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Comparative Bactericidal Activity of Ceftazidime against Isolates of *Pseudomonas aeruginosa* as Assessed in an In Vitro Pharmacodynamic Model versus the Traditional Time-Kill Method

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Bactericidal activity, historically assessed by in vitro tests which employ fixed drug concentrations, may also be evaluated in in vitro pharmacodynamic models in which in vivo pharmacokinetics and bacterial growth conditions can be simulated. However, systematic comparisons between the two methods are lacking. We evaluated the bactericidal activities of ceftazidime, at two different concentration/MIC ratios (C/MICs), against 10 clinical isolates of *Pseudomonas aeruginosa* in a two-compartment model with continuous-infusion conditions and a 2-h half-life. These values were compared to those determined by traditional 24-h time-kill (TTK) methods at the same C/MICs. Bactericidal activities were compared by using area under the colony count-time curves. Antibiotic exposure (area under the drug concentration-time curve) was also evaluated. Although bactericidal activity appeared greater by the TTK method ($P = 0.05$), when it was normalized for drug exposure, these differences disappeared ($P = 0.2$). This disparity was likely due to differences in drug exposure in the TTK method and in the peripheral compartment of the model (site of bacteria) over the first 8 h of the experiment, during which the antibiotic accumulated to target concentrations. This suggests that the bactericidal effects with constant antibiotic concentrations are similar in the two methods; however, this may not hold true with fluctuating drug concentrations. Further, results from the pharmacodynamic model may theoretically be more relevant, as in vivo pharmacokinetics and bacterial growth conditions can be more faithfully simulated.

The bactericidal activities of antimicrobials have historically been assessed by traditional time-kill (TTK) studies, in which a fixed concentration of an antimicrobial and an inoculum of bacteria are added to an enclosed container and colony counts are determined over a predetermined period of observation. However, TTK studies do not mimic in vivo conditions of exposure of a pathogen to an antimicrobial, in which drug concentrations may change with time. Additionally, TTK tests limit the availability of nutrients for microbial growth and do not allow for the elimination of bacterial waste products. In vitro pharmacodynamic models can mimic some in vivo conditions by simulating human pharmacokinetics of the antimicrobial and by varying the bacterial inoculum, strain, or culture conditions (2). While the latter two conditions can be varied in TTK experiments, models can also provide for the continuous supply of nutrients to the organism and the elimination of bacterial waste products. Studies of bacterial killing in in vitro models in which human pharmacokinetics have been simulated have produced findings similar to those found in both animal models of infection and human infections (5, 11). Limited information is available, to our knowledge, for any direct comparisons of antimicrobial activities as determined in TTK studies and in vitro model time-kill (MTK) studies.

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In a recent TTK study, we characterized the relationship of the concentration/MIC ratio (C/MIC) to the effect of ceftazidime for 10 strains of *Pseudomonas aeruginosa*, using a sigmoidal model for maximal achievable effect (E_{max}) (7). The highest C/MIC required to achieve ≥90% of the E_{max} for any isolate was 6.6. Although we were able to identify the apparent C/MIC that correlated with maximum bactericidal activity in this concentration-ranging study, it is not known if the same response (quantitatively) would be measured in an in vitro model, which, theoretically, should yield more clinically relevant results. We designed the present study to address this issue. The objective of this study was to compare the bactericidal activity of ceftazidime as measured in an in vitro pharmacodynamic model to that determined in our previous TTK study by evaluating two identical C/MICs.

MATERIALS AND METHODS

Bacterial strains. The microorganisms tested included 10 strains of *P. aeruginosa* (5 mucoid and five nonmucoid) isolated from different cystic fibrosis patients and *P. aeruginosa* ATCC 27853. Organisms were identified with the Vitek system (bioMérieux Vitek, Inc., Hazelwood, Mo.). The final inoculum was approximately 5×10^5 CFU/ml and was prepared according to National Committee for Clinical Laboratory Standard guidelines and verified with a spiral plater (Spiral Systems Inc., Cincinnati, Ohio) (9).

Antibiotic. Stock solutions of pharmaceutical- and analytical-grade ceftazidime (lots BF992 and ASW27D, respectively; Glaxo Wellcome, Research Triangle Park, N.C.) were prepared in sterile water for injection according to the manufacturer's recommendation. Pharmaceutical- and analytical-grade ceftazidime preparations were used in the MTK and high-performance liquid chromatography (HPLC) assays, respectively. Aqueous solutions of the analytical-grade ceftazidime were prepared with 10% (wt/wt) sodium carbonate (lot 34H1305; Sigma Chemical Company, St. Louis, Mo.). All working stock solutions were used to prepare the appropriate concentrations in cation-adjusted Mueller-Hin-

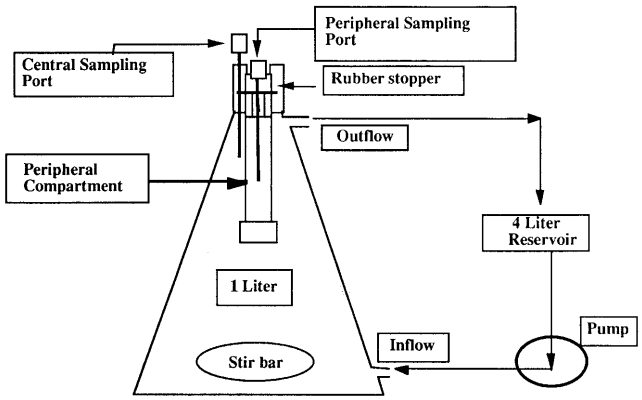

FIG. 1. In vitro pharmacodynamic model.

ton broth. These final drug solutions consisted of ≤5% (vol/vol) additional aqueous solution. Aqueous and working stock solutions were stored at −70°C and used within 7 days.

Media. Mueller-Hinton broth (lot 84585JG; Difco Laboratories, Detroit, Mich.), adjusted to 25 mg of Ca^{2+} per liter and 12.5 mg of Mg^{2+} per liter, was used for all studies. Enumeration of colonies was performed on antibiotic-free 10-cm-diameter Mueller-Hinton agar plates (BBL, Cockeysville, Md.).

In vitro model description. The in vitro pharmacodynamic model (Fig. 1) was comprised of a central compartment (dual-ported, 1-liter Erlenmeyer flask) into which a dialyzer (DisposoDialyzer; Spectrum Medical Industries Inc., San Jose, Calif.) was suspended (8). The dialyzer, representing the peripheral compartment and the site of infection, contained a final volume of 5 ml. Organisms were injected into the dialyzer, producing final concentrations ranging from 1.2×10^5 to 1.2×10^7 CFU/ml. However, values for the starting inocula for the same organism in the MTK and TTK portions of the study were all within 1 log of one another. The dialyzer's membrane material is a synthetic cellulose ester with a pore size of approximately 0.004 μm. The effective surface-area-to-volume ratio was 5.34 cm^{-1}, which is representative of that for the extravascular spaces in humans (1, 13). Both the central compartment and dialyzer (peripheral compartment) of the model were fitted with needles or catheters for convenient sample collection. Antibiotic-containing, cation-adjusted Mueller-Hinton broth was delivered from a reservoir, which was maintained at room temperature, to the central compartment of the model with a computerized peristaltic pump (Masterflex; Barnant Company, Barrington, Ill.) via silicone peroxide tubing (Masterflex). Because preliminary studies illustrated that bacterial growth and killing were not significantly different under recycled and nonrecycled conditions (probably due to dilutional effects; the reservoir holds 4 liters of broth), we chose to recycle the broth in all model experiments. During the first hour, antibiotic-free broth was pumped into the central compartment to allow the system and organism to equilibrate to the change in environment. The central and peripheral compartments were maintained at 35°C in a microbiologic warm room. Both the central compartment and reservoir were constantly stirred with magnetic stir bars.

In vitro MTK study. Two C/MICs were tested for each organism in the in vitro model (Table 1). The C/MICs tested were based on the results from the TTK study (7). The C/MICs that resulted in at least 75% of E_{max} and one twofold dilution higher were chosen so that the effects at two different and distinct parts of the C/MIC-effect relationship curve for the different methods could be compared. The desired concentrations were achieved and maintained by the administration of a bolus dose into the central compartment followed by the continuous infusion of antibiotic-containing broth into the central compartment from the reservoir. The flow rate was set to simulate a 2-h elimination half-life. Since preliminary studies showed that ceftazidime was unstable under these test conditions, small supplemental doses were administered at 12-h intervals to the reservoir to account for degradation over time. The degradation rate constants in the central compartment and reservoir were 0.023 and 0.0026 h^{-1}, respectively. The analysis was based on observations made over 24 h to allow direct comparisons to the previous TTK study.

At 0, 1, 2, 4, 8, 12, and 24 h, 0.2 ml was withdrawn from the peripheral compartment for colony count determination. Samples anticipated to contain $>4 \times 10^3$ CFU/ml, based on preliminary experiments, were serially 10-fold diluted in normal saline to facilitate the enumeration of colonies on plates prepared with the spiral plater. Undiluted samples were used when colony counts were anticipated to be $<4 \times 10^3$ CFU/ml. A 50-μl aliquot of each sample was placed onto 10-cm-diameter Mueller-Hinton agar plates with a spiral plater. The plates were incubated for 18 to 24 h at 35°C, and the surviving colonies were counted. Due to the spreading growth pattern of the organisms, colony counts were determined prior to 24 h (but not prior to 18 h) to facilitate counting. For data analysis, colony counts less than 4×10^2 CFU/ml (the limit of detection) were not plotted. Although a detailed analysis to detect antibiotic carryover was not performed, colony counts corrected for dilution were not different from counts in undiluted samples.

HPLC assay. Ceftazidime concentrations were determined in the central and peripheral compartments by withdrawing an additional 0.1 ml at 0, 8, and 24 h. Samples were either analyzed immediately or frozen at −70°C and analyzed within 7 days for ceftazidime concentration by a previously published HPLC assay adapted for a broth matrix (3). The mobile phase consisted of a mixture of 12% (vol/vol) acetonitrile–1% (vol/vol) glacial acetic acid in deionized water. The solution was adjusted to an apparent pH of 4.0 with 5 N sodium hydroxide. The mobile-phase flow rate was 2.0 ml/min through a reverse-phase C_{18} analytical column (Waters, Milford, Mass.) at ambient temperature. Ceftazidime was detected by UV light absorption at 310 nm. The retention time for ceftazidime was 3.7 min. Prior to injection, all samples were processed with 12.6 μl of acetonitrile, vortexed for 15 s, and then centrifuged for 2 min. The resultant supernatant was assayed for ceftazidime concentration. Two standard curves, encompassing concentration ranges of 5 to 120 and 0.75 to 8 μg/ml, respectively, were used. Both standard curves were linear over the concentrations tested ($r^2 >$ 0.999). For the higher-range standard curve, the inter- and intraday coefficients of variation were ≤4.6 and ≤3.0%, respectively. The inter- and intraday coefficients of variation for the lower-range standard curve were ≤5.9 and ≤3.7%, respectively.

Data analysis. (i) Ceftazidime pharmacokinetics. Ceftazidime concentrations from the central and peripheral compartments were plotted as the natural logarithm versus time. Percent error from the desired concentration and the slope from 8 to 24 h were calculated to assess the variations in drug concentrations during the study. Drug exposure was defined as the area under the concentration-time curve (AUC) and was determined for the periods 0 to 8 and 0 to 24 h.

TABLE 1. Rates and extents of killing in TTK and MTK studies

Organism and isolate no.	C/MIC	Kill rate (h^{-1})		Time (h) to 99.9% kill	
		MTK study	TTK study[a]	MTK study	TTK study
Nonmucoid					
1	2	0.09	−1.13	NA[b]	8
	1	0.06	−1.05	NA	8
2	8	−1.14	−1.11	8	8
	4	−1.05	−1.17	8	8
3	8	−1.14	−1.06	NA	8
	4	−1.14	−1.07	8	8
4	16	−1.18	−1.10	8	8
	8	−1.14	−1.07	NA	8
5	2	−0.48	−1.13	NA	8
	1	−0.61	−1.16	NA	8
Mucoid					
6	16	−0.74	−0.91	24	12
	8	−0.72	−1.00	NA	12
7	16	−1.17	−1.22	8	8
	8	−1.21	−1.14	NA	8
8	2	−0.27	−0.75	NA	24
	1	−0.30	−0.58	NA	NA
9	4	−1.05	−1.20	NA	8
	2	−1.07	−1.10	NA	8
10	2	−0.74	−1.15	NA	8
	1	−1.13	−1.34	NA	8
ATCC 27853	16	−1.12	−0.74	8	8
	8	−1.10	−1.01	NA	4

[a] Significantly different from model by Wilcoxon signed rank test ($P = 0.03$).
[b] NA, not achieved.

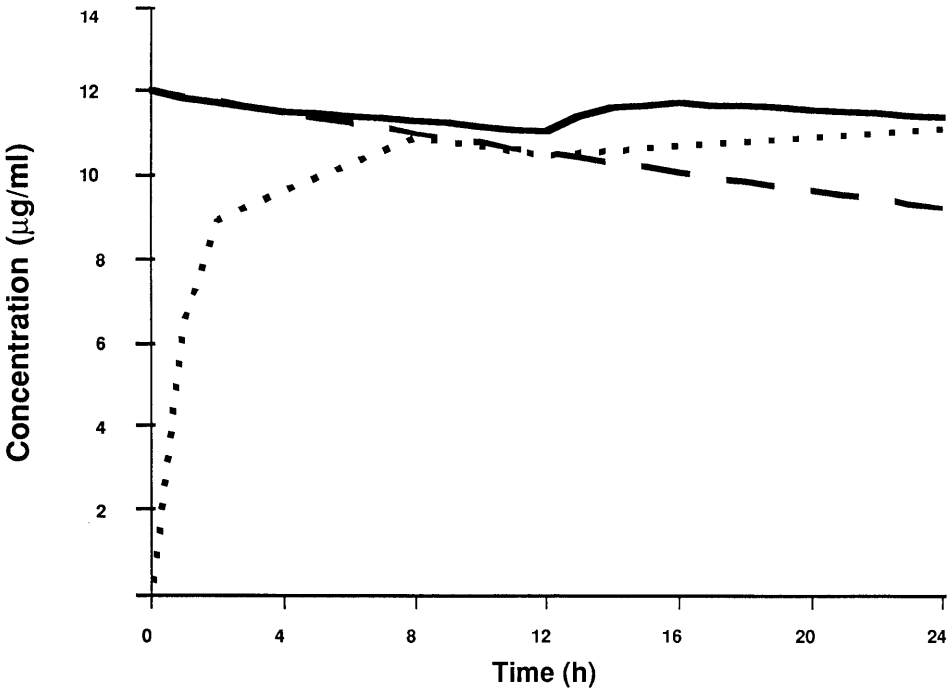

FIG. 2. Graphic representation of antibiotic concentrations over time in the central compartment of the model (solid line), the peripheral compartment of the model (dotted line), and in a TTK study (dashed line).

The AUCs were calculated by using the linear trapezoidal rule. For the MTK study, the observed concentrations were used for calculating the AUC. In the TTK study, the AUC was calculated by using concentrations extrapolated based on the degradation rate of 0.01 h^{-1} that was determined under TTK test conditions. Extrapolation of a concentration at any time point (C_t) was accomplished by using the relationship $C_t = C_0 \times e^{-kt}$, where C_0 is the concentration at time zero, k is the degradation rate constant, and t is the elapsed time.

(ii) Antibacterial activity. To assess differences in bactericidal activity, colony counts were plotted versus time and evaluated for rate and extent of killing. From these plots, the time to 99.9% killing of the initial inoculum was also determined. Kill rates between 2 and 8 h were determined by simple linear regression of the natural log of the number of CFU per milliliter versus time. In all instances in which a kill rate could be calculated, at least three data points were used. Only those kill rates with a coefficient of determination (r^2) of $\geq$0.8 were used in all subsequent statistical analyses. Further, the extent of killing was evaluated by calculating the area under the percent remaining curve (AUPR) from the percentage of the initial inoculum remaining ($\log_{10}$ colony count)-time curves by using the linear trapezoidal rule. The percent remaining was used in this calculation to account for small differences ($<$ 1 log) in the initial inoculum between the TTK and MTK studies. The AUPR from 0 to 24 h ($AUPR_{0-24}$) for each C/MIC tested was then transformed to a percent effect based on the individual growth (control) curves for each isolate by using the following equation: % effect = $(A - B)/A \times 100$, where A is defined as the area under the growth curve (transformed to AUPR) and B was defined as the $AUPR_{0-24}$ at each C/MIC tested. To compare the effects at the same drug exposure, the effects in the TTK study were calculated at the same AUC achieved in the MTK study by using the following equation: % effect = $E_{max} \times C^n/(EC_{50}{}^n + C^n) \times 100$, where C is the AUC observed in the MTK study, E_{max} is the maximum achievable effect, EC_{50} is the AUC at which 50% of E_{max} was observed, and n is the sigmoidicity factor (6). The E_{max} and EC_{50} were obtained from the previously characterized C/MIC-effect relationships in the TTK study (7).

Note that the calculated effects exhibited in both the MTK and TTK studies did not reach 100%. Total killing can be measured only if there is instantaneous and complete killing (at time zero) with no subsequent regrowth. The maximum effect that could be measured by our sampling scheme would occur when there is killing to the limit of detection by the first sample point with no subsequent regrowth over the duration of the experiment. Thus, the value of the maximum measurable effect (MME) is dependent on the initial inoculum, the sampling scheme, and the limit of quantitation. The percent MME (% MME) ranged from 68 to 71% and 65 to 74% for the TTK and MTK studies, respectively.

Statistical analysis. The outcomes described above (percent effect and kill rate) were compared at the same C/MICs tested in the TTK and MTK tests by using a Wilcoxon signed rank test. The same statistical test was used to assess differences between the two C/MICs tested. The drug exposures in the TTK and MTK studies were compared by using the paired t test. Significance was defined as $P \leq 0.05$.

RESULTS

Pharmacokinetics and drug exposure. Ceftazidime pharmacokinetics in the MTK and TTK studies are illustrated in Fig. 2. Of the 22 model runs (11 organisms; two concentrations tested per organism), the mean (standard deviation) percent error from the desired concentration at 8 and 24 h in the central compartment was 3.7 (5.1). Mean (standard deviation) concentrations in the peripheral compartment at 8 and 24 h were within 4.2% (3.9%) and 2.1% (4.2%), respectively, of those in the central compartment. Once desired concentrations were achieved, the slope between 8 and 24 h ranged from -0.008 to 0.006 h^{-1} in the peripheral and central compartments, signifying little change in ceftazidime concentration over the period of observation.

There was a significant difference between the MTK and TTK studies in drug exposure over 8 and 24 h ($P = 0.0004$ and 0.0002, respectively). Drug exposure was greater in the TTK study, but this difference was more pronounced during the first 8 h (Fig. 3 and 4). Specifically, over 0 to 8 and 0 to 24 h, the drug exposure in the peripheral compartment in the MTK study averaged 48 and 88%, respectively, of that achieved in the TTK study.

Antimicrobial activity. The kill rates and times to 99.9% killing for the MTK and TTK studies are listed in Table 1. For all kill rates in the MTK and TTK studies, r^2 was $>$0.85 except in four cases. The kill rates in the TTK study were significantly higher than those in the MTK study ($P = 0.03$). Maximum (99.9%) killing was achieved more often in the TTK study (95%) than in the MTK study (32%). In the TTK study, 99.9% killing was achieved within 8 h in the majority of cases. In 41% of instances, the colony counts at 24 h were at least 10 times greater in the MTK study. Colony counts at 24 h were at least

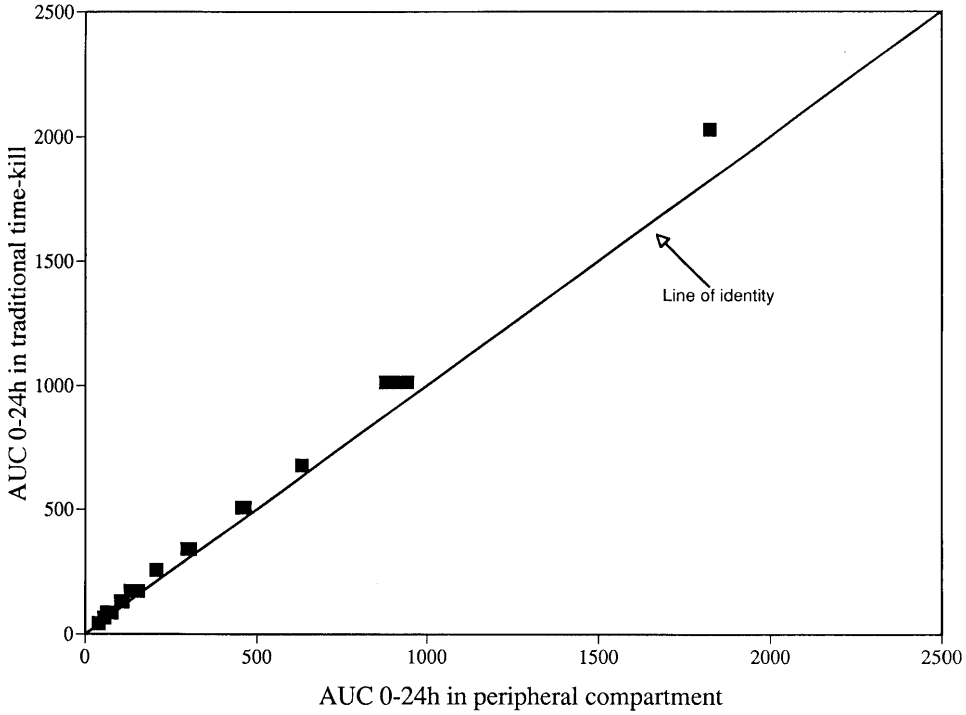

FIG. 3. Comparison of AUC_{0-24}s in the MTK (in the peripheral compartment) and TTK studies. The units for both axes are micrograms per hour per milliliter.

10 times greater in the TTK study in 14% of instances. Regrowth was noted in approximately 36% of all instances by both methodologies and, when noted, did not occur to a greater extent (higher colony counts) by one method or the other.

Overall, the average %MMEs exhibited in the MTK and TTK studies across the C/MICs tested were 73 and 80%, respectively (Table 2). There was a significant difference in the effect between the two time-kill methods ($P = 0.05$). The effect in the TTK study was higher, indicating better killing in the

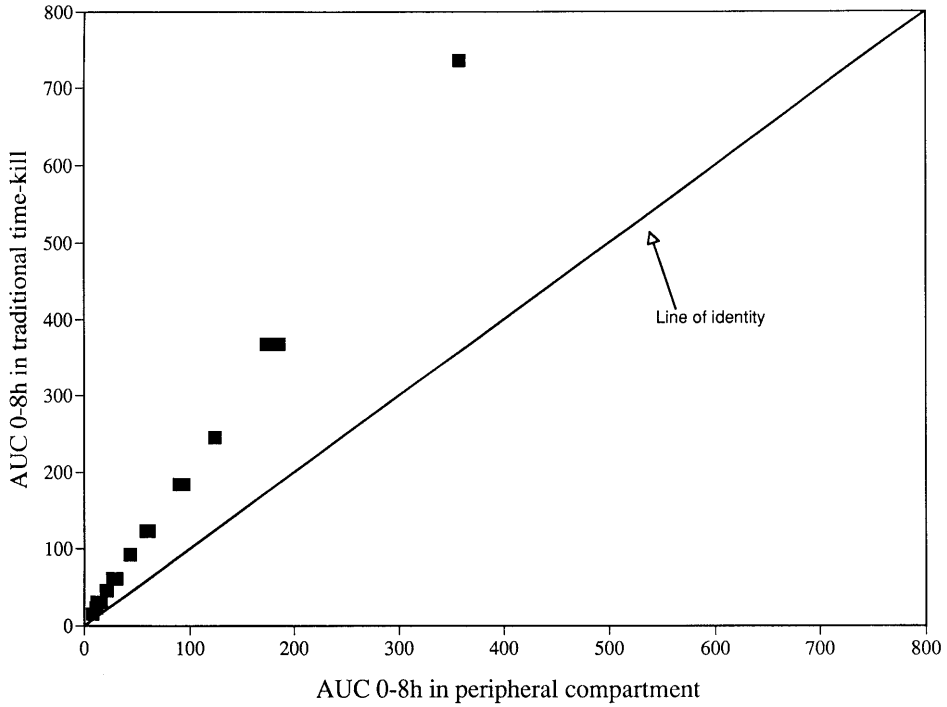

FIG. 4. Comparison of AUC_{0-8}s in the MTK (in the peripheral compartment) and TTK studies. The units for both axes are micrograms per hour per milliliter.

TABLE 2. %MMEs achieved in TTK and MTK studies

Organism and isolate no.	C/MIC	%MME		
		MTK study	TTK study[a]	TTK study at same AUC as model[b]
Nonmucoid				
1	2	45	86	82
	1	43	78	72
2	8	90	89	86
	4	92	77	81
3	8	89	82	85
	4	91	82	80
4	16	66	92	89
	8	76	92	88
5	2	70	85	83
	1	71	75	66
Mucoid				
6	16	77	79	80
	8	74	79	75
7	16	91	86	82
	8	89	85	77
8	2	53	71	65
	1	48	52	50
9	4	81	83	83
	2	61	78	76
10	2	80	65	77
	1	67	70	67
ATCC 27853	16	87	94	89
	8	74	92	87

[a] Significantly different from model by Wilcoxon signed rank test ($P = 0.05$; all isolates).

[b] MME normalized to same AUC as that achieved in the model. Not significantly different from model by Wilcoxon signed rank test ($P = 0.20$; all isolates).

TTK study than in the MTK study. By substituting the AUC for the C/MIC in the original TTK effect plots (AUC versus effect as opposed to C/MIC versus effect), one can estimate the AUC under TTK conditions for any given AUC (normalization), thus allowing a direct comparison of effects measured by the two methods. When the effect in the TTK study was normalized in this manner to the same drug exposure achieved in the MTK study, the differences in effect were no longer statistically significant ($P = 0.20$).

In the TTK study, there was a significant difference in effect between the mucoid and nonmucoid strains, with a greater effect achieved against the nonmucoid strains ($P = 0.03$). There was not a significant difference in effect between the mucoid and nonmucoid strains in the MTK study ($P = 0.80$). While there was a significant difference in effect in the TTK study associated with the two C/MICs tested ($P = 0.03$), the difference was not significant in the MTK study ($P = 0.13$).

DISCUSSION

In a recent TTK study, we tested a range of C/MICs and observed that a C/MIC of 6.6 appeared to maximize the bactericidal effect of ceftazidime against 10 strains of *P. aeruginosa* isolated from cystic fibrosis patients. Although our results are consistent with those of other investigators who addressed the C/MIC-effect phenomenon for beta-lactam antibiotics (4, 13), it was unknown if the same response or effect would be determined in an in vitro model. TTK studies, unlike the in vitro pharmacodynamic model, cannot mimic in vivo conditions. In an in vitro model, one can simulate human antimicrobial pharmacokinetics and vary culture conditions in order to more closely resemble assumed in vivo conditions. Therefore, one might expect differences in the effects measured by these two methodologies. We compared the effect achieved at two different C/MICs in the MTK and TTK experiments.

At the concentrations tested, bacterial killing (effect) was greater for the majority of *P. aeruginosa* strains tested when evaluated in the TTK studies as opposed to the in vitro model. This difference in killing was probably due to the lesser drug exposure in the model during the first 8 h of the experiment, explaining both the lower kill rates and the decreased extent of killing exhibited. This difference in drug exposure between the model and time-kill experiments is due to the difference in the initial concentrations at the site of the drug-bacterium interface at the beginning of and for the first 8 h of the experiment. It is assumed that drug concentrations are constant in a TTK study. Even though the desired test concentration was achieved immediately in the central compartment of the model by adding a bolus dose at the beginning of the experiment, the desired drug concentration in the peripheral compartment (i.e., the site of the drug-bacterium interface) was initially zero and did not achieve the target concentration for approximately 8 h. Thus, drug exposure, as reflected by the AUC for the first 8 h of the experiments, was markedly different. Another variance that may help explain the differences in results between the MTK and TTK results is the model's theoretical properties of continuously providing fresh nutrients to the organism and eliminating bacterial waste products as broth is pumped through the system. This assumes that nutrients present in broth and bacterial waste products freely pass through the dialysis membrane separating the two compartments of the model. It is also possible that the apparent difference in extent of bacterial killing measured by the two methodologies was due to bacterial adherence to the inner walls of the peripheral compartment of the model. While we were unable to take steps to exclude this possibility, it seems improbable that such a phenomenon would explain the magnitude of the difference observed.

A delay in reaching critical antibiotic concentrations at the site of infection could have important implications in the treatment of some infections. Bacterial killing may not commence until such concentrations are achieved. Although this early killing may not be crucial to treating some types of infection, it may make a difference in treating others, such as meningitis (10). Thus, results from the model may be more critical to the latter type of infection. It would obviously be desirable to determine whether differences in measured bactericidal effect as determined by TTK and MTK studies correlate to in vivo differences in antibiotic efficacy for various types of infection.

The highest C/MIC identified in our TTK study resulting in an effect that was 90% of E_{max} was 6.6. Regardless of the differences in measured effect generated by the two methodologies, it should be noted that a C/MIC of 6.6 was associated with an effect that, in either case, was 90% of the E_{max} determined in the TTK study. Beta-lactams have long been considered to possess time-dependent antibacterial activities, and it has been suggested that bactericidal activity is maximized at a C/MIC of 4 to 5 (4, 12). Our results suggest that, for at least

some bacterial isolates, a higher concentration is needed to maximize the antibacterial effect. At the two C/MICs tested in the model, killing occurred to a lesser extent than in the time-kill study, but we cannot assume that a higher concentration in the model would result in a greater effect. In fact, the effects measured in the model at the high and low concentrations were similar, suggesting that the effect may have possibly reached an upper plateau on the concentration-effect curve. Only further experiments with additional concentrations would allow full characterization of the entire concentration-effect relationship in the model.

While differences in measured bactericidal effect between the two methods were small, when normalized for drug exposure, different forms of drug administration in the model may yield differing observations. It is not surprising that continuous infusion conditions in the model yielded results similar to those in the TTK, as both expose the bacteria to a constant concentration of antibiotic. However, intermittent drug administration, which would expose the bacteria to fluctuating antibiotic concentrations, might be expected to produce altogether different results. The ability of an in vitro model to mimic in vivo bacterial growth conditions may make such differences even more likely. Moreover, because of these parallels with in vivo conditions, results from this and other in vitro models may be more clinically relevant or applicable. Nonetheless, in this continuous-infusion in vitro model study, the bactericidal effect of ceftazidime, when normalized for drug exposure, was similar to that determined by TTK methods.

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