Activity of ketoconazole against *Mycobacterium tuberculosis* in vitro and in the mouse model

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There is an urgent need for the development of new drugs that are active against drug-resistant *Mycobacterium tuberculosis* strains and can shorten tuberculosis (TB) therapy. It has previously been reported that theazole class of antifungals has anti-TB activity *in vitro*. This study evaluated ketoconazole (KTC) for activity against *M. tuberculosis*. The MIC of KTC for different *M. tuberculosis* strains ranged from 8 to 16 µg ml⁻¹ under both acidic and neutral conditions, with the minimum bactericidal concentration being about twofold higher than the MIC. KTC had enhanced activity against old, non-growing bacilli *in vitro* when combined with pyrazinamide (PZA) and rifampicin (RIF). A single oral dose of KTC at 75 mg kg⁻¹ led to an inhibitory serum concentration 2 h after administration. The *in vivo* activity of KTC was evaluated in established pulmonary TB in the murine model, compared alone and in combination with isoniazid (INH), PZA and RIF. KTC alone exhibited little effect after short-term treatment, with a borderline bacteriostatic effect on spleen colony counts but not on lung counts. KTC, when added in combination with INH, PZA and RIF, significantly improved the treatment outcome in the lungs (compared with treatment with INH, PZA and RIF). The lowest numbers of bacilli in lungs were found in mice treated with KTC, PZA and RIF. Further investigation is necessary to determine the role of KTC in the treatment of TB.

INTRODUCTION

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), remains a serious global health concern despite the widespread use of chemotherapeutic drugs and BCG (bacille Calmette–Guérin) vaccination. The development of additional methods of TB control is imperative. Of particular importance is the shortening of the duration of therapy, as well as the introduction of new compounds effective against emerging drug-resistant strains of *M. tuberculosis*.

Successful treatment of TB involves targeting the multiple populations of bacteria that reside in the host. Currently, TB is treated with a combination of drugs including isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol given for 2 months, followed by a 4 month treatment with INH and RIF (American Thoracic Society et al., 2003). The lengthy therapy is often associated with poor patient compliance and the emergence of drug resistance.

We have shown previously that members of the azole class of antifungal agents had significant anti-TB activity *in vitro* (Sun & Zhang, 1999). In addition, an interesting property of the azole drugs is that they are active against growing bacilli and, to some extent, stationary-phase bacilli. This latter property may be important for shortening the duration of TB therapy by killing a population of bacilli not readily killed by current TB drugs. Of theazole drugs, ketoconazole (KTC) was selected for examination, as it is a commonly used, systemic drug (unlike some of the other azoles that are used topically). This study evaluated KTC for its activity against *M. tuberculosis* *in vitro* and in the mouse model.

METHODS

**Bacterial strains.** *M. tuberculosis* strains H37Rv and H37Ra were obtained from the ATCC and subcultured in Middlebrook 7H9 liquid medium (Becton Dickinson) with ADC (0.5 % BSA, 0.2 % glucose, 0.0003 % catalase, 0.08 % sodium chloride), 0.2 % glycerol and 0.05 % Tween 80 (Sigma). *In vivo* studies made use of strain H37Rv, a
strain of H37Rv originally obtained from the ATCC, which was passaged twice in mice to ensure virulence and kept frozen in aliquots until infection.

**MIC and minimum bactericidal concentration (MBC) determination.** Twofold dilutions of each drug were prepared in 7H9 broth with ADC. Each tube was incubated 1:10 with a 10^{-1} dilution of a 10- to 14-day-old culture (final concentration of about 10^6 c.f.u.) of H37Rv or PAS- and streptomycin-resistant H37Rv ATCC 35824. After 14 days, tubes were inspected for visible growth and c.f.u. counts were determined for those dilutions with no visible growth by plating on 7H11 agar with ADC. The MIC value was the drug concentration that completely inhibited growth. The MBC value was the minimum concentration that killed 99% of inoculated bacteria. Determination of MIC and MBC was performed under both neutral (pH = 6.7–6.8) and acidic (pH = 5.5–5.8) conditions.

**Drug exposure assay.** Two-month-old cultures of strain H37Ra were washed and resuspended in an equal volume of 7H9 broth without ADC or glycerol supplementation and exposed for 3 days to KTC (1, 5, 10 or 50 μg ml^{-1}), RIF (10 μg ml^{-1}) or PZA (100 μg ml^{-1}), or combinations of RIF, PZA and KTC (Table 1). Drugs were dissolved in DMSO and diluted to a final concentration of 0.1% DMSO. Prior to inoculation, the medium was adjusted to a pH of 5.5–5.8 with 1 M HCl (Fisher Scientific). Solvent alone was used as a control. Following exposure for the indicated time, cells were washed and resuspended to remove the drug(s) and plated on 7H11 agar with ADC and 0.5% glycerol supplement to enumerate the remaining live cells.

**Determination of serum inhibitory titre (SIT) of the azole drug KTC.** Eight-week-old Swiss mice (NCI) were administered KTC (75 mg kg^{-1}), INH (25 mg kg^{-1}) as a control or no drug in 0.2 ml sterile water by oral gavage. Mice were then anaesthetized and exsanguinuted 1–2 h after dosing. As the SIT for KTC in relation to M. tuberculosis had not been determined previously, the serum half-life was used as a guide for determining when to collect serum.

**RESULTS AND DISCUSSION**

**MIC and MBC values of KTC against M. tuberculosis strains**

The azole class of antifungals including miconazole and clotrimazole was previously identified by our laboratory as having in vitro anti-TB activity (Sun & Zhang, 1999). As miconazole and clotrimazole are topical agents, we evaluated the activity of KTC against M. tuberculosis in this study. Itraconazole and fluconazole had no significant anti-TB activity in vitro (unpublished data). The MIC of KTC against virulent M. tuberculosis H37Rv in liquid medium was 8–16 μg ml^{-1} and the MBC was 32 μg ml^{-1}. The MIC of KTC against strain H37Ra in antibiotic-containing solid medium was found to be 8 μg ml^{-1} and the MBC was 16 μg ml^{-1}. The MIC and MBC determinations for H37Rv and H37Ra were performed at both neutral and acidic pH values and similar values were found under both conditions.

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<table>
<thead>
<tr>
<th>Exposure condition</th>
<th>Mean log_{10} (c.f.u. ml^{-1}) ± sd*</th>
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<tbody>
<tr>
<td>Control</td>
<td>5.88 ± 0.03</td>
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<tr>
<td>PZA (100 μg ml^{-1})</td>
<td>5.67 ± 0.06†</td>
</tr>
<tr>
<td>RIF (10 μg ml^{-1})</td>
<td>4.60 ± 0.24†</td>
</tr>
<tr>
<td>PZA (100 μg ml^{-1}) + RIF (10 μg ml^{-1})</td>
<td>4.67 ± 0.19†</td>
</tr>
<tr>
<td>KTC (1 μg ml^{-1})</td>
<td>5.78 ± 0.12</td>
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<tr>
<td>KTC (1 μg ml^{-1}) + PZA + RIF</td>
<td>4.14 ± 0.05‡</td>
</tr>
<tr>
<td>KTC (5 μg ml^{-1})</td>
<td>5.67 ± 0.16</td>
</tr>
<tr>
<td>KTC (5 μg ml^{-1}) + PZA + RIF</td>
<td>4.37 ± 0.28</td>
</tr>
<tr>
<td>KTC (10 μg ml^{-1})</td>
<td>5.70 ± 0.17</td>
</tr>
<tr>
<td>KTC (10 μg ml^{-1}) + PZA + RIF</td>
<td>3.80 ± 0.08§</td>
</tr>
<tr>
<td>KTC (50 μg ml^{-1})</td>
<td>4.00 ± 0.30‡</td>
</tr>
<tr>
<td>KTC (50 μg ml^{-1}) + PZA + RIF</td>
<td>&lt;2.00§</td>
</tr>
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</table>

*In this assay, the lower limit of detection was 2.00 log_{10} (c.f.u. ml^{-1}).
†P < 0.005 compared with the control group.
‡P < 0.05 compared with the PZA + RIF group.
§P < 0.005 compared with the PZA + RIF group.
Synergy of KTC with PZA and RIF in vitro

In TB infection, it is thought that a subpopulation of bacteria, originally susceptible to antibiotics, is able to persist in tissues despite exposure to drugs (McCune et al., 1957). A compound that targets this subset of cells may be able to shorten the duration of therapy. The adoption of PZA, which has been shown to be active against non-replicating persisting bacilli in vitro (Zhang et al., 2002), into the regimen for TB treatment has partly been responsible for a shortening in the duration of therapy. Through a drug exposure assay that measured activity against non-growing bacteria, KTC was found to exhibit a synergistic effect with RIF and PZA after a 3 day exposure in 7H9 broth without ADC, pH 5.5 (Table 1). Two-month-old M. tuberculosis H37Ra cultures were exposed for 3 days to KTC (1, 5, 10 or 50 μg ml⁻¹), RIF (10 μg ml⁻¹) or PZA (100 μg ml⁻¹), or combinations of KTC with RIF and PZA under acidic conditions (pH 5.5). RIF was used as a positive control instead of INH because the culture used for drug exposure was 2 months old and it is known that old cultures are not susceptible to INH but are more susceptible to RIF. No synergy was detected following short-term exposure against exponential-phase M. tuberculosis (data not shown).

KTC at a concentration of 50 μg ml⁻¹ exhibited significant anti-TB activity during a short-term exposure on non-growing cells, reducing viable bacterial counts from 5.88 log₁₀ (c.f.u. ml⁻¹) (control) to 4.00 log₁₀ (c.f.u. ml⁻¹) (Table 1). This decrease was greater than that seen with RIF or PZA alone or in combination. Furthermore, considerable anti-TB activity was observed when KTC (50 μg ml⁻¹) was combined with RIF and PZA. After exposure to these three drugs for 3 days, no viable bacteria could be cultured [lower limit of detection was 2 log₁₀ (c.f.u. ml⁻¹)]

Lower concentrations of KTC (1, 5 and 10 μg ml⁻¹) were found to have minimal effects on the non-growing population of TB after a 3 day exposure (reduction in c.f.u. values of less than tenfold). However, when combined with RIF and PZA, these lower concentrations of KTC showed greater anti-TB activity than RIF or PZA alone (Table 1).

The mode of action of KTC may be due to inhibition of M. tuberculosis cytochrome P450 mono-oxygenases (McLean et al., 2002). Azole antifungals have been shown to bind to the P450s CYP121 and CYP51 in M. tuberculosis and may exert their antimycobacterial properties by targeting these molecules (McLean et al., 2002). Azoles have also been found to inhibit the tricarboxylic acid cycle enzyme succinate dehydrogenase (Boshoff et al., 2004). The enhancement of PZA activity by KTC seen in this study could be related to the inhibition of succinate dehydrogenase by KTC such that it makes it easier for PZA, which depletes membrane energy, to kill tubercle bacilli. Further investigation into the mechanism of action of KTC on M. tuberculosis is needed.

Serum inhibitory testing

Before drug assessment in vivo, serum inhibitory testing was performed. Serum inhibitory testing is an in vitro assay that combines pharmacodynamic and pharmacokinetic principles to determine the antibacterial activity of a drug in the serum following administration (NCCLS, 1999). A single 75 mg kg⁻¹ dose of KTC resulted in a serum concentration that was able to inhibit the growth of M. tuberculosis 2 h after oral administration. The SIT is the lowest dilution of serum that inhibits growth of M. tuberculosis in vitro. At the given dose, KTC was found to have an SIT of 1:2 to 1:4 against M. tuberculosis.

Dose optimization in unestablished murine infection

To optimize oral dosing of KTC in an in vivo murine model of unestablished infection, 4-week-old, female BALB/c mice were infected with an aerosolized, exponential-phase culture of a mouse-adapted strain of M. tuberculosis H37Rv. Treatment was initiated the day after infection and continued for 5 days per week for 4 weeks. By employing a dosing study, KTC at 75 mg kg⁻¹ was found to be the lowest daily dose with activity against unestablished, murine TB infection, in a manner similar to treatment with INH alone (data not shown). Daily administration with this dose for 1 month was found to have no overt adverse effects in the infected animals.

Activity of KTC after short-duration treatment of established TB in the mouse model

The efficacy of KTC in combination with currently used TB drugs was assessed in a murine model of established pulmonary TB. KTC was co-administered daily for 1 month with various combinations of INH, PZA and RIF. Following treatment, the total number of c.f.u. in lung and spleen tissues was determined (Table 2).

On the day of treatment initiation (day 16 post-infection), signs of a well-established infection were present in the lungs and spleen of mice, and by day 44 post-infection, at treatment discontinuation, the number of bacteria increased by about 0.8 log₁₀ c.f.u. in the lungs and 1.8 log₁₀ c.f.u. in the spleen. After 1 month of treatment with the positive-control RIF only, there was a decrease in the number of bacilli in the lungs and spleen compared with untreated mice. Treatment with KTC only for 1 month led to a slight bacteriostatic effect in the spleen (P <0.01, compared with untreated mice at the conclusion of the study), but no significant change in lung counts was observed (P >0.05) (Table 2).

The group treated with the combination of INH, PZA and RIF had lung counts that were about 3 log₁₀ c.f.u. less than those of the controls at the beginning of treatment and almost 4 log₁₀ c.f.u. less than those of the controls at the end of the study (P <0.001). Mice receiving INH, PZA and
In this experiment, the lower limit of detection was 0.90 log10 c.f.u. for group, and one mouse in the group had a spleen positive. Mice given KTC, INH and PZA (replacement of RIF with essentially indistinguishable from the INH, PZA and RIF regimen of INH, PZA and RIF resulted in increased killing of bacteria. These results are preliminary in nature and further investigations are required to compensate for the pharmacokinetic interaction, even though a normal dose of RIF was shown to have an effect when combined with KTC in this study.

This account is the first report of the potential anti-TB activity of KTC in vivo. An enhancement of anti-TB activity was observed in vitro against non-growing M. tuberculosis when KTC was combined with PZA and RIF. The results from short-course treatment in this mouse model indicate that replacement of INH in the standard regimen with KTC may lead to increased killing of bacteria. These results are preliminary in nature and further investigations are underway to examine the effect of KTC in long-term treatment of M. tuberculosis infection in the mouse model.

ACKNOWLEDGEMENTS

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REFERENCES


Table 2. Lung and spleen c.f.u. counts at 44 days after treatment, which was initiated at 16 days post-inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean log10 c.f.u. ± SD*</th>
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<tbody>
<tr>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>Untreated (initial count)†</td>
<td>6.71 ± 0.16</td>
</tr>
<tr>
<td>Untreated (end count)†</td>
<td>7.52 ± 0.54</td>
</tr>
<tr>
<td>RIF</td>
<td>5.66 ± 0.26‡</td>
</tr>
<tr>
<td>KTC</td>
<td>7.37 ± 0.13</td>
</tr>
<tr>
<td>INH + PZA + RIF</td>
<td>3.63 ± 0.18‡</td>
</tr>
<tr>
<td>KTC + INH + PZA + RIF</td>
<td>3.29 ± 0.13§</td>
</tr>
<tr>
<td>KTC + INH + PZA</td>
<td>4.91 ± 0.33∥</td>
</tr>
<tr>
<td>KTC + INH + RIF</td>
<td>3.57 ± 0.09</td>
</tr>
<tr>
<td>KTC + PZA + RIF</td>
<td>2.78 ± 0.54∥</td>
</tr>
</tbody>
</table>

*In this experiment, the lower limit of detection was 0.90 log10 c.f.u.
†c.f.u. counts were determined for untreated controls at day 16 (initial count) and at day 44 (end count).
‡P < 0.01 compared with the untreated group at study conclusion.
§P < 0.05 compared with the INH, PZA, RIF group.
∥P < 0.01 compared with the INH, PZA, RIF group.
¶Positive spleen count in one animal; all other spleens in this group were negative.


