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Pharmacodynamics of Levofloxacin in a Murine Pneumonia Model of *Pseudomonas aeruginosa* Infection: Determination of Epithelial Lining Fluid Targets▽

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The dose choice for *Pseudomonas aeruginosa* remains a matter of debate. The actual exposure targets required for multilog killing of organisms at the primary infection site have not been delineated. We studied *Pseudomonas aeruginosa* PAO1 using a murine model of pneumonia. We employed a large mathematical model to fit all the concentration-time data in plasma and epithelial lining fluid (ELF) as well as colony counts in lung simultaneously for all drug doses. Penetration into ELF was calculated to be approximately 77.7%, as indexed to the ratio of the area under the concentration-time curve for ELF (AUC_{ELF}) to the AUC_{plasma}. We determined the ELF concentration-time profile required to drive a stasis response as well as 1-, 2-, or 3-$\log_{10}$(CFU/g) kill. AUC/MIC ratios of 12.4, 31.2, 62.8, and 127.6 were required to drive these bacterial responses. Emergence of resistance was seen only at the two lowest doses (three of five animals at 50 mg/kg [body weight] and one of five animals at 100 mg/kg). The low exposure targets were likely driven by a low mutational frequency to resistance. Bridging to humans was performed using Monte Carlo simulation. With a 750-mg levofloxacin dose, target attainment rates fell below 90% at 4 mg/liter, 1 mg/liter, and 0.5 mg/liter for 1-, 2-, and 3-log kills, respectively. Given the low exposure targets seen with this strain, we conclude that levofloxacin at a 750-mg dose is not adequate for serious *Pseudomonas aeruginosa* pneumonia as a single agent. More isolates need to be studied to make these observations more robust.

Nosocomial pneumonia, particularly when caused by pathogens such as *Pseudomonas aeruginosa*, remains a major cause of morbidity and mortality in intensive care units (ICUs). Part of the difficulty surrounding this disease entity is the uncertainty of the adequacy of therapy. There are many variables that have an impact on this uncertainty. In the ICU environment, bacterial burdens in patients are often high, making the selection of antibiotic dose choice important (2, 5, 13, 15).

Part of the uncertainty revolves around an understanding of the drug concentration-time profile for the primary infection site. Over the last two decades, important inroads have been made in the understanding of the relationship between drug concentration-time profile and the outcome of infections (8). This understanding has come from preclinical in vitro and animal studies and from clinical trials (1, 14, 21). Much of this insight has been guided by preclinical animal models, particularly the mouse thigh infection model (3, 6). The mouse thigh model is an excellent surrogate for exploring dose and schedule choice for infections of the skin and skin structure. Generally, drug penetration is good at this site, and the drug concentration-time profile for plasma is a good guide to the concentration-time profile for the infection site.

Much less investigation has been done for pneumonia, and little understanding is available regarding the drug concentration-time profile for the lung and how this relates to the killing of the pathogen at this site. In this investigation, we employed a well-validated murine pneumonia model (11) using strain PAO1 of *Pseudomonas aeruginosa*. We chose levofloxacin as the antibiotic probe into this system. As part of the approach, we developed a novel mathematical model where murine plasma concentrations, epithelial lining fluid (ELF) concentrations, and colony counts were simultaneously modeled for all dosing regimens examined. Drug exposure targets in ELF were then calculated and bridged to humans by employing Monte Carlo simulation and ELF penetration data for humans, which were previously reported by this laboratory (9).

(This work was recently presented at the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 25 to 28 October 2008 [10].)

MATERIALS AND METHODS

Microorganism. *Pseudomonas aeruginosa* strain PAO1 was a kind gift of Keith Poole. MICs were determined by the CLSI macrobroth methodology for levofloxacin (4). The mutational frequency to resistance was estimated by plating 1 ml of serial dilutions of strain PAO1 cultures grown overnight on agar without antibiotic and agar containing 3× the baseline MIC of levofloxacin. The ratio of these provided the estimate of the mutational frequency to resistance. This was done on at least three occasions.

Murine pneumonia model. All animal experimentation was approved by the local IACUC. The murine pneumonia model described previously by Du et al. (11) was used. Briefly, female, 24- to 26-g, outbred Swiss-Webster mice (Taconic Farms, Taconic, NY) were provided food and water ad libitum. The mice were rendered transiently neutropenic with 150 mg/kg body weight cyclophosphamide given intraperitoneally (i.p.) 4 days prior to infection and with 100 mg/kg given i.p. 1 day before infection. Anesthetized mice were infected via the intranasal route with 2×10^7 CFU of *P. aeruginosa* in 20-μl volumes. The density of the bacterial inoculum was confirmed by quantitative cultures. Five hours after bacterial inoculation, just prior to therapy initiation, five mice were sacrificed for baseline quantitative cultures of lung homogenates. Cohorts of five animals were administered levofloxacin i.p. at doses of 50, 100, 150, 200, 300, and 400 mg/kg for cell kill studies. Twenty-four hours after treatment initiation, all mice were humanely sacrificed. Lungs were aseptically collected. The tissues were homog-

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enized and washed with normal saline to prevent drug carryover. The homogenates were then quantitatively cultured on drug-free agar plates. After the plates were incubated at 35°C for 48 h, the colonies were enumerated. For drug-containing plates, colonies were enumerated at both 48 and 72 h. Means and standard deviations for the quantitative culture samples were calculated. The significance of differences in total bacterial densities associated with each drug regimen was analyzed by analysis of variance. If differences were identified, multiple pairwise comparisons were made with alpha decay by Bonferroni's adjustment. Estimates of resistant organism numbers were determined by quantitatively plating an aliquot of each homogenate of lung tissue onto plates containing 3× the baseline MIC of levofloxacin.

Pharmacokinetic studies. To correlate the doses of drug administered to mice with measures of exposure, single-dose pharmacokinetic studies were conducted with mice with *Pseudomonas aeruginosa* pneumonia. Neutropenic mice were infected via the intranasal route (see above). Single doses of levofloxacin (doses of 50, 100, 200, and 400 mg/kg/day) were given to four groups of mice 5 h after they were inoculated with *P. aeruginosa*. Pharmacokinetics in plasma and ELF were determined with eight sampling times (two mice per time point) between 0 and 6 h after an i.p. dose.

At time points between 0 and 6 h after i.p. dosing of levofloxacin, blood was collected by cardiac puncture and allowed to clot on ice. The plasma obtained by centrifugation was stored at −80°C. Bronchoalveolar lavage (BAL) fluid was simultaneously obtained by instilling 0.6 ml of water into lungs and removing 0.6 ml of water from the lungs twice. Plasma and BAL fluid were stored at −80°C until they were assayed for drug content by liquid chromatography (LC) and dual mass spectrometry (MS). The prediluted concentration of antibiotic in the BAL fluid was calculated by comparing the difference in amounts of urea measured in simultaneously collected plasma and BAL fluid.

Levofloxacin assay. Mouse plasma samples were assayed by LC/MS. Detailed methods are available from the authors upon request. The assay was linear over a range of 0.100 to 50.0 μg/ml ($r^2 > 0.998$). The interday precision (percent coefficient of variation [%CV]) ranged from 2.42 to 7.43%, with an accuracy (percent recovery) ranging from 90.2% to 105% for quality control samples at three levels (0.500 μg/ml, 1.00 μg/ml, and 10.0 μg/ml) in replicates of three at each level on each analysis day.

BAL samples (0.025 ml) were transferred into appropriately labeled autosampler vials containing 1.00 ml of high-pressure LC water. Samples were analyzed by high-pressure LC/dual MS. As described above, detailed methods are available from the authors upon request.

The assay was linear over a range of 5.00 to 1,000 ng/ml ($r^2 > 0.997$). The interday precision (%CV) ranged from 3.65 to 6.34%, with an accuracy (percent recovery) ranging from 102% to 108% for quality control samples at three levels (10.0 ng/ml, 100 ng/ml, and 500 ng/ml) in replicates of three at each level on each analysis day.

Urea assay. Assay of urea in murine plasma and ELF was performed by employing the BioAssay Systems QuantiChrom urea assay kit (DIUR-500). The kit was purchased from BioAssay Systems (Hayward, CA). The standard curve was linear from 3.1 mg/dl to 50 mg/dl ($r^2 = 0.999$). Within-day %CV values were 1.0% at 3.1 mg/dl and 1.1% at 50 mg/dl.

Population modeling approach. For the population modeling approach, the ELF was its own sampling compartment with its own apparent volume of distribution. It required 14 parameters and five differential equations to define this system:

$$dX_1/dt = -X_1 \times K_a$$

$$dX_2/dt = X_1 \times K_a - [(\mathrm{CL}/F)/(V_c/F) + K_{23} + K_{24}] \times X_2 + K_{32} \times X_3 + K_{42} \times X_4$$

$$dX_3/dt = K_{23} \times X_2 - K_{32} \times X_3$$

$$dX_4/dt = K_{24} \times X_2 - K_{42} \times X_4$$

$$dX_5/dt = K_{\mathrm{growth}} \times (1 - X_5/\mathrm{POPMAX}) \times X_5 - K_{\text{kill-max}} \times \{(X_4/V_{\mathrm{ELF}})^H/[\mathrm{EC}_{50}{}^H + (X_4/V_{\mathrm{ELF}})^H]\} \times X_5$$

The plasma concentration equals X_2/V_c, the ELF concentration equals X_4/V_{ELF}, and lung colony counts equal $\log_{10}(X_5)$. K_a is the absorption rate constant, as levofloxacin was administered via i.p. injection; CL/F is plasma clearance, where F is the bioavailability of the i.p. dose; V_c/F is the apparent volume of the central compartment, where F is the bioavailability of the i.p. dose; K_{23}, K_{32}, K_{24}, and K_{42} are all first-order intercompartmental transfer rate constants; V_{ELF} is the apparent volume of the ELF compartment; K_{growth} is the first-order growth constant of the organism; POPMAX is the maximal organism concentration (CFU/g) at stationary phase; $K_{\text{kill-max}}$ is the maximal kill rate of the organism, driven by drug concentration; EC_{50} is the drug concentration at which the organism kill rate is half-maximal; and H is Hill's constant.

The BigNPAG program described previously by Leary et al. (17) was employed for all population modeling. Weighting was the inverse of the between-day assay error variance. Bayesian estimates were obtained for each mouse using the "population-of-one" utility in BigNPAG. Model evaluation was done by predicted-observed plots. The mean error served as the measure of bias. The bias-adjusted mean squared error served as the measure of precision. As each animal had at most only one observation for plasma, ELF, and colony counts, the "adaptive γ" option was not employed. Separate cohorts of animals were studied for pharmacokinetics (plasma and ELF concentrations) and drug effects (colony counts in lung).

Lung colony counts were linked to the total drug ELF area under the concentration-time curve ($\mathrm{AUC}_{\mathrm{ELF}}$)/MIC ratio through simulation from the identified system parameters (see above). The $\mathrm{AUC}_{\mathrm{ELF}}$/MIC ratio driving a static effect as well as 1-, 2-, and 3-$\log_{10}$(CFU/g) kills from the static exposure was calculated.

Bridging to humans. We generated a 9,999-subject Monte Carlo simulation for the levofloxacin $\mathrm{AUC}_{\mathrm{ELF}}$ using a 750-mg levofloxacin dose. The mean parameter vector and full covariance matrix from data previously reported by this laboratory (9) were embedded in Subroutine PRIOR of the ADAPT II package of programs described previously by D'Argenio and Schumitzky (7). Both normal and log-normal distributions were evaluated and discriminated by the fidelity with which the measures of central tendency and dispersion were recreated by the simulation. From this, the distribution of $\mathrm{AUC}_{\mathrm{ELF}}$/MIC ratios was calculated, and the target attainments were calculated for 1-, 2-, and 3 $\log_{10}$(CFU/g) kills by employing the program SYSTAT for Windows (version 11.0).

RESULTS

MIC and mutational frequency to resistance of levofloxacin for *Pseudomonas aeruginosa* PAO1. Broth macrodilution on three separate occasions demonstrated that the MIC was 0.5 mg/liter. The mutational frequency to resistance was estimated to be $1/(6 \times 10^6)$.

Fit of the model to all the data simultaneously. The integrated model was fit to all the data simultaneously. For each of the outputs, the model fits were good, with the plasma data having a predicted-observed regression r^2 of 0.994; for the ELF concentration-time data, the r^2 was 0.976; for the colony count data, the r^2 was 0.897. The plots are displayed in Fig. 1 for each of the system outputs.

Extensive simulation indicated that the median parameter values best reflected the observed data. The median parameter values and dispersions are displayed in Table 1.

Penetration into ELF was calculated as the ratio of the levofloxacin $\mathrm{AUC}_{\mathrm{ELF}}$ to that in plasma. The dose used for simulation was 200 mg/kg. The point estimate of penetration was 77.7% (Fig. 2).

Cell kill and emergence of resistance. Simulation demonstrated that the $\mathrm{AUC}_{\mathrm{ELF}}$/MIC targets for stasis and 1-, 2-, and 3-$\log_{10}$(CFU/g) bacterial kills in lung were quite small and ranged from 12.4 to 127.6. The complete kill curve was constructed from simulation from the system parameters. The actual doses simulated were 0, 50, 100, 150, 200, 300, and 400 mg/kg. These data are displayed in Fig. 3.

While each animal had the homogenate plated for recovery of levofloxacin-resistant isolates, only three of five animals from the 50-mg/kg dosing cohort and one animal from the 100-mg/kg cohort had recovery of any resistant isolates.

Bridging to humans. A 9,999-subject Monte Carlo simulation was performed by employing the parameter vector and full covariance matrix from a study of levofloxacin penetration into

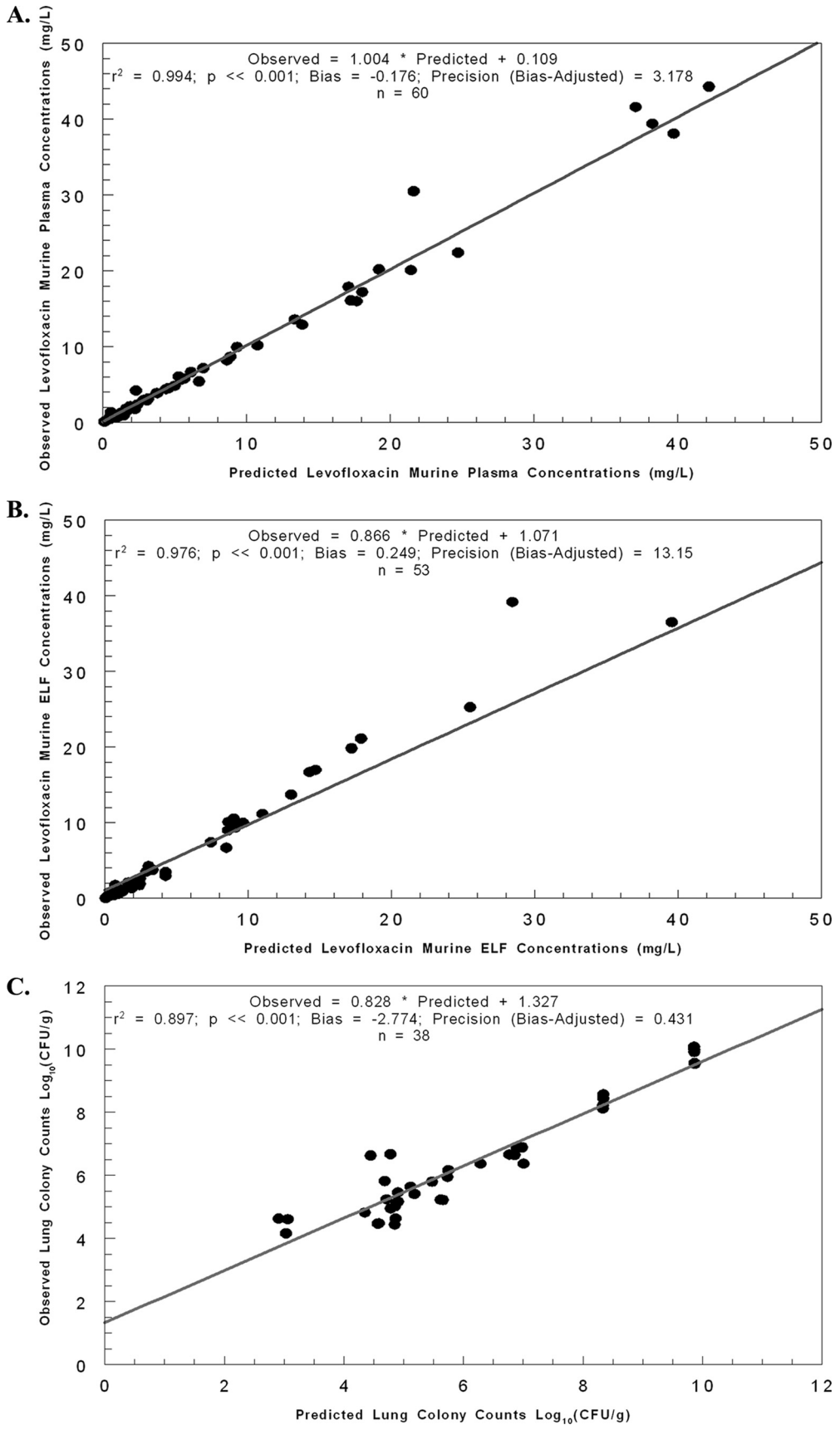

FIG. 1. Fit of the model to the data after the Bayesian step for plasma (A), ELF (B), and $\log_{10}$(CFU/g) of lung (C).

TABLE 1. Pharmacodynamic parameter values for penetration of levofloxacin into ELF of mice infected with *Pseudomonas aeruginosa* and effect parameters for cell kill

Parameter[a]	Value	
	Median	SD
V_c/F (liters)	0.044	0.18
CL/F (liters/h)	0.078	0.11
K_{23} (h^{-1})	3.33	6.17
K_{32} (h^{-1})	9.22	3.14
K_{24} (h^{-1})	7.92	3.02
K_{42} (h^{-1})	18.6	5.10
V_{ELF} (liters)	0.024	0.20
K_a (h^{-1})	7.81	5.37
K_{growth} (CFU/g/h)	0.12	3.36
$K_{kill\text{-}max}$ (CFU/g/h)	2.95	2.00
EC_{50} (mg/liter)	3.15	3.76
H	14.0	4.96
POPMAX (CFU/g)	7.2×10^9	4.0×10^9
IC_5 (CFU/g)	2.2×10^8	3.1×10^7

[a] IC_5 is the initial condition in the fifth compartment.

ELF from volunteers previously reported by our laboratory (9). The target attainments for exposure targets in ELF mediating stasis and 1-, 2-, and 3 $\log_{10}$(CFU/g) cell kills were determined. These AUC/MIC ratios were 12.4, 31.2, 62.8, and 127.6, respectively. While the MIC for this isolate was 0.5 mg/liter, we also decided to examine the cases where the levofloxacin MIC ranged from 0.5 mg/liter to 4 mg/liter. For a 1-$\log_{10}$(CFU/g) kill, MICs as high as 2 mg/liter had a target attainment exceeding 90%, while for 2- and 3-$\log_{10}$(CFU/g) cell kills, this occurred for MICs of 1 and 0.5 mg/liter, respectively (Table 2).

DISCUSSION

Pseudomonas aeruginosa remains a difficult-to-treat pathogen. ICU patients with pneumonia caused by this pathogen frequently fail therapy and often have emergence of resistance during therapy (12, 20). In the last 20 years, we have begun to understand the principles of dose choice both to optimize clinical outcome and to suppress resistance emergence (3, 14, 21, 22).

The site of infection has a major impact on the adequacy of dose choice to accomplish the stated aims of optimal outcome and resistance suppression. Certain infection sites are "privileged," meaning that because of issues like tight junctions, seen, for example, in the central nervous system and the eye, drug penetration is decreased. Therefore, to attain an exposure profile that is adequate for therapy of the infection at privileged sites, larger systemic doses need to be given. For infections in these privileged sites, the burden of microorganisms is often not very large. However, for pneumonia, particularly in the ICU, multiple lobes may be involved, with a dense population of organisms, leading to a large organism burden not present in the mouse lung infection model. This will have a major impact on the exposure required to suppress resistance.

In this investigation, we explored the penetration of the fluoroquinolone levofloxacin into the ELF of neutropenic mice with pneumonia induced by the pathogen *Pseudomonas aeruginosa* PAO1. We developed a novel integrated analysis method in which all available data (plasma concentrations, ELF concentrations, and lung colony counts) from all mice were simultaneously analyzed in a population sense, employing a system of five inhomogeneous differential equations and 14 parameters.

The model fit the data well, as evidenced by the observed-predicted plots after the Bayesian step (Fig. 1). We were able to use the identified parameter values to calculate the penetration of levofloxacin into ELF, as indexed to the ratio of AUC_{ELF} to AUC_{plasma}. The penetration rate of 77.7% (Fig. 2) was quite good and in line with the penetration rate seen for human volunteers of 116% (9). It is important that such concordance between preclinical animal models and volunteer studies may be the exception rather than the rule. Certainly, macrolides and macrolide-like drugs show a differential penetration into ELF in murine models versus volunteer studies (18, 19). β-Lactam antibiotics have shown a discordance between preclinical models and volunteer data in the opposite direction (10, 16).

We were also able to employ the model parameters to cal-

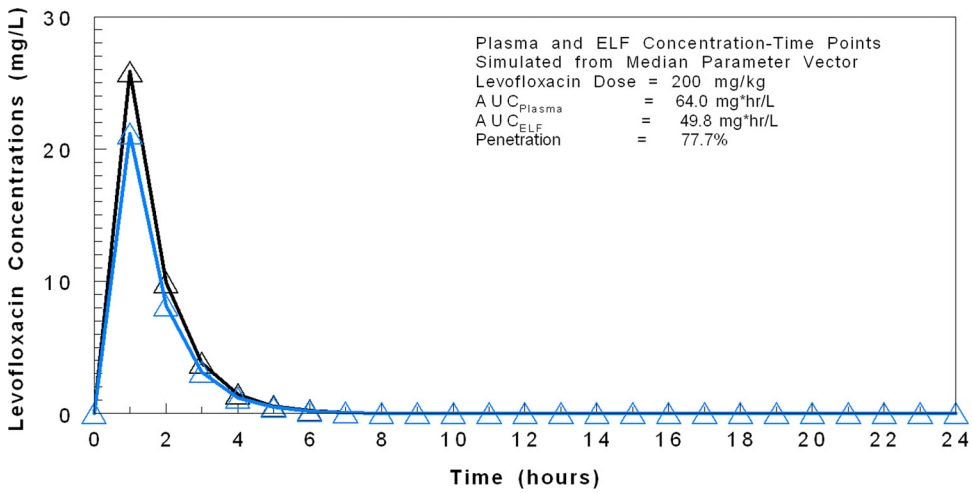

FIG. 2. Penetration of levofloxacin into murine ELF (blue) at a dose of 200 mg/kg.

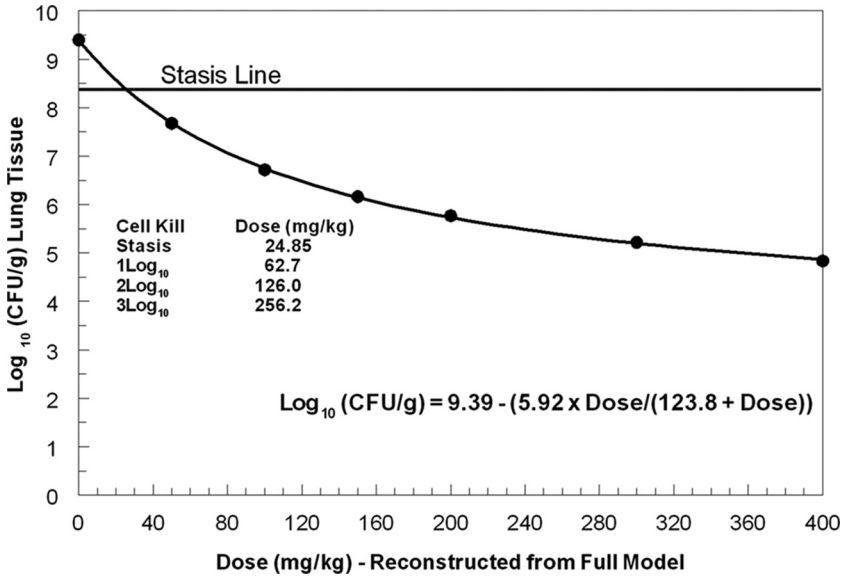

FIG. 3. Relationship between dose (mg/kg) and bacterial cell kill as reconstructed from the full model.

culate the AUC_{ELF}/MIC ratio required to kill *P. aeruginosa* PAO1 cells at the infection site. These exposures were quite modest, ranging from 12.6 to 127.6 for stasis through a 3-$\log_{10}$(CFU/g) kill, respectively. This leads to the question of why, with an initial burden exceeding 8 $\log_{10}$(CFU/g) of microorganisms (Table 1), the AUC/MIC ratio for near-maximal kill should be as low as 127.6. Part of the answer lies in the ability of the fluoroquinolone to penetrate to the infection site (Fig. 2). A more important aspect, however, resides in the mutational frequency to resistance. This is relatively infrequent for strain PAO1 for levofloxacin. At an initial inoculum of 2×10^8 CFU/g, there would be approximately 33 resistant organisms present at baseline/g. When we looked for resistance to this agent, we were able to obtain resistant organisms only on the levofloxacin-containing plates for the two lowest doses studied (AUC/MIC ratios of 32.0 for 50 mg/kg and 64 for 100 mg/kg), and even then, they were seen in only three of five animals at 50 mg/kg and one of five animals at 100 mg/kg. The resistance suppression AUC/MIC ratio can be said to be greater than 64 and no larger than 96 under this circumstance.

This finding was demonstrated previously (14). In a neutrophil-replete mouse thigh infection model, our laboratory was able to show that the AUC/MIC ratio required for different amounts of kill differed considerably, depending on the inoculum. In the instance where the organism burden was just beyond the mutational frequency to resistance at the time of therapy initiation, as was the case here, the AUC/MIC ratios required for 2- and 3-$\log_{10}$(CFU/g) kills were 26% and 20% of that needed when the initial burden was 10-fold higher. It is likely, then, that the targets seen here are applicable to circumstances where the infecting organism has a mutational frequency to resistance in the range identified here or where initial bacterial burdens are modest. Circumstances where infection involves hypermutator strains (23) or very large initial inocula, such as that seen in ventilator-associated pneumonia with multilobar involvement, would likely require larger exposures to drive the same cell kill and resistance suppression.

TABLE 2. Target attainment rates for ELF from humans after a 750-mg dose of levofloxacin[a]

MIC (mg/liter)	Target attainment (%)		
	1-Log_{10} (CFU/g) kill	2-Log_{10} (CFU/g) kill	3-Log_{10} (CFU/g) kill
0.5	99.5	97.9	92.8
1	97.9	93.0	81.4
2	93.0	81.8	63.4
4	82.0	63.6	41.7

[a] Judged by a 9,999-subject Monte Carlo simulation derived from volunteer data (9).

Using the target exposures for stasis and 1-, 2-, and 3-$\log_{10}$(CFU/g) kills, we used Monte Carlo simulation to determine how often such exposures were seen in volunteers for MICs between 0.5 mg/liter and 4 mg/liter. As can be seen, target attainments, even for these modest target exposures, fall below 90% at 4 mg/liter for a 1-$\log_{10}$(CFU/g) kill, at 1 mg/liter for a 2-$\log_{10}$(CFU/g) kill, and at 0.5 mg/liter for a 3-$\log_{10}$(CFU/g) kill. This may be interpreted as evidence that a fluoroquinolone should not be considered adequate therapy for patients with severe *Pseudomonas aeruginosa* pneumonia when used as monotherapy. Indeed, Fink and colleagues (12) previously demonstrated that ciprofloxacin at a dose of 400 mg intravenously every 8 h allowed resistance emergence during therapy for *Pseudomonas aeruginosa* in 12/36 instances (33%).

In summary, this agent penetrates well into murine ELF, to a degree slightly less than that seen in volunteer studies (9). The target exposures in ELF are modest to mediate multilog kill. Emergence of resistance was seen but was limited to the lowest doses and even then was infrequent. This is likely due to the mutational frequency to resistance seen with this strain. Bridging to humans indicated that even with the modest exposure targets, single-agent fluoroquinolone therapy would not be satisfactory for an unacceptably high percentage of patients. A hypermutator phenotype (23) and very large bacterial bur-

dens would likely lead to failure in a high proportion of cases. This investigation was done with a single isolate. More isolates of differing MICs and differing mutational frequencies to resistance need to be studied to solidify our understanding of the drug exposure targets required in ELF to drive multilog kill and suppress the emergence of resistance.

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