A Faropenem, Linezolid, and Moxifloxacin Regimen for Both Drug-Susceptible and Multidrug-Resistant Tuberculosis in Children: FLAME Path on the Milky Way

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Background. The regimen of linezolid and moxifloxacin was found to be efficacious in the hollow fiber system model of pediatric intracellular tuberculosis. However, its kill rate was slower than the standard 3-drug regimen of isoniazid, rifampin, and pyrazinamide. We wanted to examine the effect of adding a third oral agent, faropenem, to this dual combination.

Methods. We performed a series of studies in the hollow fiber system model of intracellular Mycobacterium tuberculosis, by mimicking pediatric pharmacokinetics of each antibiotic. First, we varied the percentage of time that faropenem persisted above minimum inhibitory concentration (T_{MIC}) on the moxifloxacin-linezolid regimen. After choosing the best faropenem exposure, we performed experiments in which we varied the moxifloxacin and linezolid doses in the triple regimen. Finally, we performed longer-duration therapy validation experiments. Bacterial burden was quantified using both colony-forming units per milliliter (CFU/mL) and time to positivity (TTP). Kill slopes were modeled using exponential regression.

Results. TTP was a more sensitive measure of bacterial burden than CFU/mL. A faropenem T_{MIC} > 62% was associated with steepest microbial kill slope. Regimens of standard linezolid and moxifloxacin plus faropenem T_{MIC} > 60%, as well as higher-dose moxifloxacin, achieved slopes equivalent to those of the standard regimen based by both TTP and CFU/mL over 28 days of treatment.

Conclusions. We have developed an oral faropenem-linezolid-moxifloxacin (FLAME) regimen that is free of first-line drugs. The regimen could be effective against both multidrug-resistant and drug-susceptible tuberculosis in children.

Keywords. disseminated tuberculosis; regimen design; hollow fiber model; time-to-positivity.

Treatment of tuberculosis in children, as in adults, involves using a combination of at least 3 drugs [1, 2]. We have demonstrated the efficacy of the combination of linezolid and moxifloxacin against intracellular Mycobacterium tuberculosis (Mtb) in the hollow fiber system model for pediatric intracellular tuberculosis (HFS-Peds) [3]. However, while effective, the kill rate was slower than that of the standard 3-drug regimen of isoniazid, rifampin, and pyrazinamide (herein termed “standard therapy”). Addition of a third oral agent to the backbone could improve the efficacy. One class of agents that could do this by virtue of a different mechanism of effect are β-lactams. Here, we performed a series of experiments using the HFS-Peds in which we added the β-lactam, faropenem.

Faropenem has an oral bioavailability of 70%–80% [4, 5]. The prodrug, faropenem medoxomil, results in several-fold higher exposures. Faropenem has a strong affinity for the high molecular penicillin binding proteins of the cell wall and inhibits transpeptidation, thereby interfering with cell wall synthesis [4, 5]. This differs from inhibition of bacterial protein synthesis by linezolid and gyrase inhibition by moxifloxacin, making the combination mechanistically attractive. Faropenem is more resistant to hydrolysis by β-lactamases than cephalosporins and carbapenems are, which enables it to work in β-lactamase-producing bacteria without a β-lactamase inhibitor [4–7]. This gives it an advantage over carbapenems, which have to be administered together with clavulinate (available only as Augmentin), and the associated diarrhea. Faropenem has been found to be clinically effective and safe in the treatment of upper respiratory gram-negative and gram-positive bacterial infections, bronchitis, pneumonia, otitis media, and urinary tract infections in children [8]. Recently, faropenem was also shown to possess activity against Mtb in susceptibility studies [9, 10]. Unlike the 3 first-line antituberculosis drugs of rifampin, isoniazid, and pyrazinamide, which are all associated with hepatotoxicity, faropenem is generally well tolerated, and its side-effect profile does not overlap with those of linezolid and moxifloxacin.
MATERIALS AND METHODS

Organism

*Mtb* H37Ra (ATCC 25177) was used throughout the experiments. *Mtbin culture stored at −80°C in Middlebrook 7H9 broth was thawed prior to the experiments, and grown into logarithmic phase growth in Middlebrook 7H9 broth supplemented with 10% oleic acid, dextrose, and catalase (OADC) at 37°C under 5% CO2 under slow shaking.

Materials and Drugs

Linezolid and moxifloxacin hydrochloride solution were purchased from the Baylor University Medical Center pharmacy. Faropenem sodium hydrate powder was purchased from BOC Sciences (Shirley, New York). Hollow fiber cartridges were purchased from FiberCell (Frederick, Maryland). BACTEC MGIT 960 Mycobacterial Growth Tube Indicator System (MGIT) was purchased from Becton Dickinson (Franklin Lakes, New Jersey), and MGIT tubes from BD.

Infection of THP-1 Cells With *Mtb*

Human-derived THP-1 monocytes (ATCC TIB-202) were cultured using prewarmed RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C under 5% CO2. The cultures were checked for Mycoplasma contamination using a Mycoplasma PCR detection kit. The cells were maintained by subculturing every 72 hours, and then infected with log-phase growth *Mtbin a bacteria-to-macrophage ratio of 1:1 by coincubation for 6 hours. Upon completion of the infection period, the monocytes were centrifuged at 200 g for 5 minutes and washed with warm RPMI 1640 to remove carried-over extracellular bacteria. Cells were then counted using both a hemocytometer and Coulter counter.

Determination of Minimum Inhibitory Concentration

Linezolid and moxifloxacin minimum inhibitory concentrations (MICs) were determined as described elsewhere in this supplement [3, 11]. For faropenem MIC, turbidity of *Mtbin culture in log-phase growth was adjusted to a bacterial density of 10⁵ colony-forming units per milliliter (CFU/mL). Bacterial suspension was treated with faropenem concentrations of 0, 0.007, 0.015, 0.030, 0.06, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/L in 96-well plates in triplicate, and incubated at 37°C with 5% CO2 for 7 days. Resazurin at 0.01% (v/v) was added to each well and incubated overnight under the same conditions and assessed for color development. The MIC was defined as the lowest drug concentration that prevented color change from blue to pink.

Detection of Growth by MGIT System

In a pilot experiment, serial 10-fold dilutions from 0 to −4 of turbidity-adjusted *Mtbin log-phase growth were prepared. Then, 500 µL of bacterial suspension from each dilution was inoculated into MGIT tubes purchased from BD, following which 0.8 mL of OADC was added to supplement growth. The cultures were placed in the MGIT system for detection of *Mtbin growth. Bacterial suspension from each dilution tube was also simultaneously cultured on Middlebrook 7H10 solid agar with 10% OADC growth supplement. Cultures were incubated at 37°C at 5% CO2 and CFU counted after 21 days. Exponential regression analyses of time to positivity (TTP) in days in the MGIT system vs log10 CFU/mL on agar had an r² = 0.995. This assay was used for monitoring bacterial growth in the hollow fiber.

Faropenem Effect Versus Intracellular *Mtb*

THP-1 monocytes were activated in 12-well plates with phorbol myristate acetate as described previously [3, 11]. After 72 hours, the adherent cells were infected with log-phase growth *Mtb* as described above, and cultures coincubated with faropenem concentrations of 0, 1.25, 2.5, 5, 10, 20, 40, or 80 mg/L, in triplicate. The relationship between *Mtbin log10 CFU/mL and faropenem concentration was analyzed using the inhibitory sigmoid maximal kill (Emax) model.

Linezolid, Moxifloxacin, and Faropenem Combination Study in the Hollow Fiber System

The details of the HFS-Peds have been recently described elsewhere [11, 12]. We performed 3 linezolid, moxifloxacin, and faropenem combination studies in this model. Faropenem exposures were expressed as the percentage of time concentration that persisted above the MIC in a 24-hour dosing interval (TMIC): as efficacy of this penem is linked to this parameter for other pathogens, we assumed the same for *Mtbin [13]. In the first study, we tested 3 different dosing schedules of faropenem, including once a day leading to a TMIC = 12.5%, twice a day associated with TMIC = 25%, and 3 times a day to achieve TMIC = 62.5% in combination with the dual regimen of linezolid and moxifloxacin chosen from prior studies, so as to identify the faropenem exposure associated with the best microbial response [3]. The study regimens were administered over 14 days and the study was geared toward an intensive look at the kill slopes early during therapy. The faropenem half-life was 1.0 hour [5]. Moxifloxacin was administered to achieve concentration-time profile in infants of 10 mg/kg/day and linezolid 20 mg/kg/day. We also wanted to compare the kill rates to the pediatric concentration-time profiles of drugs in standard therapy dosed as 10 mg/kg/day of isoniazid, 15 mg/kg/day rifampin, and 40 mg/kg/day pyrazinamide. Fresh media was infused into, and pumped out of, the HFS-Peds at predefined rates to mimic linezolid, moxifloxacin, isoniazid (slow acylators), and rifampin half-lives (T1/2) of 3–4 hours encountered in children aged <3.5 years [14–17]. The pyrazinamide T1/2 in children was 5.5 hours a day [16, 18]. The concentration-time profiles of each antibiotic were validated by sampling the central compartment of each HFS-Peds at 7 time points over a 24-hour dosing interval following infusion of test doses. Peripheral compartments were sampled on days 0, 3, 5, 7, 9, 12, and 14, to enable quantification of *Mtbin THP-1 cells so that the kill slopes could be examined for the different exposure regimens.
Based on the results of the first experiment, the best faropenem exposure for microbial kill was chosen for the second study. The intent of the second experiment was (i) to compare the slopes of the different experimental regimens in which the doses of moxifloxacin and linezolid combined with one faropenem exposure were varied, (ii) to add ethambutol as a fourth drug to accelerate microbial kill, and (iii) to examine the sensitivity and utility of TTP as a pharmacodynamic measure, particularly to determine limits of assay detection with $\log_{10}$ CFU/mL in a paucibacillary state. The MGIT liquid culture method has been found to be more sensitive than cultures on solid agar, and is now the standard used in the clinic and in clinical trials [19–21]. A low bacillary burden was used to mimic the paucibacillary state encountered in children. The treatment duration was for 10 days, and sampling of peripheral compartments was done on days 0, 3, 7, and 10. Samples were centrifuged at 21,000 g for 5 minutes at room temperature and supernatant was removed. Then, the pellet was suspended in equal volume of phosphate-buffered saline with 0.05% Triton X and vortexed until disappearance of the pellet. Next, 0.5 mL of this suspension was introduced into the commercial MGIT tubes as described earlier. The MGIT tubes were then loaded into the MGIT machine and incubated per the manufacturer’s instructions for detection of growth, and incubated for 56 days before being declared negative.

We performed a third hollow fiber study with drug regimen exposures identical to those of the second. This time we started with a higher bacillary burden, and extended the treatment duration for 28 days. Sampling of central compartment to validate that intended concentration-time profiles of antibiotic had been achieved was performed as described above. The peripheral compartments were sampled on days 0, 3, 7, 14, 21, and 28 for determination of both $\log_{10}$ CFU/mL and TTP. This study design allowed us to examine the longer-term bacterial decline, determine if there was total microbial kill, and ($\text{ii}$) to add ethambutol as a fourth drug to accelerate microbial kill, and (iii) to examine the sensitivity and utility of TTP as a pharmacodynamic measure, particularly to determine limits of assay detection with $\log_{10}$ CFU/mL in a paucibacillary state. The MGIT liquid culture method has been found to be more sensitive than cultures on solid agar, and is now the standard used in the clinic and in clinical trials [19–21]. A low bacillary burden was used to mimic the paucibacillary state encountered in children. The treatment duration was for 10 days, and sampling of peripheral compartments was done on days 0, 3, 7, and 10. Samples were centrifuged at 21,000 g for 5 minutes at room temperature and supernatant was removed. Then, the pellet was suspended in equal volume of phosphate-buffered saline with 0.05% Triton X and vortexed until disappearance of the pellet. Next, 0.5 mL of this suspension was introduced into the commercial MGIT tubes as described earlier. The MGIT tubes were then loaded into the MGIT machine and incubated per the manufacturer’s instructions for detection of growth, and incubated for 56 days before being declared negative.

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Regression Analysis Methods for Bacterial Burden

We examined for both linear regression, as well as an exponential decline model. Exponential decline is based on the simple exponential decay relationship:

$$\frac{dN}{dt} = kN,$$

where $N$ is $M_{tb}$ bacterial burden, $t$ is time in days, and the bacterial burden in each hollow fiber system at $t = 0$ is $N_0$. The standard solution for this is given by:

$$\log_{10} \text{CFU/mL on day } t = \left( \log_{10} \text{CFU/mL on day } 0 \right) \times e^{-kt}.$$

For the best model, we compared the $r^2$ for standard therapy, given that both exponential decline models and linear models have been applied to standard therapy in patients. We report both results; however, the model with the higher $r^2$ was chosen for the final ranking of kill rates.

Drug Concentration Assays

Concentrations of antibiotics in the samples collected from the central compartment of the HFS were analyzed using liquid chromatography–tandem mass spectrometry. The assays to measure rifampin, isoniazid, pyrazinamide, linezolid, and moxifloxacin have been described in the past [3, 11, 12]. Faropenem-d4 and sulfamethoxazole-d4 (internal standards) were purchased from Toronto Research Chemicals (Toronto, Canada). Calibrator, controls, and internal standard were included in each analytical run for quantitation. Stock solutions of faropenem and internal standard were prepared in 80:20 methanol:water at a concentration of 1 mg/mL and stored at −20°C. A 7-point calibration curve was prepared by diluting faropenem stock solution in drug-free media (0.1, 0.2, 1, 2, 5, 10, and 20 µg/mL). Quality control samples were prepared by spiking media with stock standards for 2 levels of controls of 0.8 µg/mL and 16 µg/mL. Samples were prepared in 96-well plates by the addition of 10 µL of calibrator, quality controls, or sample to 190 µL 0.1% formic acid in water containing 1 µg/mL internal standard followed by vortex. Chromatographic separation was achieved on an Agilent UPLC HSS T3 1.8-µm 50 × 2.1-mm analytical column (Waters) maintained at 30°C at a flow of 0.2 mL/minute with a binary gradient with a total run time of 6 minutes. The observed ion (m/z) values of the fragment ions were 308.0 → 177.9. Sample injection and separation was performed by an Agilent UPLC interfaced with a Xevo TQ mass spectrometer (Waters). All data were collected using MassLynx software version 4.1. The limit of quantitation for this assay was 0.1 µg/mL. The interday % coefficient of variation (%CV) for the assay was 2%–5%, which was identical to the interday %CV.
RESULTS

The faropenem MIC of Mtb H37Ra strain was 1.0 mg/L. The inhibitory sigmoid $E_{\text{max}}$ relationship between faropenem exposure and intracellular Mtb is shown in Figure 1. The figure shows that faropenem effectively kills intracellular Mtb as monotherapy.

The drug exposures achieved in the first hollow fiber study were a linezolid 0- to 24-hour area under the curve (AUC$_0$-24)/MIC of 61.93, a moxifloxacin AUC$_0$-24/MIC of 247.56, isoniazid AUC$_0$-24/MIC of 524.33, rifampin AUC$_0$-24/MIC of 113.21, and pyrazinamide AUC$_0$-24/MIC of 45.18. The faropenem T$_{MIC}$ observed after drug assays of the replicate HFS-TB in this experiment were exactly 12.5%, 25%, and 62.5%. Figure 2A shows the THP-1 counts over the 14-day experiment. The figure shows no effect by the different faropenem dosing exposures on THP-1 cell viability; indeed, the counts were similar to nontreated controls. Figure 2B shows the microbial kill slopes for each regimen over the 14 days, based on log$_{10}$ CFU/mL. Figure 2B shows that the regimen with faropenem with T$_{MIC}$ of 62.5% had the best kill rates among the faropenem regimens, based on the exponential decline model. The time required to sterilize each system by the faropenem T$_{MIC} >$ 62% triple regimen was similar to that for standard therapy.

In the next hollow fiber experiments, a faropenem T$_{MIC}$ of 62.5% was used in combination with linezolid and moxifloxacin; drug concentrations were not measured in this study. There was no change in macrophage count over the course of 10 days. Figure 3A shows the change in log$_{10}$ CFU/mL, and shows that based on colony counts, the experimental regimen of faropenem and standard doses of linezolid and moxifloxacin (FLM) had a kill rate similar to the standard therapy regimen. Since it looked as if the high-dose moxifloxacin regimen (FLMHi) plus ethambutol could have a steeper decline, we performed 2-way analysis of variance (ANOVA) to examine the effect of regimen and day of therapy, which revealed that the day of therapy accounted for 93.29% of the total variation ($P < .0001$) whereas the regimen accounted for only 0.78% ($P = .1473$). Next, we examined TTP as a pharmacodynamic measure, given that in Figure 3A the regimens seemed to have sterilized the HFS-Peds by the last day of therapy. Figure 3B shows that based on TTP, some regimens such as standard therapy had not yet sterilized the systems, whereas the FLMHi regimens with and without ethambutol were even better.

Indeed, a 2-way ANOVA revealed that while day of sampling...
exposures as in the
ments revealed linezolid, isoniazid, rifampin, and pyrazinamide
over 1 month.

Effect of a new faropenem, linezolid, and moxifloxacin (FLM) regimen

Figure 4.

Figure 4. Effect of a new faropenem, linezolid, and moxifloxacin (FLM) regimen over 1 month. A, Kill slopes of the different regimens based on a linear regression model and 95% confidence bounds. The \( r^2 \) for the FLM regimen was 0.92, for high-dose moxifloxacin (FLMHi) was 0.84, for FLMHi with ethambutol (EMB) was 0.84, and for standard therapy was 0.73. B, Exponential decline model. The decline rates of all the regimens were similar to standard therapy. C, Time to positivity (TTP) of contents from each hollow fiber system shows that there is no sterilization up to day 28 with TTP when compared to Mycobacterium tuberculosis (Mtba) log colony-forming units (CFU)/mL, which revealed sterilization. In this experiment we started with a higher bacterial burden, an acid test. Results of multiple test comparison shown as









tions, when the same regimen was compared with standard therapy over 2 months of intensive phase, we found that it actually outperformed the rifampin-pyrazinamide–based standard therapy in terms of slope (manuscripts in preparation). This suggests the regimen could work even in adult-type tuberculosis in teenagers.
The time from the first linezolid monotherapy experiment in this series to counting the last CFUs in the current study, and completion of simulations was 1 year and 4 months (with change in our laboratory location in the process). In other words, this deliberate approach is dramatically faster, cheaper, and safer than trying to perform this sequentially in clinical studies of children that would try to switch out 1 drug at a time.

Second, using this approach we have developed a faropenem-linezolid-moxifloxacin (with and without ethambutol) or “FLAME” regimen that is independent of rifampin, isoniazid, and pyrazinamide, and thus can be tested in clinical trials to treat both multidrug-resistant (MDR) tuberculosis and drug-susceptible tuberculosis, given that obtaining cultures for microbiologic diagnosis is difficult, particularly in very young children [23]. Moreover, the current treatment for drug-susceptible tuberculous meningitis is associated with death in 20% of children and probability of survival without neurologic sequelae of only 40%. In this context, the good cerebrospinal fluid (CSF) penetration by linezolid could be an advantage. Furthermore, the faropenem-linezolid portion of the regimen will also work for extensively drug-resistant (XDR) tuberculosis in children. As the microbial kill slopes were similar to those of standard short-course chemotherapy for drug-susceptible tuberculosis, this new regimen could be examined for cure of children with MDR tuberculosis in 6 months. This is more so given that we had optimized the exposures in the standard regimen (reflected by half-lives of isoniazid and rifampin), and hence AUC/MIC, on the high end of the distribution. Thus, the equivalence of effect we identified is unlikely to be due to suboptimal dosing in the standard regimen. In the future, hollow fiber studies adding another antibiotic to regimen, or switching out congeners (either oxazolidinones or quinolones or penems) to achieve faster Mtb kill rates than standard therapy could be examined. The addition of a fourth drug (especially one that also works against XDR tuberculosis) that improves the slope by a factor of 2 or 3 could create a regimen administered for shorter duration of therapy for all forms of tuberculosis, including drug-resistant ones.

Finally, in the current study we validated TTP as a pharmacodynamic measure for hollow fiber tuberculosis studies. Comparing TTP will allow easier translation to the clinic. In the clinic, liquid media have been shown to be more sensitive than solid media for culture of mycobacteria from sputum; TTP at commencement of therapy correlates with treatment outcome and mortality for pulmonary and extrapulmonary tuberculosis [24–32]. This method also gave a more sensitive index for measuring total sterilization compared with solid agar colony counts in the hollow fiber, given that positive cultures were encountered up to 28 days of therapy despite negative cultures on agar. In addition, in earlier studies we also introduced host–pathogen RNA sequencing to these HFS-Peds studies, which would provide yet another measure of total sterilization. These measures will improve sensitivity of prediction of such clinical microbial measures as relapse.

There are limitations to our study. We used an avirulent strain of Mtb in all our studies. This is because virulent strains kill macrophages within days, making it difficult to maintain a long-term co-culture system, as we demonstrated in a prior study [12]. However, the effect of drugs in this system that uses this avirulent strain and the ranking of drugs in combination regimens for the standard therapy seem to be well reflected in clinical outcomes in children with intrapulmonary, extrapulmonary, and disseminated tuberculosis [12]. Moreover, comparison of kill rates of the intracellular isogenic virulent strain in studies done by others to our current studies on adherent THP-1 cells reveals virtually the same kill curves, at least in 12-well plates, suggesting similar responses between virulent and avirulent strains [9]. We also intend to perform further validation in a mouse model of disseminated tuberculosis, still being developed and validated.

In summary, we have developed a combination FLAME regimen, for treatment of intracellular tuberculosis in children: the first steps along the mythical path created by the young Khoisan girl who gave us the Milky Way [33]. The regimen displays similar kill rates to the current standard 3-drug regimen of isoniazid, rifampin, and pyrazinamide for drug-susceptible tuberculosis. In doing so, we have created a regimen that is likely to work against both MDR tuberculosis and drug-susceptible tuberculosis, and has good CSF penetration rates for possible treatment of tuberculous meningitis.

Notes

Author contributions. T. G., and E. N. designed the study; D. D., S. S., and T. G. performed the hollow fiber studies; D. D. wrote the first draft of the manuscript; D. D., S. S., E. N., J. G. P., S. S., and T. G. wrote the manuscript.

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