

Bactericidal activity of OPC-67683 against drug-tolerant *Mycobacterium tuberculosis*

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Objectives: There is an urgent need for drugs that hasten sterilization in tuberculosis; however, we presently lack indicators of this activity to guide early drug development. We previously described a novel *in vitro* assay to study mycobacterial phenotypic drug tolerance, in which sterilizing activity could be assessed. OPC-67683 is a novel imidazooxazole that accelerates sterilization in the mouse tuberculosis model. The present study was conducted to determine the activity of OPC-67683 in the *in vitro* tolerance model using drug-tolerant clinical *Mycobacterium tuberculosis* strains.

Methods: Tolerance was assessed in Bactec radiometric culture as: (i) delayed decline in growth index during 14 days of drug exposure; (ii) shorter time to positivity of subcultures following drug exposure.

Results: Four isolates were selected from among 16 surveyed, based on delayed killing by isoniazid and OPC-67683. Unlike isoniazid and rifampicin, whose rates of killing were concentration-independent, OPC-67683 showed concentration-dependent effects that, at the highest dose levels tested (1.0 µg/mL), were superior to isoniazid and equal to rifampicin.

Conclusions: The sterilizing activity of OPC-67683 against drug-tolerant *M. tuberculosis* in the Bactec model is consistent with its activity in mice. Further studies are warranted to examine the effects of OPC-67683 on mycobacterial persistence in tuberculous patients and to determine the biological basis of tolerance in the model.

Keywords: tuberculosis, relapse, sterilization, tolerance

Introduction

It is estimated that at least 8 million new cases of tuberculosis occur each year, causing over 2 million deaths. Tuberculosis treatment presently requires 6 months of multidrug therapy. The delivery and supervision of such extended treatment place a substantial burden on public health programmes that has hindered efforts for global tuberculosis control. For this reason, shorter highly effective treatments for tuberculosis have been a high priority for tuberculosis drug development.¹

In other bacterial infections, such as streptococcal endocarditis, phenotypic drug tolerance contributes to relapse risk by reducing the rate at which non-replicating bacteria are killed.^{2,3} As in tuberculosis, relapse in endocarditis is often due to regrowth of persisting bacteria that retain the full antibiotic susceptibility of the parent strain. In 1999, a novel method was described to study mycobacterial drug tolerance,⁴ in which the Bactec-TB[®] radiometric detection system (Becton–Dickinson, Sparks, MD, USA)

was used to monitor the rate of killing. Rifampicin caused the most rapid effect of any drug tested, reducing the growth index (GI; a measure of ¹⁴CO₂ production) of most isolates from 250 (the point at which drug was added) to <20 within 1 week. Isoniazid showed intermediate activity (decreasing GI to 70 within 1 week) and ethambutol showed only bacteriostatic effects. Furthermore, isolates from patients who subsequently relapsed or whose sputum cultures cleared slowly during treatment showed delayed killing in the model, indicating that differences among strains as well as among drugs contributed to the overall effect. These data supported a role for phenotypic drug tolerance in persistence and relapse in tuberculosis.

OPC-67683 is a novel nitro-dihydro-imidazooxazole that is active against *Mycobacterium tuberculosis in vitro* and in mice, in which it hastens tissue sterilization.⁵ The present study was conducted to examine the activity of OPC-67683 against drug-tolerant *M. tuberculosis* clinical isolates and to extend experience using the Bactec tolerance model to this new compound.

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Materials and methods

Isolate selection and propagation

Banked isolates were obtained from prospective clinical trials of therapy for pulmonary tuberculosis conducted in Kampala, Uganda by the Case Western Reserve University Tuberculosis Research Unit. A representative panel was selected to include isolates from subjects whose sputum cultures cleared rapidly during treatment and did not relapse, as well as those whose response was delayed and who subsequently relapsed. In the case of subjects who relapsed, additional isolates from cultures obtained either during treatment or after relapse were included, as we had previously observed increased tolerance as treatment progressed and in relapse isolates. Isolates were given new identification numbers to preserve anonymity; however, isolates that had been studied previously in this laboratory were thus identified. Isolates from HIV-1-seropositive subjects were excluded.

Isolates were shipped from Uganda to UMDNJ as cultures on Löwenstein–Jensen medium. Isolates were then grown in Bactec 12B medium (Becton–Dickinson) to GI > 250 and frozen in aliquots. Two methods were used to prepare stock cultures of *M. tuberculosis* H37Rv: Bactec, as described earlier, and in roller bottles containing 7H9 broth, using the method of Silver *et al.*⁶ Briefly, cultures were harvested at OD = 0.7, vortexed with glass beads and frozen in aliquots. On thawing, clumps were removed by sedimentation at 400 g for 5 min prior to use.

Assessment of drug tolerance

Aliquots were thawed and propagated in Bactec 12B medium. Each isolate was studied in duplicate cultures. Inoculum volumes were selected to reach GI = 250 after 10–14 days of culture, at which time drug was added. Unless otherwise stated, 2.5 µg/mL ethambutol and 1% DMSO (final concentrations) were added to all cultures at the time of inoculation (see the Results section). In some experiments, additional 1.0 µg/mL OPC-67683 was added on day 7 of culture. GI was monitored daily for 14 days. After 14 days, 2 mL of each culture was removed. Bacteria were sedimented at 17 000 g for 15 min, resuspended and inoculated into fresh Bactec 12B medium. GI was monitored daily until positive (GI > 30) or up to 65 days.

Data analysis

GI values during the 14 days of drug exposure were adjusted by a factor of 250/(GI day 0), to account for variation in GI on the day of drug addition. The mean of duplicate cultures was taken as representing the actual value for that strain. Statistical significance was determined by repeated measures analysis of variance (RM ANOVA). This approach tests for within-strain differences among treatments. Adjustment for multiple testing was performed during *post hoc* analysis using the Holm–Sidak method. Correlations were tested by the Pearson method. Statistical tests were performed using SigmaStat (Systat, San Jose, CA, USA).

Results

Preliminary experiments

We previously had observed that in the presence of isoniazid alone, GI values for cultures of *M. tuberculosis* H37Ra declined from 250 to a nadir of 50 after 10 days, but subsequently increased due to the emergence of isoniazid-resistant clones.⁴

Emergence of resistance could be prevented by the addition of 2.5 µg/mL ethambutol, which did not otherwise affect the rate of decline due to isoniazid. For uniformity, all drugs in the present study were therefore tested in the presence of ethambutol. We also had previously reported that the rate of killing by isoniazid was concentration-independent, having tested it in concentrations ranging from 0.025 to 1.0 µg/mL. In preparation for the present study, we conducted similar experiments with rifampicin. GIs of *M. tuberculosis* H37Rv showed identical rates of decline with 0.5 and 2.0 µg/mL rifampicin, with mean values of 45, 9 and 4, and 44, 9 and 4, respectively, after 3, 7 and 10 days. All subsequent experiments were therefore conducted using only the 2.0 µg/mL concentration.

The limited solubility of OPC-67683 in aqueous solutions can be improved by the addition of 1% DMSO. Experiments were therefore conducted to determine the effects of DMSO on viability of *M. tuberculosis* H37Rv. Mean GIs of cultures without added antibiotics increased from 250 to 585 in 2 days in the presence of 1% DMSO and to 566 in control cultures, indicating that DMSO did not interfere with growth. Mean GIs declined from 250 to 42 over 14 days in the combined presence of 0.1 µg/mL isoniazid, 2.5 µg/mL ethambutol and 1% DMSO and to 43 in similar cultures without DMSO, indicating that DMSO similarly did not affect killing. All subsequent cultures in this study were therefore conducted in the presence of 1% DMSO.

Finally, two batches of *M. tuberculosis* H37Rv stock were compared, one having been propagated entirely in Bactec 12B medium and the other prepared in 7H9 broth. The 7H9 stock showed substantially greater variation in GI than the control stock after inoculation into Bactec 12B bottles (SD of replicate cultures, 83 versus 48, respectively, at 7 days), probably due to bacillary clumps that persisted despite low speed sedimentation. The 7H9-prepared cultures also showed reduced isoniazid effect when compared with Bactec control, declining from 250 to GIs of 80 and 43, respectively, after 7 days of drug exposure. This may indicate that the 7H9 culture had entered early stationary phase prior to harvesting, from which it did not fully emerge on reculture. We therefore used only culture stocks prepared entirely in Bactec in all subsequent experiments.

Selection of isoniazid-tolerant clinical isolates

Sixteen clinical isolates were screened for tolerance to isoniazid (Figure 1a). Pairs indicate isolates obtained at different time points during treatment from the same subject. These included two isolates (identified here as 103 and 116) obtained from subject 80730, whose highly tolerant strain we had previously characterized.⁴ After 2 weeks of isoniazid exposure, six clinical isolates showed mean GI values greater than that of H37Rv, whereas 10 were lower. On the basis of this experiment, isolates 102, 103, 105 and 106 were selected for further study.

Figure 1(b) shows the results of similar screening with 0.1 µg/mL OPC-67683. GIs after 2 weeks of exposure to OPC-67683 correlated very highly with those for isoniazid ($R = 0.907$, $P < 0.001$ by Spearman rank-order correlation). However, unlike isoniazid, killing of several isolates by OPC-67683 appeared to stop after 4–5 days of drug exposure. In subsequent experiments, we therefore tested OPC-67683 at higher concentrations (0.3 and 1 µg/mL) and also tested it at 1 µg/mL in cultures, in which drug addition was repeated after 1

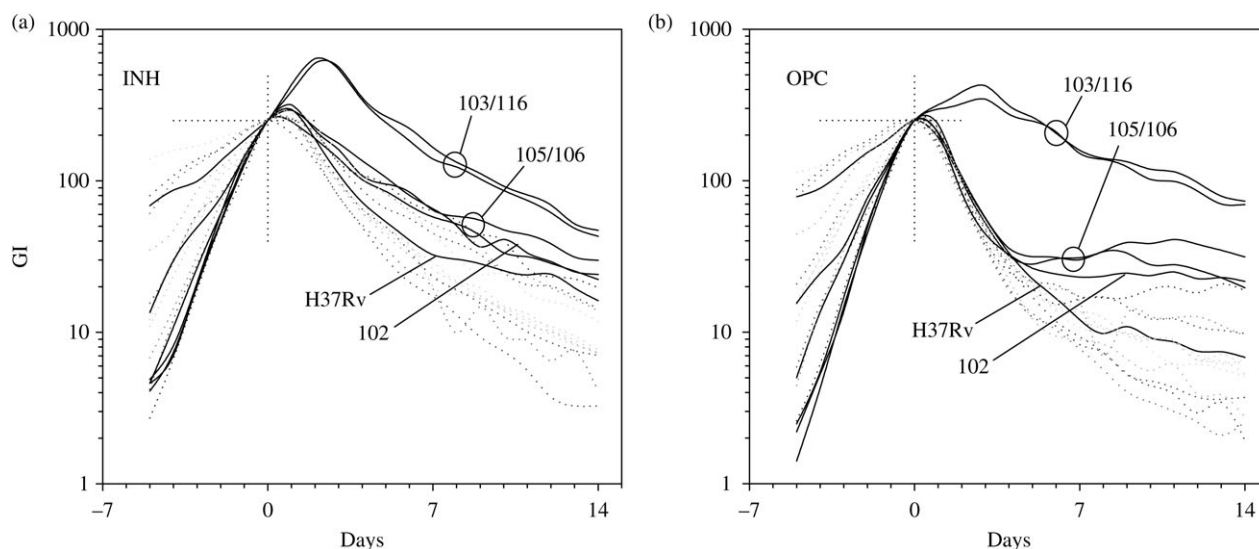


Figure 1. Screening for drug-tolerant clinical isolates of *M. tuberculosis* in the presence of 0.1 µg/mL isoniazid (INH) (a) or 0.1 µg/mL OPC-67683 (OPC) (b). All cultures were performed with added 2.5 µg/mL ethambutol (required to prevent emergence of resistance to isoniazid) and 1% DMSO (required for dissolution of OPC-67683). Paired strains were collected from the same patient at different times during treatment. A colour version of this figure is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

week, based on a report that OPC-67683 is metabolized to an inactive form by viable *M. tuberculosis*.⁵

Killing of drug-tolerant isolates

Figure 2 shows mean GIs of the four selected drug-tolerant isolates during 14 days of exposure to isoniazid, OPC-67683 and rifampicin. Statistically significant differences among the drugs emerged by day 14 ($P < 0.001$ by RM ANOVA). Killing by OPC-67683 was concentration-dependent. *Post hoc* paired testing determined that rifampicin and OPC-67683 at 1.0 µg/mL or greater differed significantly from isoniazid. At concentrations

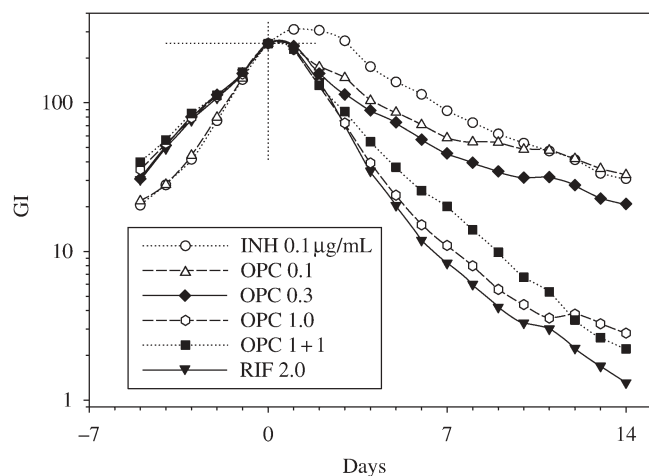


Figure 2. Killing of drug-tolerant clinical *M. tuberculosis* isolates by isoniazid (INH), OPC-67683 (OPC) and rifampicin (RIF). Data represent mean values of four isolates, each studied in duplicate. Cultures labelled 'OPC 1 + 1' had 1.0 µg/mL drug added twice on days 0 and 7. All cultures were conducted in the presence of ethambutol and DMSO.

of 1.0 µg/mL or greater, OPC-67683 did not differ from rifampicin.

The accuracy of daily Bactec readings as a measure of viability is probably reduced at low GI values. To better assess the extent of sterilization in these cultures, experiments were conducted to recover viable bacilli after 14 days of drug exposure. GIs of the subcultures were monitored daily to determine the number of days to positivity (GI \geq 30), a value that correlates with log number of initial viable bacilli.⁴ Only one culture failed to show growth in subculture (isolate 105, treated with rifampicin); its replicate became positive only after 62 days. For the purposes of statistical analysis, the sterile culture was assigned a value of 80 days. As indicated in Table 1, the number of days required to detect growth increased progressively as OPC-67683 concentration increased. At the highest dose tested (1.0 µg/mL added on days 0 and 7), its mean effects on these four isolates did not differ from rifampicin. In the case of the highly tolerant 103 strain, although mean values for OPC-67683 improved with increasing drug concentrations, they did not equal rifampicin

Table 1. Recovery of viable bacilli after 14 days of drug exposure^a

Drug	Concentration (µg/mL)	DTP (mean \pm SD)	$P < 0.05$ versus rifampicin
OPC-67683	0.1	4.2 \pm 2.0	yes
OPC-67683	0.3	5.5 \pm 2.7	yes
OPC-67683	1.0	15.4 \pm 10.4	yes
OPC-67683	1.0 + 1.0	31.4 \pm 15.7	no
Rifampicin	2.0	42.5 \pm 20.6	—

^aResults represent mean and standard deviation of days to positivity (DTP) of four drug-tolerant isolates. OPC-67683 1.0 + 1.0 indicates repeated drug addition on day 7. Statistical significance was determined by RM ANOVA, with adjustment in *post hoc* paired testing for multiple comparisons.

even at the highest level tested (values of 11 and 30 days to positivity, for OPC-67683 and rifampicin, respectively).

Discussion

There is an urgent need for new drugs to shorten therapy for tuberculosis and prevent relapse. Unfortunately, there are few mileposts to guide the discovery and early development of such drugs. The surrogate marker most closely linked to risk of relapse, sputum culture status after 2 months of therapy, cannot be assessed until Phase IIb clinical trials.^{7,8} Short-term Phase IIa studies of effects on viable sputum counts [early bactericidal activity (EBA)] do not predict sterilizing potential, as indicated by the divergent results with the sterilizing drugs rifampicin and pyrazinamide (weak or absent EBA) and the bactericidal drug ciprofloxacin (strong EBA but non-sterilizing).^{9–12} It is thought that sterilizing activity reflects effects on semi-dormant bacillary subpopulations sequestered in low-oxygen microenvironments such as granulomas.^{13,14} Its *in vitro* and pharmacokinetic correlates remain to be defined.

We have previously reported rank-order effects of rifampicin > isoniazid > ethambutol in the Bactec model.⁴ Delayed *in vitro* killing in that study was observed for isolates from 7 of the 39 subjects whose microbiological responses to treatment were delayed (defined as having sputum cultures on day 90 or later rapidly positive in Bactec). Initial and relapse organisms recovered from patient 80730 (here identified as 103 and 116) were the most tolerant of any tested. No relationship was observed between isoniazid MIC and rate of killing *in vitro*. Viable bacilli recovered after 11 days of isoniazid exposure *in vitro* showed characteristics identical to the parent strain, consistent with phenotypic tolerance. Tolerance to isoniazid and rifampicin was highly correlated, as it was in the present report with OPC-67683. These findings indicated that the Bactec tolerance model may be a useful tool in drug discovery.

The activity of OPC-67683 against *M. tuberculosis* has been reported in studies using broth culture, macrophages and mice.⁵ Like isoniazid, its mechanism of action appears to involve inhibition of mycolic acid synthesis. Unlike isoniazid, OPC-67683 has been reported to be metabolized by viable, drug-susceptible mycobacteria, to a desnitro-imidazooxazole. This may account for the loss of activity we observed in some cultures after 4–5 days at low drug concentrations in the tolerance model. Additional studies may help to determine whether killing at low OPC-67683 concentrations can be enhanced by repeated dosing or by use in combination with other tuberculosis drugs.

The main finding of the present study was that OPC-67683 showed dose-dependent killing of drug-tolerant clinical strains of *M. tuberculosis* that was superior to isoniazid and approached or equalled rifampicin, the most strongly sterilizing tuberculosis drug. To the extent that findings in the mouse model indicate potential sterilizing activity in human tuberculosis, this finding indicates a potential role for the Bactec tolerance model as an early tool to assess sterilizing activity. Inferiority of OPC-67683 to rifampicin was evident only when remaining viable bacteria were detected by subculture and when the analysis was restricted to the most tolerant strain. Sustained inhibition of growth may occur as an artefact in subculture experiments because of drug carry-over. This may lead to an over-estimation of effects, particularly for drugs such as rifampicin with high lipid solubility.

The extent of tolerance of strain 103 appears to be substantially greater than any other strains tested. Further studies are warranted to understand the genetic basis and clinical significance of this observation.

It remains uncertain why Bactec may serve as a satisfactory model of tissue sterilization. In other models, nutrient deprivation, hypoxia or dormancy induced by artificial granulomas has been required to demonstrate the superior sterilizing activity of rifampicin.^{15–17} Bactec 12B medium differs from enriched 7H9 broth in that palmitic acid is its sole carbon source. Palmitic acid induces mycobacterial expression of phosphoenolpyruvate carboxykinase and other enzymes in the glyoxylate cycle that are essential for survival under hypoxic conditions.¹⁸ Altered gene expression and metabolic profiles similar to those of hypoxia due to palmitic acid may account for the ability of the model to mimic effects in granulomas or other poorly oxygenated tissues. Further studies are warranted to examine this question.

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Transparency declarations

None to declare.

Supplementary data

A colour version of Figure 1 is available as Supplementary data at *JAC* Online (<http://jac.oxfordjournals.org/>).

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