Bacterial Replication Rate Modulation in Combination with Antimicrobial Therapy: Turning the Microbe against Itself

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ABSTRACT A major clinical challenge for treating infectious diseases is the duration of antimicrobial therapy required to eradicate the pathogen. We hypothesized that modulation of the bacterial replication rate in the context of an antimicrobial exposure is coupled with the rate and extent of bactericidal effects. Herein we describe results from in vitro infection model (one compartment, 24-h model; hollow fiber, 10-day model) studies designed to probe the relationship between the bacterial replication rate and the rate and extent of bactericidal effects in the context of an effective antibiotic exposure. The bacterial replication rate was modulated by adjusting the sodium chloride concentration (0 to 8%) in the growth media (Mueller-Hinton II broth). The study drug selected was levofloxacin, and the challenge isolate was Staphylococcus aureus ATCC 29213 (levofloxacin MIC, 0.125 mg/liter). Within each in vitro infection model, human levofloxacin concentration-time profiles (half-life, 7 h) were simulated and the challenge isolate was subjected to an effective exposure (free-drug area under the concentration-time curve over 24 h divided by the MIC [AUC/MIC ratio], 65; administered as a single dose or daily for 10 days). Over the course of each study, samples were taken from each model for bacterial density determinations and drug concentration assay using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In the 24-h one-compartment in vitro infection model studies, as the bacterial replication rate increased, so too did the rate (slope, 0 to 4 h) and extent (24-h CFU count per milliliter) of bacterial killing. In the 10-day hollow-fiber infection model studies, the times until a reduction of bacterial density to 1 \times 10^2 CFU/ml occurred were 10 days in the media in which the challenge isolate grew slowly and approximately 2 days in the media in which the challenge isolate grew rapidly. Together, these data provide a proof of concept for new adjunctive therapeutic options with respect to the use of antimicrobial agents alone that reduce treatment durations. Such adjunctive therapies hold promise for marked reductions in the tonnage of antimicrobial agents administered to patient populations and selection pressure toward antimicrobial resistance.

KEYWORDS bacterial replication, pharmacokinetics-pharmacodynamics, therapy duration

We live in an era in which antimicrobial resistance threatens modern medical practice (1, 2). Without active antimicrobial agents, many more patients would die during routine and complex surgeries and cancer chemotherapy. Additionally, many more patients would die from infectious diseases now adequately treated, such as pneumonia and intra-abdominal, urinary tract, and skin and skin-structure infections.
Antimicrobial resistance has resulted from antimicrobial use and misuse over the past 80 years. Strategies to manage and prevent antimicrobial resistance include the following: local, national, and global surveillance; isolation of those patients with infection associated with dangerous pathogens; and the introduction of novel therapeutic approaches (3–6). Therapeutic options include not only the development of new antimicrobial agents and rapid diagnostics but also improving the use of existing therapies (antimicrobial stewardship) and developing new nonantimicrobial treatments. One way to improve antimicrobial use and reduce drug resistance selective pressure is through decreasing therapy duration (7–9). Given that the majority of antimicrobial agents require replicating bacteria to exert their bactericidal effects (10), increasing the bacterial replication rate in the context of an effective antimicrobial exposure represents an unexploited path to decrease therapy duration.

To explore the relationship between bacterial replication rate and duration of antimicrobial therapy, we utilized temperature-controlled in vitro infection models in which human antimicrobial agent concentration-time profiles can be accurately simulated and the impact of drug exposure on bacterial density can be readily measured (11, 12). More specifically, the overarching objectives of these studies were to demonstrate that the bacterial replication rate was coupled to the rate and extent of antimicrobial bactericidal effects and, subsequently, that therapy duration could be modulated by altering the bacterial replication rate.

RESULTS

In vitro susceptibility studies. The levofloxacin MIC for Staphylococcus aureus ATCC 29213 was 0.125 mg/liter.

Static flask studies. Figure 1 shows the impact of sodium chloride concentration on the change in bacterial density (starting inoculum [time zero], $1 \times 10^6$ CFU/ml) in vitro over 24 h for S. aureus ATCC 29213. Note that as the sodium chloride concentration increased, the bacterial replication rate decreased.

Drug assay. The relationship between observed and targeted levofloxacin concentrations from all treatment arms is shown in Fig. 2. For Mueller-Hinton broth medium alone (Fig. 2A), Mueller-Hinton broth medium supplemented with 8% NaCl, and
Mueller-Hinton broth medium supplemented with 10% NaCl (Fig. 2B and C, respectively), the \( R^2 \) values for these relationships were 0.98, 0.98, and 0.99, respectively; the line of best fit for each did not differ from the line of identity (i.e., no regions of bias, with good precision). These data indicate that the targeted levofloxacin concentration-time profile was well simulated in both the one-compartment and hollow-fiber \textit{in vitro} infection models.

**One-compartment infection model studies.** The impact of sodium chloride concentration in the context of levofloxacin exposure (free-drug area under the concentration-time curve over 24 h divided by the MIC [AUC/MIC ratio] = 65) on the change in bacterial density \textit{in vitro} over a 24-h study period relative to the no-treatment control arms is shown in Fig. 3. Note that as the sodium chloride concentration increased over the study period, the rate (slope, 0 to 4 h) and extent (24-hour CFU/ml) of cell kill decreased. Note also that bacterial killing occurred only during bacterial replication. For example, the bacteria in the 0% sodium chloride control arm grew immediately and there was an immediate reduction in the CFU counts per milliliter in the corresponding active treatment arm. In contrast, the growth was delayed for 4 h in the 10% sodium chloride control arm and there was a similar delay in the reduction in the CFU counts per milliliter in the corresponding active treatment arm.

**Hollow-fiber infection model studies.** Fig. 4 shows the impact of the sodium chloride concentration in the context of levofloxacin exposure (free-drug AUC/MIC ratio = 65) on the change in bacterial density \textit{in vitro} over the 10-day study period.
relative to the no-treatment control arms. For the levofloxacin regimen simulated in standard growth medium, 5 log$_{10}$ CFU/ml of bactericidal activity was observed on day 1, compared to the 2 log$_{10}$ CFU/ml of activity observed when 8% sodium chloride was added to the regimen. Note that in the media in which the challenge isolate grew slowly (8% sodium chloride), the time until a reduction of bacterial density to 1/1000 CFU/ml occurred was 10 days. Conversely, in the media in which the challenge isolate grew rapidly (0% sodium chloride), the time until a reduction of bacterial density to 1/1000 CFU/ml occurred was approximately 2 days.

**DISCUSSION**

The overarching objectives of these studies were to demonstrate that the bacterial replication rate was coupled to the rate and extent of antimicrobial bactericidal effects and, subsequently, that therapy duration could be modulated by altering the bacterial replication rate.

We successfully demonstrated in a 24-h one-compartment *in vitro* infection model that the rate and extent of levofloxacin bactericidal activity were related to the bacterial replication rate. Across the treatment arms, the extent of bacterial killing was greatest when bacteria were rapidly growing and drug concentrations were highest (Fig. 3). As the majority of antimicrobial agents require replicating bacteria to exert their bactericidal effects (10), we then hypothesized that increasing the bacterial replication rate in the context of an effective antimicrobial exposure would decrease the time to significant reductions in bacterial burden.

To this end, we utilized a hollow-fiber infection model in which the bacterial burden ($1 \times 10^8$) and duration of therapy (10 days) were selected to better mimic that expected clinically in high-density infections such as pneumonia. As in the one-compartment *in vitro* infection model studies, bacterial killing was greatest when the bacteria were rapidly growing (Fig. 4). In the media in which the challenge isolate grew slowly (8% sodium chloride), the time until a $>6$ log$_{10}$ CFU/ml reduction was 10 days, while that in the media in which the challenge isolate grew rapidly (0% sodium chloride) was approximately 2 days.

Together, these data support the notion that changing the organism’s physiological state through the addition of an agent promoting bacterial replication in the context of antimicrobial therapy would result in a faster and more complete reduction of bacterial burden. Given that stimulating bacterial replication increased antimicrobial bactericidal effects (10) and that rapidly growing bacteria stimulate an improved host immune response (13), an agent stimulating bacterial replication administered adjunc-
tively to an active antimicrobial therapy may represent a new path to decreasing therapy duration due to more-rapid bacterial killing.

However, other factors are also likely important determinants of therapy duration. One likely determinant of therapy duration is bacterial burden size. It is highly likely that there is a relationship between bacterial density and the time required to reduce the bacterial burden sufficiently for the host immune system to clear remaining cells. Another likely determinant of therapy duration is the presence of drug-resistant subpopulations in high-density infections. It is likely that combination antimicrobial therapy would be required in circumstances in which the drug-resistant bacterial subpopulation is not susceptible to the exposure provided by a single agent. This is often the case in deep-seated infections, such as pneumonia or tuberculosis, for which the interpatient differences in the extent of drug penetration are large (14).

Finally, we demonstrated that the bacterial replication rate was coupled to the rate and extent of antimicrobial bactericidal effects and that therapy duration could be modulated by altering the bacterial replication rate. We conclude that changing the organism’s physiological state through the addition of an agent promoting bacterial replication in the context of antimicrobial therapy would result in a faster and more complete reduction of bacterial burden. Our findings provide a proof of concept for new adjunctive therapeutic options with respect to antimicrobial agents that reduce treatment duration. Such adjunctive therapies hold promise for marked reductions in the tonnage of antimicrobial agents administered to patient populations and selection pressure toward antimicrobial resistance.

MATERIALS AND METHODS

Bacteria and study drug. The challenge isolate utilized in these studies was *Staphylococcus aureus* strain ATCC 29213. Levofloxacin was obtained from Toronto Research Chemicals (Toronto, Canada).

Media and in vitro susceptibility studies. Susceptibility studies were performed using a microdilution method in cation-adjusted Mueller-Hinton broth (BD Laboratories, Franklin Lakes, NJ) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (15) and interpretive criterion recommendations of the United States Committee on Antimicrobial Susceptibility Testing (USCAST) (16).

PK-PD studies conducted in triplicate over a 2-day period, and results are presented as modal values.

Static flask studies. The impact of sodium chloride concentrations (0, 4, 6, 8, and 10%) on the bacterial replication rate was initially evaluated in flasks. Each flask containing Mueller-Hinton broth (pH 7.2) was inoculated with $1 \times 10^6$ CFU/ml and placed into a water bath set to 37°C and 120 rotations per min. Samples were collected from each flask hourly for 10 h and at 24 h for CFU determination.

PK-PD in vitro infection models and sample processing. Two pharmacokinetics-pharmacodynamics (PK-PD) in vitro infection models, a one-compartment model and a hollow-fiber model, each previously described (11, 12), were used in these studies. All studies were conducted in duplicate.

One-compartment infection model studies. The one-compartment model is comprised of a central infection compartment containing Mueller-Hinton broth (pH 7.2) growth media, the challenge isolate, and magnetized stir bars used to ensure homogeneity of the drug, media, and challenge isolate. The central infection compartment sits on a stir plate within a temperature- and humidity-controlled incubator set to 35°C. A computer-controlled peristaltic pump is used to infuse drug-free growth media into the central infection compartment while concurrently moving the media through an exit port to a waste container. The challenge isolate is inoculated directly into the central infection compartment, and the test compound is infused using computer-controlled syringe pumps. The peristaltic diffusion rate is set such that the resultant concentration-time profile of the study drug mimics that observed in humans. Specimens for CFU determination and drug concentration assay are collected from the central infection compartment aseptically via a needle and syringe through a rubber septum at predetermined time points.

In these experiments, initial inocula of $1.0 \times 10^6$ CFU/ml of the challenge isolate were prepared from a culture grown overnight on Trypticase soy agar–5% lysed sheep blood agar plates (BD Laboratories). Isolates were taken from the overnight cultures and grown to mid-logarithmic phase in a flask of Mueller-Hinton broth set in a shaking water bath at 35°C and 125 rotations per min. The bacterial concentration within the flask of Mueller-Hinton broth was determined by measuring the optical density and making comparisons to a previously confirmed growth curve for the challenge isolate. In the active treatment arms, bacteria were then exposed to changing concentrations of levofloxacin, simulating a half-life of 7.0 h in humans and a free-drug AUC/MIC ratio of 65 in Mueller-Hinton broth and Mueller-Hinton broth supplemented with 8 or 10% sodium chloride. Control study arms included studies using Mueller-Hinton broth or Mueller-Hinton broth supplemented with 8 or 10% sodium chloride. One-milliliter specimens were collected for CFU determination at 0, 1, 2, 3, 4, 6, 8, 12, and 24 h. Samples were centrifuged, washed, and resuspended with sterile normal saline solution twice to prevent drug carryover and then cultured onto Trypticase soy agar enriched with 5% sheep blood. Plated samples were
incubated at 35°C for 24 h. One-milliliter specimens for drug assay were collected at 1, 3, 5, 7, and 24 h and then immediately frozen at −80°C until assayed for drug concentration.

**Hollow-fiber infection model studies.** The hollow-fiber model cartridge consisted of two compartments, a central compartment and a peripheral compartment. The peripheral compartment is separated from the central compartment by semipermeable fibers, each of which has pores that are large enough to allow nutrients, drugs, and bacterial metabolites to move freely into and out of the peripheral compartment but which are too small for bacteria to leave the peripheral compartment. Fresh Mueller-Hinton broth medium (pH 7.2) was pumped into and out of the central compartment using peristaltic pumps, while the study drug was infused into the central compartment using computer-controlled infusion pumps, with the diffusion rate set such that the resultant concentration-time profile of the study drug mimicked that observed in humans. Drug concentrations equilibrate rapidly from the central to peripheral compartments due to the high surface area-to-volume ratio. Specimens for quantitative culture and drug concentration assay were removed from the peripheral compartment through sampling ports.

In these experiments, initial inocula of 1.0 × 10^8 CFU/ml of the challenge isolate were prepared from a culture grown overnight on Trypticase soy agar–5% lysed sheep blood agar plates. Isolates were taken from the overnight cultures and grown to mid-logarithmic phase in a flask of Mueller-Hinton broth set in a shaking water bath at 35°C and 125 rotations per min. The bacterial concentration within the flask of Mueller-Hinton broth was determined by measuring the optical density and by comparison to a previously confirmed growth curve for each challenge isolate. In the active treatment arms, bacteria were exposed to changing concentrations of levofloxacin simulating a half-life of 7.0 h in humans and a free-drug AUC/MIC ratio of 65 in Mueller-Hinton broth or Mueller-Hinton broth supplemented with 8% sodium chloride. The no-treatment control arms were generated using Mueller-Hinton broth and Mueller-Hinton broth supplemented with 8% sodium chloride. One-milliliter specimens were collected for CFU determinations at 0 and 5 h and on days 1, 2, 3, 4, 6, and 10. Samples were centrifuged, washed, and resuspended twice with normal sterile saline solution to prevent drug carryover and were then cultured onto Trypticase soy agar enriched with 5% sheep blood. Plated samples were incubated at 35°C for 24 h. One-milliliter specimens for drug assay were collected at 1, 3, 5, 7, 23, 25, 47, and 49 h and then immediately frozen at −80°C until being assayed for drug concentration. Hollow-fiber cartridges from Fibercell Systems Inc. (Frederick, MD) with a 12-ml volume were used in all studies.

**Drug assay.** All samples were assayed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Waters, Milford, MA). The levofloxacin standard curve was linear over a concentration range of 0.1 to 10 mg/liter for all broth matrices. The interday coefficient of variation (CV) for quality control samples ranged from 1.5 to 9.5%. The lower limit of quantification was 0.1 mg/liter.

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REFERENCES


