Pharmacokinetic-Pharmacodynamic Modeling of the In Vitro Activities of Oxazolidinone Antimicrobial Agents against Methicillin-Resistant Staphylococcus aureus

Stephan Schmidt,1 Sreedharan Nair Sabarinath,1 April Barbour,1 Darren Abbanat,2 Prasarn Manitpisitkul,2 Sue Sha,2 and Hartmut Derendorf1*

Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, Florida,1 and Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Raritan, New Jersey2

Received 11 May 2009/Returned for modification 20 August 2009/Accepted 18 September 2009

Linezolid is the first FDA-approved oxazolidinone with activity against clinically important gram-positive pathogens, including methicillin (meticillin)-resistant Staphylococcus aureus (MRSA). RWJ-416457 is a new oxazolidinone with an antimicrobial spectrum similar to that of linezolid. The goal of the present study was to develop a general pharmacokinetic (PK)-pharmacodynamic (PD) model that allows the characterization and comparison of the in vitro activities of oxazolidinones, determined in time-kill curve experiments, against MRSA. The in vitro activities of RWJ-416457 and the first-in-class representative, linezolid, against MRSA OC2878 were determined in static and dynamic time-kill curve experiments over a wide range of concentrations: 0.125 to 8 μg/ml (MIC, 0.5 μg/ml) and 0.25 to 16 μg/ml (MIC, 1 μg/ml), respectively. After correction for drug degradation during the time-kill curve experiments, a two-subpopulation model was simultaneously fitted to all data in the NONMEM VI program. The robustness of the model and the precision of the parameter estimates were evaluated by internal model validation by nonparametric bootstrap analysis. A two-subpopulation model, consisting of a self-replicating, oxazolidinone-susceptible and a persistent, oxazolidinone-insusceptible pool of bacteria was appropriate for the characterization of the time-kill curve data. The PK-PD model identified was capable of accounting for saturation in growth, delays in the onsets of growth and drug-induced killing, as well as naturally occurring bacterial death. The simultaneous fit of the proposed indirect-response, maximum-effect model to the data resulted in concentrations that produced a half-maximum killing effect that were significantly (P < 0.05) lower for RWJ-416457 (0.41 μg/ml) than for linezolid (1.39 μg/ml). In combination with the appropriate PK data, the susceptibility-based two-subpopulation model identified may provide valuable guidance for the selection of oxazolidinone doses or dose regimens for use in clinical studies.

It is critically important to develop new potent antibiotics or antibiotic classes that may successfully treat these infections. In 2000, the FDA approved the use of linezolid, the first representative of a novel class of antibiotics, the oxazolidinones. At the time of approval, linezolid was one of the few agents that showed activity against vancomycin-resistant strains (2, 5). However, resistance to linezolid was reported as early as 2002 (10, 32). In addition, toxic side effects such as reversible thrombocytopenia, neutropenia, and, rarely, neuropathy have occurred during prolonged use (4, 13, 16, 28). Due to these limitations, there is a definite opportunity to develop new oxazolidinones with optimized exposure-response relationships. RWJ-416457 is a new investigational oxazolidinone that is being developed as both an oral and an intravenous formulation for the treatment of infections caused by clinically important gram-positive bacteria. RWJ-416457 has a MIC that is frequently two- to fourfold lower than that of linezolid against multidrug-resistant gram-positive pathogenic bacteria, including methicillin (meticillin)-resistant S. aureus (MRSA), vancomycin-intermediate susceptible S. aureus, vancomycin-resistant S. aureus, vancomycin-resistant enterococci, and penicillin-resistant streptococci (8, 17). Although the MIC is routinely determined in clinical settings and has contributed much to the understanding of antibiotic dosing, it does not provide any information on the time course of bacterial growth or antibiotic-induced killing (21, 27). More detailed information can be obtained from the evaluation of growth and kill profiles over time (time-kill curves). A major strength of the time-kill curve approach is its capability of simulating the effect of changing concentrations on the antimicrobial outcome. Changing concentration time-kill curves can, subsequently, be used to evaluate the efficacies of antibiotics with different half-lives (t1/2s). Once these experiments have been performed, a mathematical model can be simultaneously fitted to the data and the respec-

1 Corresponding author. Mailing address: Department of Pharmaceutics, College of Pharmacy, University of Florida, P.O. Box 100494, Gainesville, FL. Phone: (352) 273-7856. Fax: (352) 392-3249. E-mail: hartmut@ufl.edu.
2 Published ahead of print on 28 September 2009.
tive pharmacodynamic (PD) parameters can be calculated. These PD parameters can then be linked to in vivo pharmacokinetic (PK) information for prediction of the clinical outcome.

The aims of this study were (i) to establish a general mathematical model that is appropriate for characterizing the in vitro PDs of oxazolidinones determined in static as well as dynamic time-kill curve experiments and (ii) to apply this model in order to compare the in vitro potencies of the investigational oxazolidinone RWJ-416457 and the first-in-class representative linezolid.

MATERIALS AND METHODS
Antibiotics and growth media. RWJ-416457 was provided by Johnson & Johnson Pharmaceutical Research & Development L.L.C. (Raritan, NJ). Linezolid was purchased from the manufacturer. Both compounds were stored at 4°C in the original opaque vials. RWJ-416457 and linezolid stock solutions were freshly prepared daily prior to use, kept at room temperature, and diluted to the desired concentrations with Mueller-Hinton broth (MHB; Difco, Lawrence, KS).

Organisms. MRSA strain OC2878 was obtained from Johnson & Johnson Pharmaceutical Research & Development L.L.C. The bacterial inocula used for the MIC and time-kill curve experiments were prepared in sterile saline solution and adjusted with MHB to a final concentration of approximately 5 × 10^7 CFU/mL.

MIC determination. The MICs of RWJ-416457 and linezolid against MRSA OC2878 were determined in 24-well plates (Corning Inc., Corning, NY) by a modified broth macrodilution method (31). The procedure was repeated six times per bacterial strain and antibiotic. Positive controls (with bacteria, no drug) and negative controls (no bacteria, no drug) were run simultaneously in order to assess the method.

Static time-kill curves. An in vitro model was used to investigate the effect of constant RWJ-416457 and linezolid concentrations on MRSA OC2878. This in vitro model consisted of eight 50-ml cell culture flasks (Nunc; Nunc A/S, Roskilde, Denmark). The flasks were filled with 20 ml MRS OC2878-containing MHB (~5 × 10^7 CFU/mL) and incubated for 2 h before addition of the antibiotic. Selection of the RWJ-416457 and linezolid concentration ranges tested was based on their respective MICs and included concentrations with minimum antimicrobial activity (0.25 × MIC, 0.5 × MIC, 1 × MIC), efficient bacterial killing (2 × MIC, 4 × MIC), as well as a maximum kill effect (8 × MIC, 16 × MIC) (31). In addition, a growth control (no antibiotic) was also run simultaneously. The culture flasks were incubated, and the bacterial counts were subsequently determined at predefined time points for up to 24 h. Samples (20 μL) were taken directly out of the flasks, diluted in 10-fold increments, and plated onto 5% sheep blood agar plates (defined above) after a 20-h incubation period. Every 8 h, a sample from the flask containing the highest antibiotic concentration (16 × MIC) was obtained and analyzed (see “Drug stability” below). All changing-concentration time-kill curve experiments were run in triplicate.

Drug stability. To ensure the stability of RWJ-416457 and linezolid during the 24-h course of the experiment, every 8 h samples (500 μL) were taken directly out of the flask containing the highest antibiotic concentration (16 × MIC) and were immediately frozen at −80°C. The samples were analyzed by a validated reversed-phase high-performance liquid chromatographic method with a LiChroPrep 100 RP18 column (catalog no. 1.509443) and a linear gradient from 10% acetonitrile-methanol (4:1) to 90% acetonitrile-methanol (4:1) in 5 min ammonium acetate buffer (pH 4.5) over 7 min. RWJ-416457 and linezolid peak elutions were monitored at 270 nm and 254 nm, respectively, by using a diode array detector.

Mathematical modeling. A susceptibility-based two-compartment model (Fig. 1) was used to characterize the static as well as the dynamic time-kill curve data for both RWJ-416457 and linezolid. In this model, the overall change in the experimentally determined total number of bacteria (Ntot) was defined as the sum of bacteria susceptible to antibiotic (Ns) and susceptible persister cells (Np), as shown in equation 1.

\[ N_{\text{tot}} = N_s + N_p \] (1)

Bacteria from both susceptibility stages can transform into each other with the transformation-rate constants ksp (h⁻¹) and kds (h⁻¹), respectively, as shown in Fig. 1. The initial fraction of bacteria in the susceptible or persister stage was defined as F (where 0 < F < 1).

The change in the number of susceptible bacteria over time (dN/dt) could be sufficiently described by equation 2.

\[
\frac{dN_s}{dt} = k_s \times \left(1 - \frac{N_s}{N_{\text{max}}}ight) \times \left(1 - e^{-F \times k_{d}}\right) \times \frac{C}{EC_{50} + C} \times \left(1 - e^{-F \times k_{d}}\right) \times k_{d} \times N_s \times N_p
\]

(2)

where k_s (h⁻¹) characterizes the growth-rate constant, k_d (h⁻¹) is the natural-death-rate constant, N_{max} (CFU/ml) is the maximum number of bacteria, d_{sp} (h⁻¹) is the delay in the onset of growth, t (h) is time, k_{max} (h⁻¹) is the maximum kill-rate constant, C (μg/ml) is the antibiotic concentration, EC_{50} (μg/ml) is the concentration necessary to produce 50% of the maximum effect, and d_{sp} (h⁻¹) is the delay in the onset of kill. Due to the fewer number of data points for the dynamic time-kill curves compared to the number for the static time-kill curves within the first 2 h of the experiment, d_{sp} was fitted to the data for the static time-kill curves and then fixed when d_{sp} was estimated for the full data set.

In comparison, the change in the number of persister cells over time (dN/dt) could be described as a function of the number of susceptible cells entering the persister stage, as well as the number of persistent bacteria reentering the susceptible stage and the number of bacteria in the persister stage undergoing natural death (equation 3).

\[
\frac{dN_p}{dt} = k_p \times N_s - k_s \times N_p - k_d \times N_p
\]

(3)

In order to account for decreasing drug concentrations during the 24-h course of the static as well as the dynamic time-kill curve experiments due to degradation
and/or elimination, a first-order degradation and/or elimination rate constant was incorporated into the PK-PD model.

Data analysis. This susceptibility-based two-compartment model was simultaneously fitted to the log-transformed data of the static as well as the dynamic time-kill curve experiments by using a first-order conditional estimation method algorithm, as implemented in the NONMEM VI program (ADVAN6; Globomax, Hanover, MD). The between-experiment variability of the model parameters was estimated by using exponential error models. The residual variability, which includes the within-experimental variability and model misspecification, was estimated by using a log error model.

EC_{50}s were compared by using a two-sided, two-sample t test. A P value of <0.05 was considered statistically significant.

Model validation. Evaluation of the model performance included the preparation of standard diagnostic plots, calculation of the objective function value and the precision of the parameter estimates, as well as visual inspection of the data for the quality of fit. Decreases in the objective function value of 6.63 for forward inclusion (P < 0.01 for 1 degree of freedom) and of 10.8 for backward exclusion (P < 0.001 for 1 degree of freedom) were considered significant.

The robustness of the final model was assessed by using the Wings module of the NONMEM VI program by nonparametric bootstrap analysis. In the nonparametric bootstrap procedure, bacterial samples corresponding to one strain and concentration were sampled 1,000 times with replacement from the original data set in order to obtain a new data set containing the same number of samples. The final model was individually fitted to each of these new data sets, and all population model parameters were estimated. Results from the successful runs were determined, and the median bootstrapped parameter values (including a 95% bootstrap confidence interval [CI]) were compared to the final model-predicted parameter estimates (6).

RESULTS

MICs. By using a modified broth macrodilution method, twofold differences in the MICs (modes) against MRSA OC2878 were determined for RWJ-416457 (0.5 μg/ml) and linezolid (1.0 μg/ml).

Static time-kill curves. Constant-concentration time-kill profiles for both RWJ-416457 and linezolid are shown in Fig. 2A and Fig. 2C, respectively. After an initial lag phase of about 2 h, an ~2- to 2.5-log reduction in bacterial counts could be observed after 24 h of antibiotic exposure at concentrations greater than 8× MIC. The concentrations necessary to produce this maximum kill effect were approximately twofold lower for RWJ-416457 (4 μg/ml) than for linezolid (8 μg/ml).

Dynamic time-kill curves. Changing-concentration time-kill profiles (Fig. 2D) showed that after an initial ~2-log decrease in the numbers of CFU at 16× MIC, all concentrations of linezolid tested failed to prevent bacterial regrowth within 8 h of exposure. In contrast, the RWJ-416457 concentration of 16× MIC exhibited sufficient bacteriostatic activity after 24 h of exposure to prevent regrowth over that time period (Fig. 2B).

Drug stability. While linezolid was completely stable, approximately 10% of the RWJ-416457 degraded over the 24-h time course of the experiment (Fig. 3). Hence, the degradation-rate constant of RWJ-416457 was determined (by assuming first-order degradation kinetics) and incorporated into the mathematical model.

Mathematical modeling. The final PK-PD model was capable of describing the static time-kill curves (Fig. 2A) as well as the dynamic time-kill curves (Fig. 2B) of RWJ-416457 and linezolid (Fig. 2C and D) against MRSA OC2878 reasonably well. The corresponding model parameters estimates (±stand-
dard errors (SEs) are listed in Table 1. In the final model, $N_{max}$, $d_{s0}$, and $k_{sp}$ were estimated from the growth control data and were assumed to be constant. Furthermore, the value of $k_{sp}$ was fixed equal to 0 (22). Since the addition of a Hill factor did not significantly improve the overall fit, a maximum-effect model rather than a sigmoidal maximum-effect model was used. In addition, allowing for between-experiment variability ($\eta$) on $k_s$, $d_{s0}$, $d_{k}$, and $N_{max}$ by use of a log-error model did significantly improve the final model fit. 

**Model validation.** The mean final model-predicted parameter estimates (±SEs) and the results of the nonparametric bootstrap runs ($n = 1,000$) were in good agreement, as shown in Table 1. All parameter estimates from the final model lay within the 95% bootstrap CI. When the population predicted values were plotted against the data (Fig. 4A), the population predicted values as well as the individual predicted values (Fig. 4B) were uniformly and randomly distributed around the line of identity. In addition, no trend was observed when weighted residual versus individual model predicted (Fig. 4C) and weighted residuals versus time (Fig. 4D) were plotted. In combination with the results of the nonparametric bootstrap run, the diagnostic plots indicate that the model is robust and shows good predictability.

**DISCUSSION**

The selection of an appropriate dose and dosing regimen is a fundamental step for therapeutic success with any pharmacological agent (19). For antimicrobial agents, the selection of the best drug and dosing scheme for a specific pathogen not only increases the chances of cure while preventing toxic side effects but also decreases the probability that the infecting pathogen will become resistant to the antimicrobial agent (11, 30). With a good understanding of the dose-exposure relationship, or PKs, and the exposure-response relationship, or PDs, it may be possible to identify a quantitative link between the dose or dosing regimen on the one hand and the desired as well as the undesired drug effects on the other. For antibiotics, this link has been established by correlating the PK parameters that are based on free plasma or serum concentrations to the MIC of the respective pathogen. To date, three main MIC-based PK-PD indices have been identified for antimicrobials: the cumulative percentage of the dosing interval that the free drug concentration exceeds the MIC under steady-state conditions, the area under the free concentration-time curve at steady state divided by the MIC, and the free peak level divided by the MIC (20). However, the estimate of the MIC at a single point in time is not capable of characterizing the time course of either growth or antibiotic-induced kill or the effect of the antibiotic at concentrations besides the MIC (21, 22, 27). In addition, the methodology used to determine the actual MIC has not yet been internationally standardized and is a source of variability between different MIC determination methods (20). To overcome these limitations, other susceptibility breakpoints, such as the $EC_{50}$ have been suggested to be the PD input for PK-PD indices (25). The $EC_{50}$ can be obtained, together with parameters characterizing bacterial growth and maximum antibiotic-induced kill, from continuous measure-
ment of the antibiotic concentration-effect relationship over time (time-kill curves) (1, 22, 31). In general, there are two different types of time-kill curves, based on the concentration profile used in these in vitro models: static and dynamic time-kill curves (26). While constant-concentration models represent the steady-state concentrations obtained after constant-rate infusion, changing-concentration models try to simulate the change in antibiotic concentrations that occurs in vivo. During the changing-concentration experiments, the desired concentration-time profile can be generated either manually by using syringes or automatically by employing pump systems. However, it has been shown in previous experiments that the flow rate necessary to simulate a RWJ-416457 $t_{1/2}$ of approximately 24 h is so slow that bacteria can actually grow back into the broth reservoir and cause contamination. In this case, the use of the pump systems is not desirable, and consequently, syringes have been used.

Once the time-kill curve experiments have been performed, evaluation of the respective outcomes allows the activities of RWJ-416457 and linezolid against MRSA OC2878 to be compared over a wide range of concentrations. Qualitative analysis of the constant concentration time-kill curves revealed that the bacterial counts in these experiments were reduced by less than 3 log steps, indicating that both RWJ-416457 and linezolid are bacteriostatic rather than bactericidal antibiotics (23). It could be further shown that the linezolid concentrations (8 $\mu$g/ml) necessary to reach the maximum kill effect were approximately twofold higher than the RWJ-416457 concentrations necessary to achieve the same effect (4 $\mu$g/ml). These twofold differences in the concentration-effect relationship were consistent with the observed twofold differences in the MICs (linezolid MIC, 1.0 $\mu$g/ml; RWJ-416457 MIC, 0.5 $\mu$g/ml). In theory, the increased antimicrobial activity could be explained by a higher potency or a larger number of molecules at the effect site, yet the molecular weights of linezolid (337.35 g/mol) and RWJ-416457 (377.41 g/mol) are not substantially different. As a result, the increased activity of RWJ-416457 against MRSA OC28768 is explained by a higher potency rather than the number of molecules.

In order to evaluate the effects of decreasing concentrations (according to the physiological $t_{1/2}$) on the antimicrobial outcome, changing-concentration time-kill curve studies were performed. The findings indicated that initial RWJ-416457 concentrations of 4 $\mu$g/ml were sufficient to obtain bacteriostatic activity, whereas even fourfold higher initial linezolid concentrations (16 $\mu$g/ml) failed to prevent bacterial regrowth after 24 h of incubation. These findings imply that the increased potency and prolonged $t_{1/2}$ of RWJ-416457 compared to those of linezolid may support the use of a lower dose and/or increased dosing interval. In order to identify an appropriate dosing regimen for RWJ-416457, time-kill curve-based modeling and simulation approaches can be used. Although the description of time-kill curves is mathematically somewhat more complex, the PD parameters derived from this in vitro model.
can then be combined with in vivo PK data to simulate the antimicrobial efficacy of the respective dosing regimen(s). Once a general mathematical model is established, it can be applied to the time-kill curve data for other investigational oxazolidinones and the respective outcome parameters between drugs and/or dosing regimens can be compared. Modeling and simulation approaches are, consequently, very valuable for dose selection and have been recommended by the FDA as tools that may be used to streamline the drug development process (7). In fact, a model-based comparison of a new drug candidate and the approved first-in-class representative may allow the demonstration of superiority rather than noninferiority.

The model that was found to be appropriate for the simultaneous characterization of the static as well as the dynamic time-kill curve data for both RWJ-416457 and linezolid has structural similarities to previously described models (14, 22, 28). A persistence rather than a resistance model was chosen due to the fact that (i) the starting inoculum was less than the natural mutation rate (1 in $10^7$ to $10^9$) and (ii) there was no significant regrowth of the bacteria over the course of 24 h at constant concentrations of $\geq 2 \times$ MIC in the static time-kill curve experiment, whereas regrowth should have been observed in the case in which resistant mutants were present (29). However, bacterial regrowth in the dynamic time-kill curve experiments could be attributed to decreasing antibiotic concentrations.

In this susceptibility-based model, bacteria can exist in two different metabolic stages: an active, growing, antibiotic-susceptible stage and an insusceptible persister stage, as shown in Fig. 1. In addition, the model allows naturally occurring deaths, a saturation of growth, and a delay in the onset of antibiotic-induced killing to be accounted for. Although one would expect that a 2-h preincubation time would be sufficient for the bacteria to reach the logarithmic growth phase, a delay in the onset of growth was observed (Fig. 2). Accounting for this delay in the onset of growth significantly improved the model fit. When it was simultaneously fitted to the data, the final model was capable of describing the experimentally determined time-kill curve data reasonably well (Fig. 2). The overall model fit could be improved by incorporating drug degradation during the 24-h course of the experiment. At this point, it should be mentioned that the model fit could be slightly improved when the model was fitted to the individual set of data for the static and dynamic time-kill curves for RWJ-416457 and linezolid with separate sets of parameters. However, due to nonsignificant differences in the parameter estimates, the model was fitted with one set of parameters (except the EC$_{50}$), which allowed inferences about the potencies of RWJ-416457 and linezolid to be made.

The final model was internally validated by nonparametric bootstrapping and showed good robustness and predictability (Fig. 3). Comparison of the EC$_{50}$ obtained revealed that RWJ-416457 (EC$_{50}$, 0.41 mg/mL) is approximately 3.4-fold more potent than the first-in-class representative, linezolid (EC$_{50}$, 1.39 mg/mL). The parameter estimates obtained from this in vitro model can then be used in combination with PK data to predict the clinical outcome and provide guidance for the selection of the appropriate doses or dosing regimens.

In conclusion, a general PK-PD model that is appropriate for characterization of the in vitro time-kill curve data for oxazolidinone antibiotics has been developed. The simultaneous fit of the developed model to static as well as dynamic time-kill curve data revealed that RWJ-416457 has a 3.4-fold increased in vitro potency compared to the potency of the first-in-class representative, linezolid. Combined with appropriate PK data, this model may provide valuable guidance for the selection of doses or dosing regimens for new, investigational oxazolidinones.

ACKNOWLEDGMENTS

We thank Johnson & Johnson Pharmaceutical Research & Development L.L.C. for financial support of this study. We also thank Oliver Ghoibrial (Department of Pharmacueticals, College of Pharmacy, University of Florida, Gainesville) for his support with the time-kill curve experiments and Navin Goyal (Department of Pharmacueticals, College of Pharmacy, University of Florida, Gainesville) for his technical support with the NONMEM program as well as the bootstrap analysis.

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