decrease the expression of the AmpC enzyme. Table 1 shows the PCR data. It is clear that the aminoglycoside decreases the AmpC expression, and this is at least a part of the mechanism of interaction between these agents for resistance suppression.

To provide guidance to clinicians, we performed a Monte Carlo simulation for the ability to attain the lowest tested drug exposures of cefepime plus tobramycin in combination that successfully shut off resistance emergence. Since we were aiming for therapy of patients with ventilator-associated pneumonia (VAP), we corrected the exposures for the ability of the two drugs to penetrate into ELF (3, 6). For a regimen of 2 g of cefepime every 8 h plus 7 mg/kg of tobramycin daily, cefepime exposures were attained at a high rate even at an MIC value of 8 mg/liter (99.5%). The tobramycin target attainment was excellent at MIC values of 0.25 and 0.5 mg/liter (100%) and acceptable at an MIC of 1 mg/liter (70%) but was unacceptable at tobramycin MIC values of 2 and 4 mg/liter (<1 and 0%, respectively). Clearly, the utility of the combination is promising if the tobramycin MIC is 1 mg/liter or less.

In summary, patients with high-inoculum *P. aeruginosa* infections, as may be seen in VAP, require chemotherapy that will both provide excellent bacterial cell kill and suppress resistance emergence. In this instance, combination chemotherapy has been demonstrated to be able to provide both these endpoints and to do so at quite small exposures (achieving a percentage of the time that the free drug concentration in ELF is greater than the MIC of 24.7% for cefepime plus an ELF AUC/MIC value of 55 for tobramycin). Combination chemotherapy with modest drug exposures was able to attain the desired endpoint of resistance suppression when even supraphysiological doses of either drug alone allowed resistance emergence. We need to attain a better understanding of combination therapy and the mechanisms underlying resistance suppression.

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We have no conflicts to disclose.

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