Pharmacokinetics and Efficacy of Linezolid in a Gerbil Model of *Streptococcus pneumoniae*-Induced Acute Otitis Media

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Received 28 June 2002/Returned for modification 22 October 2002/Accepted 13 January 2003

The oxazolidinone linezolid represents a new antibacterial class of potential benefit in managing multidrug-resistant gram-positive infections, including those caused by *Streptococcus pneumoniae*. In a gerbil model of acute otitis media (AOM) induced by either penicillin-resistant *S. pneumoniae* (PRSP; amoxicillin MIC = 8 µg/ml, linezolid MIC = 1 µg/ml) or penicillin-susceptible *S. pneumoniae* (PSSP; amoxicillin MIC = 0.015 µg/ml, linezolid MIC = 1 µg/ml), we explored the plasma and ear fluid levels of linezolid required to demonstrate efficacy. Threshold pathogen doses required to induce bilateral AOM (1,500 CFU/ear with PRSP; 30 CFU/ear with PSSP) were administered to gerbils by intrabullar injection on day 0. At peak infection (~10⁶ to 10⁷ CFU/ear flush; day 2 for PRSP-AOM and day 3 for PSSP-AOM), twice-a-day oral doses of linezolid, amoxicillin, or vehicle were administered over 4.5 days prior to collection and assay of middle ear effluents for *S. pneumoniae* content. Linezolid doses of ≥10 mg/kg of body weight induced significant cure rates of ≥72% versus both PRSP and PSSP infections, whereas amoxicillin at ≤100 mg/kg was consistently effective only versus PSSP-AOM. Plasma and ear fluid levels of linezolid necessary to elicit pneumococcal eradication from the middle ear were measured by high-performance liquid chromatography-tandem mass spectrometry and found to be similar both within and between each infection protocol. The plasma-ear fluid pharmacodynamic profile associated with linezolid efficacy was a $T > \text{MIC}$ of ≥42%, a $C_{\text{max}}/\text{MIC}$ ratio of ≥3.1, and a (24-h area under the curve)/MIC ratio of ≥30 h. Application of this model will be useful in defining preclinical pharmacodynamic relationships of novel antibiotics necessary to cure *S. pneumoniae*-induced AOM.

Streptococcus pneumoniae is an obligate parasite in humans that often becomes the causative organism of upper respiratory tract infections, including pneumonia, sinusitis, and acute otitis media (AOM) (2, 3). Of particular concern has been the increase in the frequency of antibiotic-resistant isolates of this ubiquitous pathogen. Since the late 1980s, a steady rise in the incidence of beta-lactam- and macrolide-azalide-resistant pneumococcal strains has been well documented in epidemiological surveillance studies (13, 21, 22). Currently, 40 to 50% of *S. pneumoniae* clinical isolates have demonstrated reduced susceptibility to penicillin while exhibiting increased cross-resistance to other antibiotic classes, such as macrolides, trimethoprim-sulfamethoxazole, and tetracyclines (13, 21). In terms of treating AOM, this resistance development has been associated with clinical failures of drug therapy (10, 11, 29).

AOM is diagnosed over 25 million times annually in the United States, and in approximately 40% of cases the infection is caused by *S. pneumoniae* (4). One approach to overcoming the difficulties inherent in managing AOM induced by multi-drug-resistant *S. pneumoniae* is the discovery of new antibiotics with improved antipneumococcal activity (23, 29). The oxazolidinone linezolid (Zyvox) represents a novel class of antibiotic with proven efficacy in treating drug-resistant, gram-positive infections in both preclinical models (12, 15) and human subjects (30, 33). Linezolid acts against such pathogens by inhib-

iting bacterial protein synthesis via prevention of the formation of the initiation complex (24, 34). With a MIC for 90% of strains tested for *S. pneumoniae* of 1.0 µg/ml (25, 32, 38), linezolid has been evaluated for efficacy in treating AOM in both the experimental and clinical settings. In chinchillas (27), an oral twice-a-day (b.i.d.) linezolid dose of 25 mg/kg of body weight effected cures in 100% of middle ear infections caused by penicillin-resistant *S. pneumoniae* (PRSP). In pediatric patients, a twice-daily oral linezolid dose of 10 mg/kg was successful in curing 84% of AOM cases known to be caused by *S. pneumoniae* (D. L. Fleishaker, D. C. Anderson, J. B. Bruss, W. H. Chang, W. M. Todd, and B. Hafkin, abstract, Clin. Infect. Dis. 312:224, 2000).

As new oxazolidinones become available, a thorough understanding of the pharmacodynamic relationships necessary for effecting cures of *S. pneumoniae* infections becomes critically important in setting appropriate susceptibility breakpoints and optimizing applied dosing regimens (8, 17). In this study, we investigated the pharmacokinetic and pharmacodynamic relationships of linezolid necessary to effect cures in a newly characterized gerbil model of AOM, in which infections were induced with either a penicillin-susceptible or penicillin-resistant strain of *S. pneumoniae*.

(This paper was presented in part at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 16 to 19 December 2001.)

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MATERIALS AND METHODS

Animal considerations. Female Mongolian gerbils (35 to 50 g; Charles River Laboratories, Kingston, N.C.) were used in this study. All animal procedures

were performed in compliance with the Animal Welfare Act regulations, 9 CFR Parts 1, 2, and 3 and with the 1996 *Guide for the Care and Use of Laboratory Animals*, issued by the ILAR Commission of Life Sciences, National Academy Press (Washington, D.C.), as well as with all internal policies set forth by Pharmacia Corporation.

Infecting organisms, inoculum preparation, and MIC determinations. *S. pneumoniae* strains UC15087 (clinical isolate; serotype 19F) and ATCC 6305 (serotype 5 strain; American Type Culture Collection, Rockville, Md.) were chosen for use in this study based on prior susceptibility and virulence profiling. Strain UC15087 was designated as PRSP, as it has been shown to be resistant to penicillin and cephalosporin in prior testing (14), while strain ATCC 6305 was designated as penicillin-susceptible *S. pneumoniae* (PSSP), as it is known to be a penicillin-susceptible strain of *S. pneumoniae* (37). Cultures of each strain were grown overnight at 35°C on blood agar plates (brain heart infusion agar [BHI agar]; Difco Laboratories, Sparks, Md.) supplemented with 5% (vol/vol) digested sheep's blood (Becton Dickinson Microbiology Systems, Sparks, Md.). Aliquots of these cultures, suspended in BHI broth (Difco Laboratories) containing a 20% (vol/vol) glycerol suspension, were maintained in the vapor phase of a liquid nitrogen freezer until needed for testing. PSSP and PRSP inocula suspensions used for in vivo testing were prepared by diluting thawed aliquots directly with BHI broth supplemented with 5% (vol/vol) horse serum (Gibco BRL, Paisley, United Kingdom). The MIC susceptibility profiles for these strains to linezolid and other selected antibiotics were determined using a broth microdilution assay described previously (38) that followed guidelines set forth by the National Committee for Clinical Laboratory Standards (26).

Induction and measurement of AOM. The techniques used to induce bilateral *S. pneumoniae*-induced AOM in gerbils were similar to those described previously by Barry and colleagues (3) with some modifications. Animals anesthetized with Ketamine (40 mg/kg intramuscular) and Xylazine (13 mg/kg intramuscular) received intrabullar injections of bacteria in both ears by using 28-gauge insulin syringes. The inoculum volume was standardized at 30 µl/ear. Inoculations were deemed successful if the investigator heard a popping sound, indicative of a sudden increase in middle ear air pressure. With this technique, >99% of inoculated ears had intact tympanic membranes upon visual inspection with an operating microscope. Gerbils were allowed to recover in a dorsal recumbent position after the inoculation procedure and monitored for up to 14 days postinfection. Middle ear infection development was monitored periodically as follows. Gerbils were reanesthetized as above and euthanized by cervical dislocation, and the external ears were removed for visual inspection of the tympanic membranes. Middle ear *S. pneumoniae* levels were then monitored by microbiological assay. Each middle ear chamber was flushed with 30 µl of BHI broth that was delivered and immediately withdrawn via direct tympanic membrane puncture with a 26-gauge needle. In pharmacokinetic experiments (described below) and in early model development, direct removal of middle ear fluid from each infected bullae revealed pooled volumes ranging from immeasurable to 30 µl. The subsequent injection and withdrawal of BHI flush most likely represented a ≥ 2 -fold dilution of ear fluid prior to its contact with the needle dead-space volume of BHI (approximately 40 µl). Final effluent volumes assayed for bacterial content usually approximated 60 µl/ear. Hence, with this flushing technique, it was estimated that collected ear flush samples represented a ≥ 6 -fold dilution of constitutive ear fluid. Once collected, the samples were serially diluted with BHI broth, and 20-µl aliquots of each dilution were subsequently plated on blood agar. Plates were incubated at 35°C for 18 to 20 h prior to CFU determination. Bacterial counts for each ear were reported as the CFU per ear flush. The lowest detectable bacterial count was 1 CFU/20-µl drop of each ear flush sample collected (ca. 3 CFU/ear flush). Ears were considered uninfected or cured if *S. pneumoniae* counts in their respective effluents were below this detection limit.

AOM model development and in vivo antibiotic efficacy studies. Virulence testing of PRSP and PSSP were performed by inoculating gerbils with intrabullar doses ranging from 3 to 1.5×10^6 CFU/ear and evaluating the frequency and intensity at which AOM was established 48 h later. For each strain, the lowest inoculum inducing AOM in 100% of treated ears was then used to define the time course of infection and applied during in vivo testing of antibiotics. Gerbils receiving this inoculum were examined between 6 h and 14 days later to monitor progression or regression of the established infection in terms of middle ear bacterial content and tympanic membrane appearance (subjectively determined if opaque or clear). For each specific AOM model, the postinfection period where middle ear bacterial levels peaked was determined and used to establish a standardized, multiday dosing interval in subsequent antibiotic efficacy trials.

Antibiotic efficacy testing was performed in each AOM model using a nine-dose, oral b.i.d. treatment regimen (every 12 h for 4.5 days) given during the time of peak infection. For dose-response testing, middle ear chambers were evaluated for *S. pneumoniae* content 4 h after the last dose of drug or corresponding

vehicle. Dose-response curves to linezolid and amoxicillin were generated versus both PRSP-induced and PSSP-induced AOM. The dose levels necessary to cure 50% of infected ears (50% effective dose [ED₅₀]) were calculated from these curves using the Spearman-Kärber test, while the dose regimens curing 100% of infected ears (ED₁₀₀) were also noted. In addition, time courses of antibacterial efficacy associated with therapeutic linezolid regimens were evaluated in both infections. To this end, middle ear *S. pneumoniae* levels in vehicle- and linezolid-treated gerbils were measured at three time points: 4 h after the fifth b.i.d. dose (2.5 days into the treatment protocol), 4 h after the ninth and final dose (day 4.5 of treatment), and 4 days after cessation of therapy. No drug inactivation or removal procedures were applied during analysis of the collected ear effluents, as it was determined that the dilution effect inherent with the collection process (noted above) would sufficiently minimize any antibiotic carryover effects. Reverse-dilution effects were not observed in any of the antibiotic efficacy trials.

During the early stages of AOM model development, we evaluated the presence of pneumococci in host brains in some experiments to monitor the frequency by which the infections became disseminated. Whole brains were aseptically removed from euthanized animals and homogenized in 3 ml of BHI broth. The homogenates were analyzed for the presence of *S. pneumoniae* by using the same drop-plate method noted above (detection limit, ≈ 100 CFU/organ).

Pharmacokinetic studies. Gerbils given bilateral PRSP-AOM or PSSP-AOM were placed on either partially effective or maximally effective antibacterial dosing regimens of linezolid and amoxicillin. Total linezolid levels (protein bound plus unbound or free) in plasma and ear fluid were evaluated simultaneously in samples collected before and up to 12 h after the third scheduled b.i.d. dose, before and 1 h after dose 5, and prior to the last dose (dose 9). Total amoxicillin levels in plasma samples were evaluated using the same experimental time course. Concentrations of free drug were not determined. In ultrafiltration studies, the level of protein binding of [¹⁴C]linezolid was demonstrated to be relatively low and consistent across species (24 to 32%) and not concentration dependent (31). This finding was extended to gerbils, where equilibrium dialysis experiments performed over a concentration range of 5 to 50 µg/ml revealed a linezolid plasma protein binding level of $28\% \pm 4\%$ ($n = 6$ samples). Similarly, amoxicillin has been shown to have relatively low protein binding across species (19, 37). Thus, we considered the impact of not correcting for protein binding to be minimal in this study. At each scheduled time point, three gerbils were anesthetized with 3% isoflurane-oxygen, bled via cardiac puncture, and euthanized by cervical dislocation. The blood samples, which were anticoagulated with EDTA (EDTA Microtainer tubes; Becton Dickinson and Co., Franklin Lakes, N.J.), were immediately centrifuged at $18,000 \times g$ for 4 min at 4°C to prepare plasma for analysis. An attempt was then made to obtain ear fluid directly from each infected ear ($n \leq 6$ ears/time point) by insertion of a 28-gauge insulin syringe through the tympanic membrane. All ear fluids thus collected were centrifuged as noted above to remove cellular content. The plasma and ear fluid samples were stored at -20°C until assayed for drug content.

Amoxicillin and linezolid levels in biological fluids were measured via high-performance liquid chromatography-tandem mass spectroscopy (HPLC-MS/MS) analysis using a Perkin-Elmer SCIEX API 3000 quadrupole mass spectrometer and a Hewlett-Packard 1100 HPLC apparatus as the solvent delivery injection system. Turbo Spray was used as the ion source for performance of amoxicillin MS, the data from which were acquired in the multiple reaction monitoring (MRM) scan mode with a dwell time of 250 ms, a scan speed of 0.51 s, and a pause time of 5.0 ms. The MRM ion pair for amoxicillin was 366 (Q1) and 349 (Q3). HPLC-MS/MS analysis of linezolid in plasma and ear fluid samples was performed as described previously (12). HPLC mobile-phase solutions were prepared according to standard practices. As required, standard antibiotic solutions used for these analyses were prepared separately in pooled plasma (amoxicillin and linezolid) and in pooled ear fluid (linezolid). The lower limits of quantitation for amoxicillin and linezolid were 0.013 and 0.001 µg/ml, respectively.

Pharmacokinetic and pharmacodynamic calculations. Pharmacokinetic calculations of the plasma and ear fluid concentration-time curve data collected following the third b.i.d. dose of linezolid were performed using the software package WinNonLin Professional (Scientific Consulting Inc., Apex, N.C.). Calculated parameters included peak drug level ($C_{\max}$), time to $C_{\max}$ ($T_{\max}$), apparent terminal disposition half-life ($t_{1/2}$), and the area under the concentration-time curve from 0 to 12 h (AUC_{0-12}). Pharmacodynamic relationships such as percentage of time drug concentrations met or exceeded the MIC threshold ($T > \text{MIC}$), the $C_{\max}/\text{MIC}$ ratio, and the $\text{AUC}_{0-24}/\text{MIC}$ ratio [$(\text{AUC}_{0-12} \times 2)/\text{MIC}$] were determined by directly relating the pharmacokinetic data to the MIC of linezolid towards each infecting *S. pneumoniae* strain.

Antibacterial agents and dosing preparation. Linezolid was synthesized in our laboratories (Pharmacia Corp., Kalamazoo, Mich.). Amoxicillin, penicillin G,

TABLE 1. In vitro antibiotic susceptibility profiles of infecting *S. pneumoniae* strains used to develop gerbil models of AOM

Strain ^a	Test agent	MIC ($\mu\text{g/ml}$) ^b
PRSP (serotype 19F)	Penicillin G	4
	Amoxicillin	8
	Cefotaxime	8
	Vancomycin	0.5
	Linezolid	1
PSSP (serotype 5)	Penicillin G	0.015
	Amoxicillin	0.015
	Cefotaxime	0.015
	Vancomycin	0.5
	Linezolid	1

^a PRSP, penicillin-resistant *S. pneumoniae* strain UC15087 (15); PSSP, penicillin-susceptible *S. pneumoniae* strain ATCC 6305 (38).

^b Values represent modes of MIC assays performed in triplicate.

and vancomycin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Cefotaxime (sodium salt of acetate ester) was obtained from Calbiochem (La Jolla, Calif.).

For in vivo studies, stock suspensions of linezolid were formulated at 10 mg/ml in an Avicel vehicle containing 1% Avicel RC-591 (FMC Corp., Philadelphia, Pa.), 5% polysorbate 80, and 50 mM acetate buffer (pH 4.5). Stock suspensions of amoxicillin were prepared using 0.1 M NaH_2PO_4 –0.1 M NaHPO_4 phosphate buffer (pH 6) as vehicle. For each experiment performed, a mean body weight was determined from 12 to 18 gerbils randomly selected at the time of pathogen inoculation. Drug concentrations in the final dosing suspensions were adjusted by vehicle dilution to provide a final dosing volume of 0.2 ml, which was given by oral gavage. After preparation, drug and vehicle suspensions were used throughout each protocol and kept at 4°C when not in use.

Data presentation and statistical analysis. All data represent means $\pm$ standard deviations of means unless otherwise noted. Treatment group geometric means of middle ear bacterial levels included those ear flush samples that were culture negative. In such instances, a value of 3 CFU/ear flush (detection limit of assay) was given to each sample. Differences between drug- and time-matched vehicle groups in terms of AOM frequency and intensity were analyzed using Fisher's exact test and the Kruskal-Wallis one-way analysis of variance with post hoc application of a pair-wise Dunnett's test, respectively. Group differences were considered statistically significant at a *P* level of <0.05 .

RESULTS

In vitro susceptibility tests. The in vitro antibiotic susceptibility profiles of the two infecting strains of *S. pneumoniae* are summarized in Table 1. The divergence in MICs of the beta-lactams versus PRSP and PSSP was consistent with the penicillin-resistant and penicillin-susceptible designations of these strains, with PRSP demonstrating a high level of amoxicillin resistance (amoxicillin MIC = 8 $\mu\text{g/ml}$). These tests confirmed the lack of cross-resistance to linezolid, which had an MIC of 1 $\mu\text{g/ml}$ for both pneumococcal isolates.

AOM model development. In initial dose-response testing, the threshold inocula required by the differing serotype strains to induce a reproducible AOM response—an opaque tympanic membrane with confirmed middle ear presence of pneumococci in 100% of ears ($n = 8$ to 10 ears/dose) within 2 days of infection—were 1,500 and 30 CFU/ear for PRSP (serotype 19F) and PSSP (serotype 5), respectively. These inoculum levels became the standard doses used in all subsequent studies. Differential leukocyte counts performed on fixed samples of ear effluent collected on or after day 5 postinfection revealed a high percentage of neutrophils ($>80\%$) intermingled among ubiquitous amounts of cellular debris indicative of an active inflammatory response.

The infection time courses of AOM induced by PRSP (serotype 19F) and PSSP (serotype 5) are illustrated in Fig. 1. Middle ear *S. pneumoniae* levels rose ≥ 2 logs/ear flush within 48 h of infection in both models, peaking to approximately 10^6 to 10^7 CFU/ear flush between days 2 and 5 of PRSP infection (Fig. 1A) and between days 3 and 7 of PSSP infection (Fig. 1B). Evidence of resolution of middle ear infections appeared between days 10 to 14 in both models, but more so in PRSP-infected animals (Fig. 1). From these data, we chose to administer 4.5-day antibiotic regimens between days 2 and 6 of PRSP infection and between days 3 and 7 of PSSP infection. This

FIG. 1. Infection time courses of AOM in gerbils following bilateral intratubular inoculation of either a PRSP strain (serotype 19F) (A) or a PSSP strain (serotype 5) (B). Inoculum doses delivered were 1,500 and 30 CFU/ear for the PRSP and PSSP strains, respectively. Pneumococcus levels in terminal ear flush effluents (solid circles) were measured periodically postinfection by microbiological assay. Ear effluents that were culture negative (open circles) were given a value of 3 CFU/ear flush (assay detection limit [D.L.]) for determination of the geometric mean $\pm$ standard deviation of CFU data for each time point (open bars).

FIG. 2. Antibacterial effects of amoxicillin (A) and linezolid (B) as well as the antibacterial time course of selected oxazolidinone treatments (C) in a gerbil model of AOM induced by PRSP. Two days following bilateral intrabullar inoculation of PRSP (1,500 CFU/ear), subgroups of gerbils were given either no treatment, 4.5-day oral antibiotic therapy, or corresponding antibiotic vehicle. (A and B) In the standard dose-response protocol, middle ear *S. pneumoniae* levels in terminal ear flush samples (solid circles) were measured by microbiological assay on day 6 postinfection, 4 h after the last drug dose. (C) In the time course experiment, middle ear bacterial levels (solid circles) were determined on the third day of linezolid therapy (day 4 postinfection) as well as at the end of the drug treatment period (day 6 postinfection). Open bars and open circles represent group geometric mean CFU data and culture-negative ear flush samples, respectively. The *S. pneumoniae* detection limit (D.L.) was 3 CFU/ear flush. *, $P < 0.05$ versus the respective vehicle group.

slight shift in treatment schedule was applied to more closely match the plateau phase of each respective infection time course.

The divergent serotypes of the two *S. pneumoniae* strains were accompanied by divergent pathogenicities of the two *S. pneumoniae* strains following intrabullar inoculation. During AOM induced by PSSP (serotype 5), the mortality rate of untreated animals was approximately 50% (9 of 17), with the majority of deaths occurring between days 2 and 6 of infection. Of those animals surviving the protocol, approximately 50% (4 of 8) showed signs of systemic infection (ruffled fur, reduced body weight) which was confirmed by the detection of pneumococci in the brain. In contrast, no infection-related deaths were observed after AOM induction with PRSP (serotype 19F), and only 6 of 28 infected animals (21%) sacrificed between days 1 and 14 had detectable brain levels of *S. pneumoniae*.

Antibiotic efficacy testing with PRSP-AOM. Figure 2 sum-

marizes the antibacterial effects of amoxicillin and linezolid in gerbils with PRSP-AOM. Pushing the amoxicillin b.i.d. dose to relatively high levels (≥ 60 mg/kg) was partially successful at curing the middle ear infections ($\text{ED}_{50} = 54$ mg/kg). However, even at the highest dose tested (100 mg/kg), an inconsistent antibacterial response was still observed in the gerbil middle ear (Fig. 2A). In comparison, consistent antibacterial effects were observed with linezolid at a threshold oral dose of 10 mg/kg, which lowered mean middle ear *S. pneumoniae* levels by over 4 logs/ear flush ($P < 0.05$) and restored 50% (5 of 10) of infected ears to a culture-negative status. At 30 mg/kg, linezolid restored 100% of infected ears to a culture-negative status (Fig. 2B). Clearing of the tympanic membrane was observed upon visual inspection of ears that subsequently expressed microbiological cures. Figure 2C summarizes the antimicrobial time course of therapeutic linezolid regimens versus PRSP-AOM. In this follow-up trial, a 100% cure rate was observed with both 10-mg/kg and 30-mg/kg linezolid doses as early as 2.5 days into therapy. Separation of efficacy of these two regimens did not occur until a "test-of-cures" analysis was performed 4 days after completion of treatment. At that time, gerbils given 10 mg of linezolid/kg had an ear infection rate of 60% (6 of 10, versus 80% in vehicle group; $P > 0.05$), whereas those animals receiving 30 mg/kg had an infection rate of only 10% (1 of 10; $P < 0.05$ versus vehicle group). *S. pneumoniae*

FIG. 3. Antibacterial effects of amoxicillin (A) and linezolid (B) in a gerbil model of AOM induced by PSSP. Infected animals received either antibiotic or corresponding vehicle for 4.5 days beginning 3 days after bilateral intrabullar inoculations of PSSP (30 CFU/ear). In the standard dose-response model, middle ear *S. pneumoniae* levels (solid circles) were measured on day 7 postinfection, 4 h after the last drug dose. In evaluating the time course of linezolid efficacy (B), middle ear bacterial levels in terminal ear flush samples were also determined on day 5 postinfection, 4 h after the fifth of nine scheduled drug doses. Open bars and open circles represent group geometric mean CFU data and culture-negative ear flush samples, respectively. The *S. pneumoniae* detection limit (D.L.) was 3 CFU/ear flush. *, $P < 0.05$ versus the respective vehicle group.

colonies collected from those ears expressing infection in the test-of-cure period were reassayed for linezolid susceptibility. In each case, there was no evidence of development of resistance to the oxazolidinone.

Antibiotic efficacy testing with PSSP-AOM. The antibacterial effects of amoxicillin and linezolid in gerbils with PSSP-AOM are summarized in Fig. 3. As predicted from the *in vitro* susceptibility profile of PSSP, the beta-lactam effectively cured the middle ear infections. The oral b.i.d. ED_{50} and ED_{100} of

amoxicillin in this protocol were 0.2 and 0.3 mg/kg, respectively (Fig. 3A). In a separate trial, the dose response and antimicrobial time course of linezolid were examined in the same experiment (Fig. 3B). Significant antibacterial effects were observed within 2.5 days at linezolid doses of ≥ 10 mg/kg (>5 -log reduction in CFU/ear flush compared to the vehicle group; $P < 0.05$), with a 100% cure rate being achieved by the 30-mg/kg b.i.d. regimen. A test-of-cures analysis performed 4 days after completion of linezolid therapy revealed cure rates consistent with those observed within 4 h of treatment cessation (seven of eight ears cured in the 10-mg/kg group, eight of eight ears cured in the 30-mg/kg group; $P < 0.05$ versus vehicle group infection rate of 86%). In all cases, the microbiological cures achieved with these antibiotic regimens were again accompanied by tympanic membrane clearing.

The mortality rates of vehicle-treated animals with PSSP-AOM during the 4.5-day dosing period ranged between 33% (4 of 12 in the linezolid experiment) and 40% (6 of 15 in the amoxicillin experiment). These rates were significantly lower in animals placed on antibiotic therapy. Overall mortality rates in those groups receiving amoxicillin doses of ≥ 0.3 mg/kg or linezolid doses of ≥ 3 mg/kg were reduced to 8% (2 of 26; $P < 0.05$) and 3% (1 of 31; $P < 0.05$), respectively.

Pharmacokinetics of oral antibiotic treatments. Studies of the pharmacokinetics associated with selected doses of linezolid and amoxicillin in gerbils with *S. pneumoniae* AOM focused primarily on the plasma and/or ear fluid drug levels achieved after the third of nine scheduled b.i.d. doses (between 24 and 36 h of drug treatment). This protocol was applied in part to allow time for drug levels to reach steady state and in part because measurable ear fluid samples (≥ 10 μ l/ear) were readily collectable during this period of the infection time course.

Plasma and concomitant ear fluid drug concentration-time curves generated from infected gerbils receiving either 10- or 30-mg/kg repeat oral doses of linezolid are illustrated in Fig. 4. The patterns of drug exposure achieved with these doses were similar regardless of whether PRSP (Fig. 4A and B) or PSSP (Fig. 4C and D) was the infectious agent. Additionally, there was little evidence of drug accumulation with either dosing regimen. Specifically, mean trough levels of linezolid, which were equivalent in plasma and ear fluid, remained relatively steady over the course of the 4.5-day treatment period used in this study (Fig. 4).

Third-dose pharmacokinetic parameters determined from the linezolid exposure experiments are listed in Table 2. In each of four pharmacokinetic trials, linezolid readily and rapidly distributed between the plasma and middle ear fluid compartments. Overall, mean ear fluid C_{max} levels ($102\% \pm 35\%$ of mean plasma levels; $n = 4$) and AUC_{0-12} levels ($113\% \pm 16\%$ of mean plasma levels; $n = 4$) were equivalent to those observed in host plasma, while the delay in T_{max} observed in ear fluid was ≤ 0.5 h (Table 2). Under both experimental conditions (PRSP or PSSP infection), the threshold linezolid antibacterial regimen of 10 mg/kg was associated with mean plasma and ear fluid drug C_{max} levels of 3.1 to 5.1 μ g/ml and mean AUC_{0-12} levels of 14.9 to 20.4 μ g $\cdot$ h/ml. Increasing the b.i.d. dose to 30 mg/kg resulted in a linear increase in linezolid exposure, as mean plasma and ear fluid drug C_{max} and AUC_{0-12} levels rose to between 10.9 and 16.7 μ g/ml and 53.7 to

FIG. 4. Plasma and ear fluid drug concentration-time curves of linezolid in gerbils with AOM induced by either PRSP (A and B) or PSSP (C and D) that were placed on 4.5-day b.i.d. oral dosing therapy of the antibiotic at 10 mg/kg (A and C) or 30 mg/kg (B and D). Mean drug levels in each fluid compartment were detected periodically throughout the dosing period by HPLC-MS/MS analysis. Experimental $n = 3$ to 6 samples/time point. PD, predose.

75.6 $\mu\text{g} \cdot \text{h}/\text{ml}$, respectively. This half-log dose increase was also associated with a modest 1.3- to 2.1-fold increase in the $t_{1/2}$ of linezolid in these two fluid compartments (Table 2).

Plasma pharmacokinetic parameters associated with amoxicillin treatment are also summarized in Table 2. In PRSP-infected gerbils, the 100-mg/kg b.i.d. therapy was associated with high plasma drug levels ($C_{\max} = 30.2 \pm 0.8 \text{ mg/ml}$; $T_{\max} = 0.25 \text{ h}$) that cleared rapidly ($t_{1/2} = 2.2 \text{ h}$). In PSSP-infected gerbils, the narrow detection window that existed between the relatively low plasma drug levels and the detection limit of the HPLC-MS/MS assay complicated pharmacokinetic analysis of the maximally effective 0.3-mg/kg dose of amoxicillin. From the limited data thus collected, only mean plasma $C_{\max}$ (0.042 $\mu\text{g/ml}$; $n = 2$) and $T_{\max}$ (0.25 h) could be estimated for this dosing regimen (Table 2). With both treatment regimens, there was no indication of drug accumulation with each successive dose. The pharmacokinetics of amoxicillin, observed here, are in line with those reported in humans receiving multiple-dose oral therapy (5).

Pharmacodynamics of oral antibiotic treatments. Table 3 summarizes the estimated plasma and ear fluid drug pharmacodynamics as well as the antibacterial effects that accompanied selected doses of linezolid and amoxicillin in this model.

Linezolid antibacterial potency, efficacy, and concomitant pharmacodynamic profiles were similar regardless of the infecting *S. pneumoniae* strain. Additionally, ear fluid pharmacodynamic parameters estimated for this agent usually met or exceeded those determined in the plasma compartment. In the combined AOM experiments, the threshold antibacterial regimen of 10 mg/kg was accompanied by plasma and ear fluid drug $C_{\max}/\text{MIC}$ ratios ranging from 3.1 to 5.1, $T > \text{MICs}$ ranging from 42 to 63%, and $\text{AUC}_{0-24}/\text{MIC}$ ratios ranging from 30 to 41 h. Increasing the linezolid dose threefold caused a similar increase in most of the parameters that were monitored (Table 3). In PRSP-infected animals, 100-mg/kg b.i.d. amoxicillin therapy was associated with a $C_{\max}/\text{MIC}$ ratio of 3.8 and a $T > \text{MIC}$ of 22%. Stringent analysis of the pharmacodynamics of the 0.3-mg/kg regimen was not attempted due to the limited pharmacokinetic data (noted above) and the lack of separation between the amoxicillin MIC level (for PSSP) and the detection limit of the HPLC-MS/MS assay (Table 3).

DISCUSSION

Mongolian gerbils are often used to establish antibiotic screening models of AOM due to their enlarged middle ear

TABLE 2. Third-dose plasma and ear fluid drug pharmacokinetics associated with selected regimens of linezolid and amoxicillin in a gerbil model of *S. pneumoniae*-induced AOM

Drug and oral b.i.d. dose ^a (mg/kg)	Sample type	$C_{\max}^b$ (μg/ml)	$T_{\max}$ (h)	$t_{1/2}^c$ (h)	AUC ₀₋₁₂ (μg · h/ml)
PRSP					
Linezolid, 10	Plasma	3.1 ± 1.2	0.5	1.7	14.9
	Ear fluid	4.6 ± 1.2	1.0	2.0	20.0
Linezolid, 30	Plasma	10.9 ± 2.4	0.5	3.1	53.7
	Ear fluid	11.4 ± 4.2	1.0	2.7	55.4
Amoxicillin, 100	Plasma	30.2 ± 0.8	0.25	2.2	63.3
PSSP					
Linezolid, 10	Plasma	5.1 ± 1.8	1.0	2.4	17.6
	Ear fluid	4.7 ± 3.8	0.5	2.6	20.4
Linezolid, 30	Plasma	16.7 ± 1.2	0.5	3.2	76.5
	Ear fluid	10.7 ± 1.7	1-4 ^d	5.5	75.6
Amoxicillin, 0.3	Plasma	0.02, 0.07	0.25	ND ^e	ND

^a Nine-dose regimen given over 4.5 days; drug exposures measured after third scheduled dose.

^b Mean ± standard deviation ($n = 3$ for plasma; $n = 4$ to 6 for ear fluid) or actual levels when $n < 3$.

^c Apparent terminal disposition half-life.

^d Two-hour time point missed.

^e ND, not determined due to a lack of time points with detectable drug levels.

chambers or bullae and the fact that common human pathogens normally do not reside in either the bullae or the interconnected nasopharynx (16, 36). In this study, we have characterized a gerbil model of *S. pneumoniae*-induced AOM that should prove useful in evaluating the antimicrobial efficacy of multiday antibiotic dosing regimens. Once experimental conditions were established to elicit reproducible infections with either a PRSP or PSSP strain, the model was validated by

the success (or lack thereof) of amoxicillin and linezolid therapies in eradicating pneumococci from the gerbil middle ear. Variations in virulence and infection invasiveness between PRSP- and PSSP-AOM were not unexpected, considering their respective serotype differences (2). These variations were accounted for (during the creation of a standardized protocol) by adjusting the intrabullar inoculum doses of each strain as well as the time frame after which antibiotic treatments were initiated. During the design phase, a concerted effort was made to implement a protocol that not only matched the clinical situation, i.e., antibiotic therapy initiated at peak infection, but also one that allowed simultaneous measurement of drug exposures achieved in plasma and ear fluid during efficacious drug treatment.

The objective of this study was to define the overall pharmacodynamic profiles of linezolid associated with efficacy in a clinically relevant model of *S. pneumoniae* infection. Using a chinchilla model of AOM, Pelton and colleagues (27) were the first to demonstrate that orally administered linezolid readily distributes into ear fluid at concentrations sufficient to cure *S. pneumoniae* infections of the middle ear. In their study, a twice-daily linezolid dose of 25 mg/kg cured 100% of infected ears within 2 days of treatment while maintaining an ear fluid drug $T > \text{MIC}$ of 100% (27). In this gerbil study, we have confirmed and extended this observation through a more extensive pharmacodynamic evaluation. Significant middle ear eradication of *S. pneumoniae* was achieved with linezolid therapy when the ear fluid drug $T > \text{MIC}$, $C_{\max}/\text{MIC}$, and $\text{AUC}_{0-24}/\text{MIC}$ ratios reached thresholds of $\approx 60\%$, 4 to 5, and 40 h, respectively. The in vitro and in vivo antibacterial potency of linezolid was similar for the PRSP and PSSP strains, as was the ear fluid drug pharmacodynamic profile that accompanied pneumococcal eradication from the gerbil middle ear.

Pharmacodynamic profiling of linezolid has been performed in other *S. pneumoniae* infection models. In a rat model of

TABLE 3. Estimated pharmacodynamic profiles associated with selected linezolid and amoxicillin regimens in a gerbil model of *S. pneumoniae*-induced AOM

Infectious strain	Drug and oral b.i.d. dose ^a (mg/kg)	Reduction in log ₁₀ CFU/ear flush ^b (cure rate) ^c	Sample type	$T > \text{MIC}$ (%)	$C_{\max}/\text{MIC}$	AUC _{0-24}/\text{MIC} (h)}
PRSP	Linezolid, 10	$>4.5 \pm 1.1^{*d}$ (13/18)	Plasma	45	3.1	30
			Ear fluid	57	4.6	40
	Linezolid, 30	$>5.1 \pm 0.3^{*}$ (18/18)	Plasma	90	10.9	107
			Ear fluid	84	11.4	111
	Amoxicillin, 100	$>3.6 \pm 2.6$ (5/8)	Plasma	22	3.8	8
PSSP	Linezolid, 10	$>5.1 \pm 0.2^{*}$ (6/8)	Plasma	42	5.1	35
			Ear fluid	63	4.7	41
	Linezolid, 30	$>5.2 \pm 0.0^{*}$ (8/8)	Plasma	100	16.7	153
			Ear fluid	100	10.7	151
	Amoxicillin, 0.3	$>5.6 \pm 0.0^{*}$ (8/8)	Plasma	ND ^e	2.8	ND

^a Nine-dose oral b.i.d. regimen given over 4.5 days.

^b Geometric mean reduction in ear flush CFU levels versus respective vehicle group measured 4 h after the last drug dose.

^c Frequency of ear flush samples within each treatment group that were culture negative.

^d *, $P < 0.05$ versus respective vehicle group.

^e ND, not determined due to lack of time points with detectable drug levels in pharmacokinetic experiments and insufficient separation between amoxicillin MIC (0.015 μg/ml for PSSP) and the lower limit of quantitation of the HPLC-MS/MS assay (0.013 μg/ml).

lethal pneumonia, significant reductions in mortality (42% reduction) and concomitant bacteria levels (>5-log reduction after five doses) were observed with an oral b.i.d. regimen of 25 mg/kg that maintained the total serum drug $T > \text{MIC}$, mean $C_{\text{max}}/\text{MIC}$, and mean $\text{AUC}_{0-24}/\text{MIC}$ at 36%, 16.9, and 119 h, respectively (18). In a neutropenic thigh infection model in mice, Andes and colleagues (1) demonstrated that the serum drug $\text{AUC}_{0-24}/\text{MIC}$ ratio was the best predictor of antibacterial efficacy of linezolid and that, for eight different *S. pneumoniae* strains, bacteriostatic doses of linezolid maintained mean $\text{AUC}_{0-24}/\text{MIC}$ ratios between 22 and 97 h. In this AOM study, antibacterial efficacy was associated with a plasma drug $T > \text{MIC}$ of 42%, a $C_{\text{max}}/\text{MIC}$ of 3, and an $\text{AUC}_{0-24}/\text{MIC}$ of 30 h. Determination of the specific pharmacodynamic parameter most predictive of efficacy in this model is a topic of future study. Such a determination will require a comparison of activities of similar daily doses applied at various dosing intervals.

The similarities of the plasma and serum drug pharmacodynamic profiles of linezolid associated with efficacy against preclinical pneumococcal infections, regardless of infection site, strongly reflects the relatively high volume of distribution of the drug, which approximates total body water (6, 31). The ability of linezolid to penetrate soft tissues and/or cure localized infections has been well demonstrated in both animals (15, 18) and humans (7, 30, 33). In terms of treating upper respiratory tract infections, Pelton and colleagues (27) showed that linezolid eradicated *S. pneumoniae* not only from the middle ear but also from the nasopharynx, which can become an important reservoir for resistant bacteria. The ability of an agent to distribute throughout the upper respiratory tract at antibacterial concentrations decreases the chances of rebound middle ear infection caused by resistant strains of targeted organisms, and it also decreases the potential spread of such infections via nasopharynx carriage and transmission (10, 27).

The test-of-cure data generated 4 days posttherapy in the gerbils infected with either PRSP or PSSP revealed only one case of potential AOM relapse (6% of all ears examined) in those animals given the ED_{100} of linezolid. In this single case, the persistent *S. pneumoniae* isolate had retained its susceptibility to the oxazolidinone. When interpreting relapse data in this model, it is important to realize inherent protocol limitations, such as an abbreviated dosing schedule, and the fact that time-dependent infection frequencies were monitored in separate populations of animals. Nonetheless, it is interesting that in various clinical trials, between 24 and 58% of bacterial isolates collected from persistent cases of AOM were shown to be susceptible to the previously prescribed antibiotics (20, 28, 35). The exact cause of this phenomenon is unknown but may include such factors as the brevity of the dosing regimen, poor compliance, poor accessibility of drug-containing ear fluid secretions to the site of infection origin, pathogen biofilm production, and/or variability in the host immune response necessary for proper infection clearance.

Amoxicillin, given alone or in combination with clavulanic acid, is prescribed often for the empirical treatment of AOM because of its effectiveness against both *S. pneumoniae* and the common gram-negative pathogens *Haemophilus influenzae* and *Moraxella catarrhalis*. It has been well characterized that a serum drug $T > \text{MIC}$ approaching 40% is a key pharmacodynamic determinate of in vivo efficacy for this and other beta-

lactams (8, 9). Amoxicillin regimens were applied in this study as a means of confirming the transference of the differential susceptibilities of the PRSP and PSSP strains from the in vitro to the in vivo setting. In PRSP-infected animals, administration of a 100-mg/kg b.i.d. dose of amoxicillin was associated with a $T > \text{MIC}$ of only 22% despite achieving markedly elevated plasma drug levels, a pharmacodynamic insufficiency caused by the high level of pathogen resistance. In turn, the inconsistent antibacterial effects obtained with high-dose amoxicillin against PRSP-AOM were most likely due to the suboptimal $T > \text{MIC}$ profile of the treatment regimen (Fig. 2; Table 3).

The continuing increase in the frequency of drug-resistant clinical isolates of *S. pneumoniae* poses concern towards achieving pharmacological cures of AOM. Complications associated with unresolved cases of *S. pneumoniae*-induced AOM can be severe and include meningitis, mastoiditis, and persistent effusion with hearing loss (2, 4, 29). In treating these infections, there has been much debate as to how best to minimize the development of resistant strains while improving clinical outcomes of antibiotic intervention. Besides the need to continue the ongoing search for drugs active against resistant bacteria (11, 29), it is apparent that breakpoint determinations guiding current and future antibiotic usage must take into account the respective pharmacokinetic and pharmacodynamic properties of such agents (8, 14, 21). In this study, we defined the pharmacodynamic relationships of linezolid necessary to effect preclinical cures of *S. pneumoniae*-induced AOM. Application of this methodology will prove beneficial in the determination of appropriate breakpoints for other novel antibiotics.

ACKNOWLEDGMENTS

We acknowledge the receipt of *S. pneumoniae* strain UC15087 from Edward O. Mason, Jr., at Texas Children's Hospital and Baylor College of Medicine, Houston, Tex. We also acknowledge the assistance of Daniel G. Miley in HPLC-MS/MS sample preparation and analysis and that of Janice M. Friis, Maria Courtney, Michael T. Verburg, and Wade J. Adams in the performance of gerbil plasma protein binding experiments. We further acknowledge the laboratory of Gary V. Doern, University of Iowa Hospitals and Clinics, Iowa City, for serotyping of the *S. pneumoniae* isolates.

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