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## ***Mycobacterium tuberculosis* sterilizing activity of faropenem, pyrazinamide and linezolid combination and failure to shorten the therapy duration**

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### **Abstract**

**Background:** Faropenem (F), an orally bioavailable  $\beta$ -lactam, kills *Mycobacterium tuberculosis* (*Mtb*) without the help of a  $\beta$ -lactamase inhibitor. This study explored the sterilizing effect of adding F once or twice daily to a linezolid (L) plus pyrazinamide (Z) backbone regimen.

**Methods:** *In vitro* studies were performed using the hollow fiber model of tuberculosis (HFS-TB) to compare the kill rates of: 1) ZL two-drug combination; 2) F administered once daily plus ZL (F<sub>1</sub>ZL); 3) F administered twice-daily plus once daily ZL (F<sub>2</sub>ZL); 4) F<sub>2</sub>ZL with high-dose Z (F<sub>2</sub>Z<sub>hi</sub>L); 5) standard therapy of isoniazid, rifampin and Z; and 6) non-treated controls. The study was performed over 56 days with three HFS-TB replicates for each regimen.

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Author contributions

Conceptualization and design, SS and TG; HFS-TB experiments, SS, DD, CMS; data analysis, SS and TG. SS wrote the first draft of the manuscript; JWA provided clinically relevant comments and edited the manuscript. All authors reviewed and approved the final version of the manuscript.

Conflict of interest

TG is the founder and CEO of Praedicare Laboratories Inc. CMS is an employee of Praedicare Laboratories Inc. All authors have no conflict of interest.

Ethical approval

Not applicable.

**Results:** *Mtb* in the non-treated HFS-TB grew at a rate of  $0.018 \pm 0.007 \log_{10}$  CFU/mL/day. The exponential kill rates for standard therapy were 6.6–13.2-fold higher than ZL dual therapy. The F<sub>1</sub>ZL and F<sub>2</sub>ZL regimens ranked third. The pre-existing isoniazid-resistant sub-population in the inoculum ( $1.34 \pm 0.57 \log_{10}$  CFU/mL) grew to  $4.21 \pm 0.58 \log_{10}$  CFU/mL in 56 days in non-treated HFS-TB. However, no isoniazid-resistant sub-population was recorded in any of the FZL combination regimens.

**Conclusion:** Due to the slow kill rate compared to standard therapy, FZL regimens are unlikely to shorten therapy duration. Efficacy of these regimens against drug-resistant tuberculosis needs to be determined.

## Keywords

Sterilizing activity;  $\beta$ -lactam; Tuberculosis; Therapy duration

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## Introduction

The treatment of tuberculosis [TB] has begun to evolve with several studies to shorten therapy duration, and with the design of regimens that can work in both drug-resistant and drug-susceptible TB, termed “pan-TB” regimens (Dhedha et al., 2018a; Wallis et al., 2018). Recently, the combination of bedaquiline, pretomanid and linezolid (L) was shown to cure >90% of patients with multidrug-resistant TB (MDR-TB) within 6 months; however, the main limitation was L-based toxicity, which increased with prolonged duration of therapy (Conradie et al., 2020). Elsewhere, the role of L and L-based combinations with moxifloxacin and faropenem (F) in children and adults (FLAME regimen) has been examined (Bolhuis et al., 2018; Srivastava et al., 2017a). This combination’s kill rate equaled those for standard therapy in the hollow fiber system model of TB (HFS-TB), suggesting a 6-month duration (Deshpande et al., 2016a, 2016b, 2016c). While other  $\beta$ -lactam antibiotics – such as benzylpenicillin and ceftazidime/avibactam, ertapenem and ceftriaxone – have higher kill rates than F in the HFS-TB, F has the major advantage of being orally bioavailable (Deshpande et al., 2018b, 2017; van Rijn et al., 2017).

In order to assess the possible contribution of F to efficacy of L plus a pyrazinamide (Z) backbone, this study compared the kill rates of: 1) ZL; 2) F administered once a day plus ZL (F<sub>1</sub>ZL); 3) F administered twice a day plus ZL (F<sub>2</sub>ZL); 4) F<sub>2</sub>ZL with high-dose Z (F<sub>2</sub>Z<sub>hi</sub>L); 5) standard therapy of isoniazid, rifampin and Z; and 6) non-treated controls in three HFS-TB replicates for each regimen.

## Materials and methods

### Bacteria, materials and reagents

The *Mycobacterium tuberculosis* (*Mtb*) laboratory strain H37Ra (ATCC #25177) was used in the experiments, with culture media and bacterial growth conditions described in previous publications (Srivastava et al., 2011a; Srivastava et al., 2011b). Linezolid was purchased from Baylor Medical Center pharmacy, F sodium hydrate from BOC Sciences (Shirley, NY, USA), and isoniazid, rifampin, and Z were purchased from Sigma Aldrich (St Louis, MO, USA). Hollow fiber cartridges were procured from FiberCell Systems Inc (New Market,

MD, USA). BACTEC MGIT 960 mycobacterial growth tube indicator system (MGIT) and MGIT tubes were supplied from Becton Dickinson (Franklin Lakes, NJ, USA). Analytical standards of all the drugs were purchased from Sigma, and stable isotope labeled standards were purchased from CDN isotopes (Quebec, Canada). All chemicals used in the drug concentration measurement were chromatographic or LC-MS/MS grade.

### **In vitro HFS-TB model and transformation of Mtb into a semidormant state**

The detailed description of the HFS-TB has been published elsewhere (Gumbo et al., 2009; Srivastava and Gumbo, 2011). Briefly, there are two compartments in the HFS-TB: the central compartment, where the drugs are administered *via* a programmable syringe pump to achieve the time to maximum concentration ( $T_{max}$ ), as seen in patients to reach the peak concentration ( $C_{max}$ ). The dilution rate of the fresh media into the central compartment, *via* a peristaltic masterflex pump, is set to achieve a different half-life ( $t_{1/2}$ ) of the drugs in the same compartment. The drug containing media is then circulated, using a duet pump, in to the peripheral compartment that contains the semipermeable hollow fiber membranes with pore sizes big enough to allow the nutrients and drugs to cross the membrane but small enough to keep the bacteria in the peripheral compartment. Thus, bacteria always remain in contact with fluctuating drug concentrations, as the waste media is pumped out of the central compartment *via* a second set of masterflex pumps.

To perform the sterilizing effect studies in the HFS-TB (Gumbo et al., 2009), 4-day-old log-phase growth *Mtb* cultures were transferred to media acidified using citric acid at a pH 5.8. The cultures were incubated at 37 °C for 4 days, which transformed the cultures into slowly growing bacteria representing a semidormant state at an acidic pH. The cultures were then either diluted or concentrated by centrifugation to achieve the intended bacterial density in the inoculum before the start of the experiment.

### **Linezolid, pyrazinamide and faropenem combination in the HFS-TB**

The experiments were performed with three HFS-TB replicate units per regimen, treated daily with each drug combination for 56 days. The non-protein bound (free) L and Z exposures (24-h area under the concentration-time curve,  $fAUC_{0-24}$ ) as well as F exposure (%time concentration persisting above the MIC,  $\%T_{MIC}$ ) were selected from previously published studies (Bolhuis et al., 2018; Deshpande et al., 2016b; Gumbo et al., 2009; Srivastava et al., 2017a). The inflow media dilution rate was set to mimic an 8-h half-life of L and Z, and 1-h half-life for F (Bolhuis et al., 2018; Gettig et al., 2008; Pasipanodya et al., 2013; Srivastava et al., 2017a). While we took into account the drug penetration ratios of L, Z, rifampin, and isoniazid, as observed in TB cavities in patients (Dheda et al., 2018b; Ordonez et al., 2020), the F penetration ratio was unknown. The experimental regimens were those as shown in Table 1. Briefly: L was administered equivalent to 600 mg twice daily; Z at a standard 40 mg/kg/daily and high-dose (to mimic the concentration in the epithelial lining fluid of the patients with TB) (Conte et al., 1999); and F at a 300 mg dose (dosing schedule as detailed in Table 1). The standard three-drug combination regimen consisted of 300 mg isoniazid, 600 mg rifampin and 1.5 g Z, administered once daily. The non-treated HFS-TB units served as controls.

For the pharmacokinetic analysis, the concentration time profile of each drug was validated by multiple sampling of the central compartment, using the previously described assays, without any modification (Deshpande et al., 2016b), and used to calculate the  $AUC_{0-24}$  shown in Table 1. As a pharmacodynamic measure, the bacterial burden was estimated on days 0, 3, 7, 14, 21, 28, 35, 42, 49, and 56. Samples from the peripheral compartment were cultured on Middlebrook 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) for 21 days at 37 °C for estimation of the colony forming unit (CFU) counts. In addition, the samples were inoculated on agar containing  $2\times$  MIC of each drug to estimate the drug-resistant subpopulation (not the change in MIC). A portion of the samples in to the MGIT was also inoculated to determine if the sterilization of *Mtb* in the HFS-TB seen with agar culture was true or if the bacterial burden in those systems was simply below the limit of detection by the agar culture method ( $0.69 \log_{10}$  CFU/mL). The time-to-positive (TTP) was recorded using EpiCenter software and used for subsequent pharmacodynamic analysis.

## Results

Table 1 show the pharmacokinetic parameters, calculated using the measured drug concentrations, which were similar to those observed in the cavities of patients with TB on standard doses (Dheda et al., 2018b; Kempker et al., 2018; Ordonez et al., 2020). The MICs of the *Mtb* isolate used (ATCC #25177) for L, Z and F were 1 mg/L, 25 mg/L and 1 mg/L, respectively.

Repetitive sampling of the peripheral compartment of the HFS-TB was performed to determine the change in the bacterial burden over 56 days of the study. The results shown in Fig. 1A are for the TTP readout recorded using the MGIT system, whereas Fig. 1B shows the results based on CFU readouts on Middlebrook 7H10 agar. The non-treated control achieved a flat growth by TTP (the higher the TTP, the lower the bacterial burden) in Fig. 1A and a minimal growth of  $0.018 \pm 0.007 \log_{10}$  CFU/mL/day over 56 days in Fig. 1B. This slow growth rate validated the semidormant metabolic state of *Mtb* growing under an acidic pH of 5.8. While the  $\log_{10}$  CFU/mL readout demonstrated sterilization by the ZL regimen on day 56 (Fig. 1B), the more sensitive MGIT assay showed (Fig. 1A) that his dual regimen failed to sterilize the HFS-TB units; ZL was the worst regimen by time to negative culture. The best performing regimen was standard therapy, while  $F_1ZL$  and  $F_2ZL$  were worse than standard of therapy. However, all F-containing regimens were demonstrably better than ZL, which means that F contributes to the sterilizing effect of an LZ backbone regimen.

The kill rates with each treatment regimen were analyzed using exponential decline *versus* growth regression slopes from day 0 to the first negative culture, for TTP/day and for  $\log_{10}$  CFU/mL/day, to give a maximum kill rate for each regimen, with results shown in Fig. 2. Standard therapy had the highest slopes (best kill rate) either by TTP-based (Fig. 2A) or CFU/mL-based kill rates (Fig. 2B) at 6.59-fold (TTP slopes) and 13.22-fold (CFU/mL/day) that of ZL.  $F_2Z_{hi}L$  was ranked second best by TTP, while both  $F_1ZL$  and  $F_2ZL$  ranked third. This means the experimental regimens are unlikely to shorten therapy duration.

It was observed that there was a pre-existing isoniazid-resistant sub-population in the inoculum ( $1.34 \pm 0.57 \log_{10}$  CFU/mL) that grew to  $4.21 \pm 0.58 \log_{10}$  CFU/mL in 56 days in the non-treated HFS-TB. However, no isoniazid-resistant subpopulation was recorded in any of the L, Z and F combination regimens. Thus, these regimens likely perform well in the clinical setting for treating drug-resistant TB. There was a transient isoniazid subpopulation recorded on day 3 in the HFS-TB treated with the standard regimen, which was killed by study day 7.

## Discussion

The acidic pH in the caseum renders many antibiotics ineffective and Z, which penetrates the caseum, is one of the antibiotics that has efficacy against slowly growing organisms under acidic pH as well as non-replicating persisters. Recently, HFS-TB studies showed that L has sterilizing activity and identified the PK/PD optimized clinical dose for the treatment of TB (Srivastava et al., 2017b). In addition, F was found to have *Mtb* killing efficacy (Srivastava et al., 2016) independent of the beta-lactamase inhibitor that is used to optimize other antibiotics from the same class (Deshpande et al., 2018a; Deshpande et al., 2017; Srivastava et al., 2020; van Rijn et al., 2017). Thus, it was hypothesized that these three drugs (F, L and Z) in combination may have better sterilizing efficacy – in other words faster kill slopes – compared with standard therapy, hence have the potential to shorten therapy duration.

However, in the present study, as opposed to the current hypothesis, the F-Z-L combination failed to sterilize the HFS-TB in a shorter time compared with the standard regimen. The only consolation was the comparable kill rate with high-dose Z in combination with FL, when compared with the current standard therapy. It should be noted that the L exposure achieved in the HFS-TB was higher ( $AUC_{0-24}/MIC$  of  $137.2 \pm 8.21$ ) in the current study than the optimal exposure target identified elsewhere (Srivastava et al., 2017b). Considering the meta-analysis of Millard et al. (Millard et al., 2018) where an L MIC distribution of 0.125 mg/L to 0.5 mg/L was used in 78 clinical isolates to run Monte-Carlo simulations, the L  $AUC_{0-24}/MIC$  of  $137.2 \pm 8.21$  identified in the HFS-TB could be achieved with a 600 mg twice daily dose. However, it may also increase the possibility of L toxicity.

In the HFS-TB model, F improved the *Mtb* killing efficacy of the Z-L two-drug combination but at a slower kill rate compared with the isoniazid-rifampin-Z combination regimen. While the tested experimental combinations showed that these regimens will likely not shorten the therapy duration, the  $F_2Z_{hi}L$  regimen may provide an isoniazid and rifampin-free regimen that can be used for the treatment of MDR-TB and in select patients with drug-susceptible TB where standard drugs are intolerable.

This pre-clinical *in vitro* study had some limitations. While in the HFS-TB experiments it was found that the F-L-Z combination can kill isoniazid-resistant *Mtb*, experiments with drug-resistant clinical strains with varying MICs were not performed; with an increase in MIC, the probability of achieving the optimal exposure target decreases. Being exploratory in nature, this study did not test the combination across the MIC range of clinical isolates.

Such HFS-TB studies with MDR-TB strains are needed to confirm the sterilizing efficacy of the F-Z-L combination.

## Conclusion

This study is instrumental in identifying regimens that should not be taken further in attempts to create shorter therapy duration regimens. It highlights the role of HFS-TB in reducing the complexity of a study design to identify short treatment duration regimens for TB.

## Acknowledgments

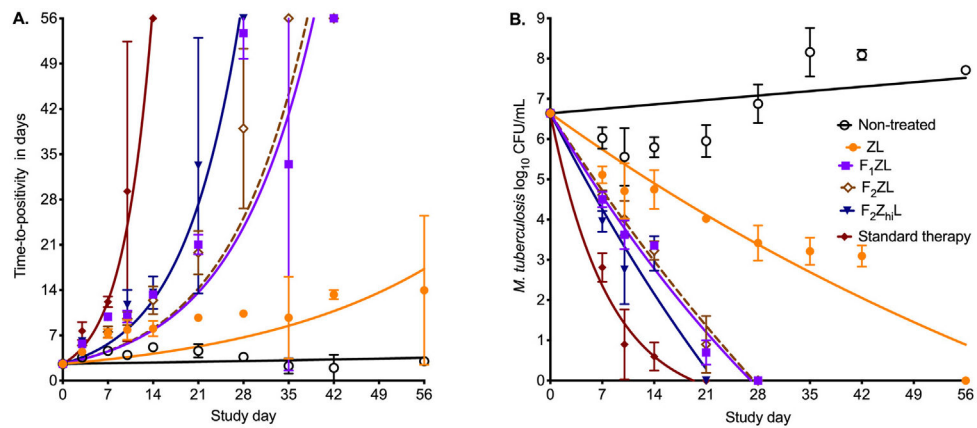
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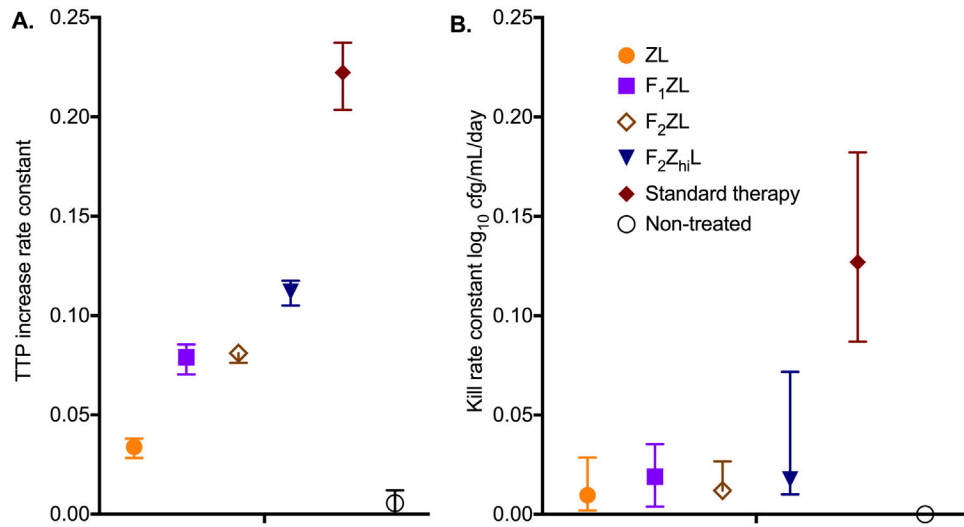


**Fig. 1.**

*Mycobacterium tuberculosis* time-to-positive and kill curves in the HFS-TB. (A) On day 56, except the ZL two-drug combination, there was no growth unit recorded by the mycobacterial growth tube indicator system (MGIT) machine in any of the hollow fiber model of tuberculosis (HFS-TB) units treated with any of the FZL experimental regimens as well as the standard therapy, hence sterilization. (B) As opposed to the TTP results, there was no *Mtb* colony recorded on agar plates inoculated with samples from the HFS-TB treated with the ZL combination. However, the bacterial burden could have simply been below the limits of detection ( $0.69 \log_{10}$  CFU/mL) because MGIT liquid culture is more sensitive than solid agar culture. All three FZL combinations as well as the standard therapy sterilized the HFS-TB by day 28.

Abbreviations: HFS-TB, hollow fiber model of tuberculosis; F, faropenem; Z, pyrazinamide; L, linezolid; TTP, time-to-positive; ZL, two-drug combination; F<sub>1</sub>ZL, F administered once daily plus ZL; F<sub>2</sub>ZL, F administered twice-daily plus once daily ZL; F<sub>2</sub>Z<sub>hi</sub>L, F<sub>2</sub>ZL with high-dose Z.





**Fig. 2.**

Kill slopes of experimental regimens compared to standard therapy. The error bars are 95% confidence interval; there were three replicates. The rate of change in non-treated controls had a 95% confidence interval that encompassed zero, which means that they did not grow.

The time-to-positive (TTP)-based kill rates had better discrimination or resolution.

Abbreviations: F, faropenem; Z, pyrazinamide; L, linezolid; ZL, two-drug combination; F<sub>1</sub>ZL, F administered once daily plus ZL; F<sub>2</sub>ZL, F administered twice-daily plus once daily ZL; F<sub>2</sub>Z<sub>hi</sub>L, F<sub>2</sub>ZL with high-dose Z.

Table 1

Combination regimens and drug exposure achieved in the HFS-TB.

	Drugs (dosing frequency)	Observed AUC <sub>0-24</sub> (mg* <i>h</i> L <sup>-1</sup> )
Regimen #1 (ZL)	Linezolid (BID)	137.2 ± 8.21
	Pyrazinamide (OD)	687 ± 92.61
Regimen #2 (F <sub>1</sub> ZL)	Linezolid (BID)	137.2 ± 8.21
	Pyrazinamide (OD)	687 ± 92.61
	Faropenem (OD)	4.23 ± 0.76
Regimen #3 (F <sub>2</sub> ZL)	Linezolid (BID)	137.2 ± 8.21
	Pyrazinamide (OD)	687 ± 92.61
	Faropenem (BID)	8.46 ± 1.52
Regimen #4 (F <sub>2</sub> Z <sub>hi</sub> L)	Linezolid (BID)	137.2 ± 8.21
	Pyrazinamide Hi dose (OD)	3587 ± 42.2
	Faropenem (BID)	8.46 ± 1.52
Regimen #5 (Standard therapy)	Isoniazid (OD)	22.05 ± 2.71
	Rifampin (OD)	11.79 ± 0.44
	Pyrazinamide (OD)	687 ± 92.61
Regimen #6	Non-treated Control	0

Abbreviations; OD, once daily; BID, twice daily; F, faropenem; Z, pyrazinamide; L, linezolid; ZL, two-drug combination; F<sub>1</sub>ZL, F administered once daily plus ZL; F<sub>2</sub>ZL, F administered twice-daily plus once daily ZL; F<sub>2</sub>Z<sub>hi</sub>L, F<sub>2</sub>ZL with high-dose Z.