Correlation of *in vitro* activity and *in vivo* efficacy of itraconazole intravenous and oral solubilized formulations by testing *Candida* strains with various itraconazole susceptibilities in a murine invasive infection

Katsuhisa Uchida¹, Kosuke Shimogawara² and Hideyo Yamaguchi¹*

¹Teikyo University Institute of Medical Mycology, Hachioji, Tokyo 192-0395, Japan; ²Laboratory of Chemistry, Teikyo University School of Medicine, Hachioji, Tokyo 192-0395, Japan

*Corresponding author. Tel: +81-42-676-3003; Fax: +81-42-678-3285; E-mail: hyamaguc@main.teikyo-u.ac.jp

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**Objectives:** To examine whether *in vitro* antifungal susceptibility test results correlate with *in vivo* efficacy of two cyclodextrin-solubilized itraconazole formulations (intravenous and oral) against *Candida* in a murine model of invasive infection.

**Methods:** A selected set of 12 *Candida* spp. strains with various itraconazole susceptibilities were tested. We studied the efficacy of intravenous and oral itraconazole administered once daily at dosages of 0.63, 2.5, 10 and 40 mg/kg body weight in mice lethally infected with each tested strain. Survival of mice in each treated group was monitored daily until the death of all control mice and compared between groups.

**Results:** Survival of mice infected with 9 of 12 *Candida* strains with itraconazole MICs of ≤0.016–2.0 mg/L was significantly prolonged by treatment with intravenous itraconazole at dosages of 2.5 or 10 mg/kg and above. In contrast, the other three strains resistant to 8 mg/L itraconazole *in vitro* were refractory to the therapy, even at the highest itraconazole dosage (40 mg/kg). Closely similar *in vivo* data were obtained with the oral itraconazole therapy. The effective doses of the two itraconazole formulations increased with increasing itraconazole MICs for the infecting strains.

**Conclusions:** The *in vivo* efficacy of intravenous and oral itraconazole correlated with the *in vitro* susceptibility data.

**Keywords:** itraconazole, intravenous formulation, oral solution, *in vitro* antifungal activity, *in vivo* efficacy, *Candida*-infected mice

**Introduction**

Itraconazole is a triazole antifungal agent with potent *in vitro* activity against a wide range of pathogenic fungi, including *Candida* spp.¹,² Initially, itraconazole was used as a capsule formulation for the treatment of various types of superficial and systemic fungal infections. However, erratic absorption and decreased bioavailability of itraconazole have been reported in some patients, particularly in immunosuppressed patients, and low blood drug levels have been associated with therapeutic failure.³,⁴

Since improved absorption of itraconazole is considered to result in its increased therapeutic efficacy, two solubilized itraconazole formulations (oral and intravenous) were developed. Both formulations contain a combination of lipophilic itraconazole and hydroxypropyl-β-cyclodextrin (a ring of substituted glucose molecules), which improves the solubility of the drug.⁵,⁶ The enhanced absorption and bioavailability of itraconazole in these two newer formulations make them potentially applicable for the treatment and prophylaxis of invasive fungal infections in a wide range of patient populations, including high-risk populations for which the capsule formulation may be impractical.⁷ In fact, a number of large, multicentre prospective studies thus far performed to evaluate the prophylactic usefulness of intravenous and/or oral itraconazole in patients with haematological malignancies or those receiving allogeneic stem cell transplants gave some promising results⁸–¹⁰ although the success was limited by drug-related toxicities.⁹–¹¹

In general, adequately measured *in vitro* antifungal activity in terms of MIC is considered to be of clinical relevance, and often gives useful therapeutic clues if there is a good correlation between the *in vitro* MIC of an antifungal agent and its efficacy...
in vivo. Intensive efforts to develop standardized, reproducible and clinically relevant methods for susceptibility testing of Candida spp. and other yeasts have resulted in the development of the CLSI M27-A methodology (now updated with the essentially identical M27-A3 methodology).12,13 Data-driven interpretive breakpoints in three categories, namely, susceptible (S), susceptible-dose/delivery dependent (S-DD) and resistant (R), are currently available for testing the susceptibility of Candida spp. to itraconazole and two other triazoles (fluconazole and voriconazole).14 The data for the tentative establishment of itraconazole interpretive breakpoints for Candida spp., or their supportive data, were limited to those regarding clinical responses for oropharyngeal candidiasis in HIV-related patients treated with itraconazole capsules or oral solution.15–19 The itraconazole interpretive breakpoints derived from data on the treatment of mucosal candidiasis with only itraconazole formulations for oral use may not be adequate for interpreting the MICs of itraconazole for Candida spp. as the causative agent of invasive fungal infections. However, few studies have reported experimental or clinical data on the association between in vivo test results and the in vivo efficacy of the drug, particularly of its soluble formulations for oral or intravenous use.

In this study, we used 12 Candida spp. strains of three different CLSI susceptibility categories for itraconazole, i.e., S, S-DD and R, and a murine model of invasive candidiasis to examine the correlation between MICs determined according to the CLSI methodology and therapeutic outcome when Candida-infected animals were treated with intravenous or oral itraconazole. The tested strains also included a Candida strain that showed a ‘trailling’ growth phenomenon1,2,3 when tested against itraconazole—the strain was classified as a trailing strain (TR strain).

### Materials and methods

#### Strains

A selected set of 12 Candida spp. strains (mostly clinical isolates) belonging to different M27-A susceptibility categories for itraconazole were tested in a murine model of invasive candidiasis. Seven strains, consisting of three S strains, two S-DD strains, one R strain and one TR strain, were collected between June 2001 and June 2005 as part of the nationwide Japan Antifungal Surveillance Program 200121 and 2005.22 One S-DD strain and three R strains were selected from the collection at our institution (TIMM Collection). One R strain of Candida albicans (ATCC 64124) was purchased from the ATCC. Species identification was confirmed using the yeast identification system. The 12 strains comprised 11 C. albicans and 1 Candida tropicalis strains.

#### Itraconazole preparations

A bulk powder sample of itraconazole was provided by Janssen Research Foundation, Beerse, Belgium. Two itraconazole formulations, one for intravenous infusion and the other for oral intake (Janssen Pharmaceutical K.K., Tokyo, Japan) were used. In both formulations, itraconazole was solubilized in 40% (w/v) hydroxypropyl-β-cyclodextrin (Acros Organics, NJ, USA).

#### Broth microdilution susceptibility testing

MICs were determined according to the CLSI M27-A3 microdilution procedure.13 Itraconazole (powder) was dissolved in and diluted with RPMI 1640 medium (with L-glutamine and without bicarbonate) (Sigma Chemical Co., St Louis, MO, USA), and buffered to pH 7.0 with 0.165 M MOPS. The final itraconazole concentration range was 0.016–8 mg/L.

Testing was performed in 96-well round-bottom microtitration plates. The plates were incubated at 35°C, and were read spectrophotometrically after incubation for 24 and 48 h. The MIC of itraconazole was the lowest concentration at which 50% reduction of turbidity was produced, as compared with that for the drug-free control. Itraconazole MICs of ≤0.125, >0.125 to ≤1 and >1 mg/L determined after 48 h of incubation were defined as S, S-DD and R, respectively.14 When a strain exhibited trailing growth the MICs at 24 h and 48 h were S and R, respectively, the strain was considered to be a TR strain.

#### In vivo study in an animal model

Four-week-old male ICR SPF mice (Charles River Japan Inc., Kanagawa, Japan) were used in the study. In preliminary experiments, groups of five mice were intravenously infected with various inocula of each tested Candida strain to determine the inoculum of each strain that led to most deaths between days 2 and 5. In the treatment studies, randomly selected groups of five to seven mice were inoculated with the lethal inoculum for each strain via the caudal vein on day 0. The treated mice were administered intravenous or oral itraconazole (diluted with saline) at doses of 0.625, 2.5, 10 and 40 mg per kg body weight once a day in a volume of 0.2–0.25 mL, 1 h after intravenous infection; treatment of mice was continued until death for up to 5 days. Intravenous itraconazole solution was administered via the caudal vein and oral itraconazole solution was orally administered with the aid of gastric gavage under fasting conditions. The untreated control animals were intravenously or orally administered 40% hydroxypropyl-β-cyclodextrin solution without itraconazole. Following infection, the mice were observed twice daily and deaths were recorded. The mice were observed for survival over a period of 9 days. At the time-point at which no animal survived in the untreated control group, the numbers of surviving animals in each treated group were counted to

### Table 1. Characterization of the in vitro itraconazole susceptibility levels of 12 Candida isolates used in this study on the basis of the CLSI M27-A broth microdilution method

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Species</th>
<th>MIC (mg/L) at 24 h</th>
<th>MIC categories of CLSI interpretive guidelines</th>
<th>MIC (mg/L) at 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 (JASP 01001)</td>
<td>C. albicans</td>
<td>≤0.016</td>
<td>S</td>
<td>≤0.016</td>
</tr>
<tr>
<td>#2 (JASP 02963)</td>
<td>C. albicans</td>
<td>≤0.016</td>
<td>S</td>
<td>≤0.016</td>
</tr>
<tr>
<td>#3 (JASP 05003)</td>
<td>C. albicans</td>
<td>≤0.016</td>
<td>S</td>
<td>0.032</td>
</tr>
<tr>
<td>#4 (JASP 01244)</td>
<td>C. albicans</td>
<td>0.125</td>
<td>S-DD</td>
<td>0.5</td>
</tr>
<tr>
<td>#5 (JASP 05363)</td>
<td>C. tropicalis</td>
<td>0.25</td>
<td>S-DD</td>
<td>0.5</td>
</tr>
<tr>
<td>#6 (TIMM 3165)</td>
<td>C. albicans</td>
<td>0.25</td>
<td>S-DD</td>
<td>0.5</td>
</tr>
<tr>
<td>#7 (TIMM 3163)</td>
<td>C. albicans</td>
<td>1</td>
<td>R</td>
<td>1</td>
</tr>
<tr>
<td>#8 (JASP 05539)</td>
<td>C. albicans</td>
<td>1</td>
<td>R</td>
<td>2</td>
</tr>
<tr>
<td>#9 (TIMM 3314)</td>
<td>C. albicans</td>
<td>≥8</td>
<td>R</td>
<td>≥8</td>
</tr>
<tr>
<td>#10 (TIMM 3969)</td>
<td>C. albicans</td>
<td>≥8</td>
<td>R</td>
<td>≥8</td>
</tr>
<tr>
<td>#11 (ATCC 64124)</td>
<td>C. albicans</td>
<td>≥8</td>
<td>R</td>
<td>≥8</td>
</tr>
<tr>
<td>#12 (JASP 01215)</td>
<td>C. albicans</td>
<td>≤0.016</td>
<td>S-DD</td>
<td>≥8</td>
</tr>
</tbody>
</table>

*a* Institutional strain names are in parentheses.  
*b* Values to be adopted as MICs according to the CLSI M27 guideline.  
*c* MICs at 24 h and 48 h were defined as S and R, respectively.
determine the survival rate. The experiments were repeated two or three times and the survival data were pooled for statistical analysis. All animal care procedures were supervised and approved by Teikyo University Animal Welfare Committee.

**Statistical methods**

Survival data for each group of treated mice were compared with those for a group of untreated mice and among groups of treated mice using the log rank test. In addition, the 50% effective dose (ED$_{50}$) was calculated by logistic regression using maximum likelihood estimation, derived from the survival rates for each group of treated animals determined at the timepoint at which no surviving animal was observed in the untreated control group. Correlations between individual MICs and ED$_{50}$ were examined using Spearman’s rank correlation coefficient.

**Results**

**In vitro susceptibility testing results**

The results of itraconazole MIC testing of 12 Candida strains used to infect mice are presented in Table 1. Each strain was tested at least twice by using the M27-A3 microdilution procedure. The results obtained for all strains were basically identical to the previously recorded results, indicating the stability of their itraconazole susceptibilities. On the basis of these in vitro testing results, the following itraconazole susceptibility categories for the 12 tested strains were confirmed: 3 strains with low MICs ($\leq 0.016$–$0.032$ mg/L) at both 24 and 48 h (Strains #1, #2 and #3) were categorized as S; 5 strains with intermediate MICs (0.125–0.5 mg/L) at both 24 and 48 h (Strains #4, #5 and #6) were categorized as S-DD; and 5 strains with high MICs ($\geq 1.0$ mg/L) at both 24 and 48 h (Strains #7, #8, #9, #10 and #11) were categorized as R. The residual strain was a TR strain (Strain #12).

Response to therapy with different doses of intravenous and oral itraconazole

After establishing the proper inoculum size for each of the 12 strains, two survival treatment experiments were performed with all of the strains. Since there were no intertreatment differences with regard to the survival times of each strain in the untreated control group ($P>0.1$), the data from the replicate treatment experiments were pooled to determine a single estimate of survival for each strain with each dose of both itraconazole formulations.

Treatments with intravenous itraconazole at doses of 2.5 or 10 mg/kg/day resulted in a significantly prolonged survival of the mice infected with all three S and all three S-DD strains compared with survival of the control group (Figure 1). This was also the case for the two R strains with relatively low MICs of 1–2 mg/L (strains #7 and #8) and the one TR strain. In contrast, survival was not prolonged in the mice infected with the three R strains with higher MICs of $\geq 8$ mg/L (strains #9, #10 and #11) even after treatment with the highest dose (40 mg/kg/day) of intravenous itraconazole (Figure 1). Virtually the same survival data were obtained when the infected animals were treated with oral itraconazole (Figure 2).

**Relationship between in vitro MIC and in vivo response to therapy**

The ED$_{50}$ of intravenous and oral itraconazole for animals infected with each of the 12 tested strains were calculated on the basis of survival data by using the cumulative distribution function (Table 2). The ED$_{50}$ of intravenous itraconazole for the three S strains were in the range 2.43–4.45 mg/kg/day (mean, 3.8 mg/kg/day), and were similar to those for the three S-DD strains (2.14–4.02 mg/kg/day; mean, 3.0 mg/kg/day) and also that for the one TR strain (5.00 mg/kg/day). Compared with these values, the ED$_{50}$ for the two R strains with relatively low MICs (i.e. Strains #7 and #8, 7.07 and 7.69 mg/kg/day (mean, 7.4 mg/kg/day), respectively) seemed to be similar or only slightly greater, whereas those for the three R strains with higher MICs amounting to $\geq 40$ mg/kg/day increased by almost one order of magnitude. The ED$_{50}$ data on oral itraconazole were almost comparable (Table 2).

With the use of these ED$_{50}$ values, we examined their association with in vitro itraconazole susceptibilities on the basis of scatter plots of itraconazole MIC results for 12 Candida strains versus in vivo responses to itraconazole treatment. As shown in Figure 3(a), the Spearman’s rank correlation coefficient between MICs and ED$_{50}$s of intravenous itraconazole was 0.669 ($P=0.017$). The pattern of correlation between MICs and therapeutic outcome of oral itraconazole was virtually the same (0.856; $P=0.00038$) (Figure 3b).

**Discussion**

On the basis of the clinical and pharmacokinetic data available, the CLSI interpretive breakpoints of itraconazole for in vitro susceptibility testing of Candida spp. are defined as follows: S, $\leq 0.125$ mg/L; S-DD, 0.25–0.5 mg/L; and R, $\geq 1.0$ mg/L. However, such data are derived entirely from experience with response of mucosal candidiasis, in particular oropharyngeal candidiasis, to orally administered itraconazole capsules. No clinical data supporting the breakpoints for invasive Candida infections in cases in which intravenous or oral itraconazole was used for treatment have been reported.

Although itraconazole has a potent and broad-spectrum in vitro activity against Candida spp., itraconazole capsule administration has been reported to lead to erratic absorption and low serum drug levels, and this has been associated with clinical failure of the drug. To improve the kinetic drawback of itraconazole capsules, two newer itraconazole formulations with better bioavailability, i.e. intravenous and oral itraconazole, have been developed. Thus far, a number of pharmacokinetic studies conducted in healthy volunteers as well as in various patient populations, including HIV-infected patients, neutropenic patients and bone marrow transplant recipients, have demonstrated that when compared with capsule itraconazole, the bioavailability of oral itraconazole is increased by $\geq 60\%$. The intravenous formulation of itraconazole circumvents all the problems of bioavailability. Furthermore, excellent pharmacokinetic properties of intravenous itraconazole were confirmed in several different patient populations, such as those with haematological malignancy, deep mycoses and persistent neutropenic fever. Therefore, improved bioavailability of both intravenous and oral itraconazole formulations can make them effective in the
Figure 1. Cumulative mortality of mice infected with 12 Candida strains with various in vitro itraconazole susceptibilities (Strain #1–Strain #12) and treated with intravenous itraconazole at 0.63, 2.5, 10 or 40 mg/kg/day. *P < 0.05 versus the control. †P < 0.05 versus itraconazole 0.63 mg/kg/day. ‡P < 0.05 versus itraconazole 2.5 mg/kg/day. §P < 0.05 versus itraconazole 10 mg/kg/day. Numbers of mice contained in all treatment groups and the control group were: 10 each for Strains #4, #8, #9, #10 and #11; 12 each for Strains #1, #2, #3, #5 and #12; and 14 each for Strains #6 and #7.
Figure 2. Cumulative mortality of mice infected with 12 Candida strains with various in vitro itraconazole susceptibilities (Strain #1–Strain #12) and treated with oral itraconazole at 0.63, 2.5, 10 or 40 mg/kg/day. \(^a^P<0.05\) versus the control. \(^b^P<0.05\) versus itraconazole 0.63 mg/kg/day. \(^c^P<0.05\) versus itraconazole 2.5 mg/kg/day. \(^d^P<0.05\) versus itraconazole 10 mg/kg/day. Numbers of mice contained in all treatment groups and the control groups were 10 each for all of the tested strains.
In vitro–in vivo correlation for itraconazole

In agreement with our expectation, intravenous itraconazole therapy increased the survival of mice infected with 9 of the 12 tested Candida strains to a similar extent and in a dose-dependent manner; only the 3 residual Candida strains with the highest itraconazole MIC (≥8 mg/L) were completely refractory to the highest dose of the drug (40 mg/kg/day). Oral itraconazole had virtually the same therapeutic efficacy. In addition, significant correlation was found between in vitro test results (MICs) and in vivo therapeutic outcome (according to ED50) in mice treated with intravenous or oral itraconazole. It is noteworthy that the strains that were responsive to therapy with each formulation included not only the S and S-DD strains, but also the R strains with relatively low MICs (1 or 2 mg/L). The ED50 for these R strains increased only 2- or 3-fold as compared with those for the S or S-DD strains, thereby indicating that in vivo outcome for the R strains with MICs of 1–2 mg/L is distinct from that for the R strains with MICs of ≥8 mg/L, and closer to that for the S and S-DD strains. This suggests that invasive infections with such low resistance Candida strains might respond to itraconazole therapy.

In conclusion, the results of our study show that both intravenous and oral itraconazole formulations can protect not only mice infected with Candida spp. strains with an itraconazole MIC range of ≤0.016–0.5 mg/L (CLSI category S or S-DD), but also those infected with strains with an itraconazole MIC range of 1–2 mg/L (CLSI category R) to virtually the same extent and in a dose-dependent fashion. The results also show that mice infected with R strains non-susceptible to 8 mg/L itraconazole are refractory to therapy with both itraconazole formulations. Furthermore, it appears that effective doses of intravenous or oral itraconazole in infected mice are correlated with itraconazole MICs for Candida strains challenged.

| Table 2. Effects of intravenous and oral itraconazole (ITC) in terms of ED50 on the survival of mice infected with 12 Candida strains with various in vitro ITC susceptibilities |
|---------------------------------|-----------------|-----------------|-----------------|
| Infecting Candida strain (strain no.) | MIC of ITC (mg/L) | ED50 (mg/kg/day) of intravenous oral ITC ITC |
| #1 | ≤0.016 | 4.44 | 3.26 |
| #2 | ≤0.016 | 4.45 | 5.01 |
| #3 | ≤0.016 | 2.43 | 2.70 |
| #4 | 0.5 | 2.14 | 3.10 |
| #5 | 0.5 | 2.79 | 5.94 |
| #6 | 0.5 | 4.02 | 7.92 |
| #7 | 1.0 | 7.07 | 10.0 |
| #8 | 2.0 | 7.69 | 15.3 |
| #9 | ≥8.0 | >40 | >40 |
| #10 | ≥8.0 | >40 | >40 |
| #11 | ≥8.0 | >40 | >40 |
| #12 | ≤0.016 | 5.00 | 5.78 |

In vitro–in vivo correlation for itraconazole therapy in murine models of invasive candidiasis caused not only by the S or S-DD Candida strains, but also by some of the R strains of the CLSI interpretive guidelines. However, no clinical study has dealt with the clinical relevance of in vitro itraconazole susceptibility with these two itraconazole formulations for the treatment of patients with invasive candidiasis.

Animal studies are potential substitutes for clinical studies in humans to evaluate the correlations between MIC and in vivo outcome, and, eventually, to determine the interpretive breakpoints. Intravenously infected murine models of invasive candidiasis have been frequently used to study the efficacy of antifungal drugs. However, Candida infections in humans cannot be perfectly mimicked in murine models, and, moreover, itraconazole kinetics seem to substantially differ in humans and experimental animals. Despite such limitations, the results of animal studies in murine models of haematogenous candidiasis have provided useful information on the in vitro–in vivo correlation of fluconazole. The lack of clinical data in both humans and animals on infections in humans cannot be perfectly mimicked in murine models, and, moreover, itraconazole kinetics seem to substantially differ in humans and experimental animals. However, no such information is available on any itraconazole formulation.

The selected set of Candida strains used in the present study included one TR strain (Strain #12). Almost all published studies on trailing growth phenomena have dealt with the phenomenon observed in the fluconazole test. Such trailing isolates of C. albicans have been shown to respond to fluconazole therapy in murine models of invasive candidiasis and, therefore, these strains are classified as S rather than R strains, in terms of susceptibility to fluconazole. In contrast, few studies have reported on isolates of Candida spp. exhibiting trailing growth for itraconazole. The results of our study show that, similar to the trailing isolates for fluconazole, a C. albicans strain displaying trailing growth for itraconazole is as susceptible to the drug in vivo as the S and S-DD strains of C. albicans.

In conclusion, the results of our study show that both intravenous and oral itraconazole formulations can protect not only mice infected with Candida spp. strains with an itraconazole MIC range of ≤0.016–0.5 mg/L (CLSI category S or S-DD), but also those infected with strains with an itraconazole MIC range of 1–2 mg/L (CLSI category R) to virtually the same extent and in a dose-dependent fashion. The results also show that mice infected with R strains non-susceptible to 8 mg/L itraconazole are refractory to therapy with both itraconazole formulations. Furthermore, it appears that effective doses of intravenous or oral itraconazole in infected mice are correlated with itraconazole MICs for Candida strains challenged.

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References


Figure 3. Correlation between in vitro antifungal susceptibility test results for 12 Candida strains and in vivo therapeutic outcome of administration of intravenous itraconazole (a) and oral itraconazole (b) in a murine model of invasive candidiasis. Spearman’s rank correlation coefficients and P values versus MIC for (a) and (b) are 0.669 and $P = 0.017$, and 0.856 and $P = 0.00038$, respectively. #Plots for three different strains.


