Antifungal effect of voriconazole on intracellular *Candida glabrata*, *Candida krusei* and *Candida parapsilosis* in human monocyte-derived macrophages

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Infections caused by *Candida* species other than *Candida albicans* are increasingly common, and decreased susceptibility to azoles has made them more difficult to treat. Since phagocytic killing is important in elimination of *Candida* infections, intracellular killing of fluconazole-resistant *Candida glabrata*, *Candida krusei* and *Candida parapsilosis* (four strains each) by voriconazole was investigated in human monocyte-derived macrophages (MDMs). MDMs were infected with *Candida*, and voriconazole was then added. MDMs were lysed at 0, 24 or 48 h after infection, and viable *Candida* in the lysates enumerated. Compared to the starting inoculum, the number of viable intracellular *C. parapsilosis* and *C. glabrata* in untreated MDMs increased to 28 121 and 351 %, respectively, in 48 h. In contrast, the number of *C. krusei* decreased to 42 %. In MDMs treated with voriconazole, the decrease in viable count was dependent upon drug concentration. At 48 h, *C. glabrata* was killed only at $5 \times \text{MIC}$ ($P < 0.05$), *C. krusei* was killed at all voriconazole concentrations, while *C. parapsilosis* was inhibited at 0.5 and $1 \times \text{MIC}$ and killed at $\geq 2.5 \times \text{MIC}$ ($P < 0.05$). The data show that intracellular growth and survival of these *Candida* species in the absence or presence of voriconazole vary markedly. The activity of voriconazole depends on the concentration of the drug and the time of exposure. For the 12 *Candida* strains studied, regression curves show that the maximum intracellular anticandidal activity of voriconazole was reached at $3.5\text{--}5 \times \text{MIC}$.

Received 1 November 2005

Accepted 22 March 2006

INTRODUCTION

Candidaemia caused by *Candida albicans* and other *Candida* species is known to be an important cause of morbidity and mortality, especially in debilitated and/or immunocompromised hosts (Anaissie *et al.*, 1998; McNeil *et al.*, 2001). Treatment of invasive tissue infections caused by *Candida*, including osteomyelitis, endocarditis, endophthalmitis and hepatic candidiasis, can be difficult and prolonged, and frequently results in recurrence or failure (Anaissie *et al.*, 1998; McNeil *et al.*, 2001). The development of resistance to fluconazole among *C. albicans*, *Candida glabrata*, *Candida parapsilosis* and other *Candida* species, as well as the innate resistance of *Candida krusei* to fluconazole, has made the treatment of these infections increasingly difficult (Loeffler & Stevens, 2003; Ostrofsky-Zeichner *et al.*, 2003; Pfaller *et al.*, 2002). Furthermore, serious infections caused by species other than *C. albicans*, especially *C. glabrata*, *C. parapsilosis* and *C. krusei*, have increased in

hospitalized patients (Bodey *et al.*, 2002; Ostrofsky-Zeichner *et al.*, 2003).

Voriconazole, a novel triazole antifungal agent (Sanati *et al.*, 1997; Sheehan *et al.*, 1999), is known to be active against *Candida* species, including those resistant to fluconazole. Since phagocytic killing of *Candida* species by human neutrophils and monocytes/macrophages is the major host defence in serious fungal infections (Maródi *et al.*, 1991a, b), it is important to demonstrate the activity of voriconazole against intracellular *Candida* in phagocytic cells. It is known that fluconazole can enter phagocytes (Pasqual *et al.*, 1993), and the intracellular activity of voriconazole against *C. albicans* in phagocytes has already been demonstrated (Vora *et al.*, 1998; Baltch *et al.*, 2001, 2005). However, there is limited knowledge about its intracellular activity against *C. glabrata*, *C. parapsilosis* and *C. krusei*.

Our study describes the growth characteristics of intracellular fluconazole-resistant *C. glabrata*, *C. parapsilosis* and *C. krusei* in untreated human monocyte-derived macrophages (MDMs), and the effect of increasing concentrations

Abbreviation: MDM, human monocyte-derived macrophage.

of voriconazole on the intracellular growth and survival of these *Candida* species. It also provides data suggesting that maximal effective intracellular concentrations of voriconazole can be reached in these phagocytic cells.

METHODS

Yeast strains. All yeast strains used in this study were clinical isolates. Four strains each of *C. glabrata*, *C. krusei* and *C. parapsilosis* were tested. *C. krusei* strain 6 and *C. parapsilosis* strain 25 were obtained from the University of Iowa, Iowa City, IA. *C. krusei* strains 1, 2 and 4, *C. glabrata* strains 11, 12, 14 and 20, and *C. parapsilosis* strains 21, 29 and 30 were obtained from the Wadsworth Center, New York State Department of Health, Albany, NY. These strains were chosen because they are moderately resistant to fluconazole (MICs ranging from 16 to 64 µg ml⁻¹) and are susceptible to voriconazole. The Clinical Microbiology Laboratory at the Stratton Veterans Affairs Medical Center verified the species of each yeast strain using the RapID Yeast Plus Identification System (Remel) and microscopic examination of yeast grown on corn meal agar. For preparation of inocula, *Candida* from stock cultures frozen at -80 °C in 15% (v/v) glycerol were streaked for isolation on Sabouraud dextrose (SD) agar and incubated overnight at 37 °C. Four to five well-isolated colonies from a plate were used to make a suspension in sterile 0.85% (w/v) NaCl. The absorbance at 530 nm was determined and adjusted to yield a final cell density of approximately 2 × 10⁶ c.f.u. ml⁻¹. The cells were then diluted 100-fold to 2 × 10⁴ c.f.u. ml⁻¹. The cell density of each inoculum was verified using the standard plate count method.

Antimicrobial compounds and MIC testing. Laboratory-standard powders of voriconazole and fluconazole were provided by Pfizer. Voriconazole and fluconazole solutions were prepared according to the supplier's directions and filter-sterilized (0.45 µm pore-size) immediately prior to use. MICs of voriconazole and fluconazole for all *Candida* strains used in this study were determined in triplicate using the National Committee for Clinical Laboratory Standards-approved macrodilution method M27-A2 (National Committee for Clinical Laboratory Standards, 2002). MICs (in µg ml⁻¹) of fluconazole/voriconazole for *C. glabrata* strains 11, 12, 14 and 20 were 32/1, 16/0.5, 64/2 and 16/1, respectively. For *C. krusei* strains 1, 2, 4 and 6, MICs (in µg ml⁻¹) of fluconazole/voriconazole were 16/0.5, 16/0.5, 64/0.5 and 32/0.25, respectively. For *C. parapsilosis* strains 21, 25, 29 and 30, MICs (in µg ml⁻¹) of fluconazole/voriconazole were 8/0.25, 32/0.5, 64/1 and 16/0.5, respectively.

Human monocytes. Monocytes were obtained from heparinized blood of healthy human donors who had signed the informed-consent form approved by the Institutional Review Board of the Stratton Veterans Affairs Medical Center. Mononuclear cells were separated from whole blood by using Histopaque 1077 (Sigma). The resulting mononuclear cell preparation was more than 98% pure. The separated cells were resuspended at a concentration of 2 × 10⁶ cells ml⁻¹ in RPMI + [RPMI 1640 medium (Sigma) supplemented with 15% heat-inactivated fetal bovine serum (F-2442, Sigma)]. Cell viability, determined by the trypan blue exclusion test, was at least 98%.

Time-kill studies. Time-kill assays were run for voriconazole. Mononuclear cells prepared as described above were added to the wells of 48-well culture plates (Corning/Costar) at 10⁶ cells per well and allowed to adhere for 2 h. Monocytes adhered to the wells in contiguous monolayers, while non-adherent cells, including lymphocytes, were removed by aspiration. The adhered cells were considered to be MDMs. A suspension of *Candida* cells in RPMI + (500 µl at 2 × 10⁴ c.f.u. ml⁻¹) was then added to each well. Cell monolayers

were incubated for 1 h at 35 °C in an atmosphere containing 5% CO₂ in order to allow phagocytosis of the yeast to occur. Following phagocytosis, the medium was removed by aspiration and the MDM monolayers were washed once with RPMI + (additional washing did not further decrease the number of yeast in the well). The intracellular location of the yeast was verified by differential fluorescent staining of intracellular and extracellular yeast and examination by fluorescence microscopy. Following phagocytosis and washing, approximately 5 × 10³ intracellular yeast per well remained. After washing, RPMI + containing voriconazole at concentrations from 0.1 to 5 × MIC was added. Incubation was at 35 °C in an atmosphere containing 5% CO₂. Following incubation (0, 24 and 48 h), the medium was removed from each well by aspiration and the MDMs were lysed using sterile distilled water in order to release the yeast. The number of viable yeast in each sample was determined by using SD agar and the standard plate count method. In each experiment, each experimental condition was run in duplicate (two wells) and the number of surviving yeast in each well was determined in duplicate. Each assay was repeated at least three times. Kill curves represent geometric means of the numbers of surviving organisms at 24 and 48 h, expressed as a percentage of the c.f.u. ml⁻¹ at 0 h (number of organisms at 24 or 48 h divided by the number of organisms for the same experimental condition at 0 h, multiplied by 100). It should be noted that since the ordinates of these graphs are logarithmic, differences that appear small may still be significant. For time-kill curves, inhibition is defined as the number of viable yeast being less than the number in the untreated control but greater than the number at 0 h, and killing (fungicidal activity) is defined as the number of viable yeast being lower than the number at 0 h.

Statistical methods. The analysis of variance (Stuart & Ord, 1991a) was used to analyse the observed c.f.u. ml⁻¹ transformed by log₁₀. Post hoc comparisons were made under the Dunn procedure (Stuart & Ord, 1991b). For presentation of results, the mean log₁₀ values were converted to the geometric mean numbers of c.f.u. ml⁻¹, expressed as a percentage of the geometric mean c.f.u. ml⁻¹ at 0 h. Regression curves were fitted to the observed data using our adaptation of the von Bertalanffy model (Draper & Smith, 1981a) and the least squares method (Draper & Smith, 1981b). The regression equation used was:

$$y = m + 10^{a+bx+cx^2}$$

where y is the percentage of viable counts at 24 or 48 h (i.e. geometric mean number of c.f.u. ml⁻¹ at 24 or 48 h, expressed as a percentage of the geometric mean number of c.f.u. ml⁻¹ at 0 h), m is the minimum percentage viable count attainable, and x is the concentration of voriconazole, expressed as a multiple of the MIC. The parameters a , b and c determine the configuration of the exponential curve, which depicts the percentage viable counts as a function of the voriconazole concentration, specific for each *Candida* species. The level of significance was 0.05.

RESULTS

Growth and survival of *Candida* in untreated MDM

Fig. 1 and Table 1 demonstrate that the growth and survival of intracellular yeast in untreated controls were specific for each *Candida* species tested. The greatest increase in the viable count at 48 h was observed for *C. parapsilosis* (28 121%), while *C. glabrata* increased to 351% during the same period. In contrast, there was a continuous decline in

Fig. 1. Intracellular killing of *C. glabrata* (A), *C. krusei* (B) and *C. parapsilosis* (C) by voriconazole in MDMs. The geometric means are shown of four strains per species and three assays per strain expressed as a percentage of the c.f.u. ml⁻¹ at 0 h. ●, control; ○, 0.1 × MIC voriconazole; ▼, 0.5 × MIC voriconazole; ▽, 1 × MIC voriconazole; □, 2.5 × MIC voriconazole; ■, 5 × MIC voriconazole. For all figures, the error bars represent 1 × SEM, and the dotted line at 100% represents the number of viable organisms at 0 h.

Table 1. Effect of voriconazole on growth and survival of three *Candida* species in MDMs

Viable count values are geometric means of c.f.u. ml⁻¹ at 48 h expressed as percentages of c.f.u. ml⁻¹ at 0 h.

<i>Candida</i> species and treatment	Viable counts
<i>C. glabrata</i>	
Untreated	351
5 × MIC voriconazole	28
<i>C. krusei</i>	
Untreated	42
5 × MIC voriconazole	13
<i>C. parapsilosis</i>	
Untreated	28 121
5 × MIC voriconazole	5

the number of viable *C. krusei* over 48 h to 42% of the number initially present.

Effect of voriconazole on growth and survival of *Candida* in MDM

Fig. 1(A) shows the combined results of time-kill assays for four strains of *C. glabrata*. Compared to the untreated control, voriconazole had significant intracellular activity against *C. glabrata* at all concentrations tested ($P < 0.05$), and this activity was concentration dependent. At 24 h, the number of surviving yeast was significantly lower than the number at 0 h (100%) at all concentrations except 0.1 × MIC ($P < 0.05$). At 48 h, there was inhibition at 0.1 × MIC ($P < 0.05$). At 0.5 and 1 × MIC at 48 h, the numbers of surviving yeast were not significantly different from the numbers at 0 h. However, the values were significantly lower than in the untreated controls ($P < 0.05$). At 5 × MIC and 48 h, there was fungicidal activity against *C. glabrata* ($P < 0.05$).

Results for *C. krusei* are shown in Fig. 1(B). At 24 h, voriconazole had significant intracellular activity against *C. krusei* at all concentrations tested ($P < 0.05$), and the activity was concentration dependent. The numbers of viable organisms at 24 h for 0.1 and 0.5 × MIC were lower than for the control, but did not differ from one another. Numbers of viable yeast at 24 h for 1, 2.5 and 5 × MIC were lower than they were for 0.1 or 0.5 × MIC, and numbers of surviving organisms for 2.5 and 5 × MIC were significantly lower than for 1 × MIC ($P < 0.05$). At 48 h, all of the viable counts, including those for the untreated control, were significantly lower than they were at 0 h ($P < 0.05$), and decreased as the voriconazole concentration increased. Fungicidal activity was not different for the untreated control, 0.1 or 0.5 × MIC of voriconazole at 48 h. Activity was greater at 1, 2.5 and 5 × MIC than at 0, 0.1 and 0.5 × MIC ($P < 0.05$), but activities at 1, 2.5 and 5 × MIC did not differ significantly from one another.

Fig. 1(C) shows the results for *C. parapsilosis*. At 24 h, voriconazole had significant intracellular activity against *C. parapsilosis* at all concentrations except $0.1 \times \text{MIC}$, and the number of surviving yeast decreased with respect to the untreated control as the voriconazole concentration increased ($P < 0.05$). The number of surviving yeast at 24 h was significantly lower for 2.5 and $5 \times \text{MIC}$ than for $1 \times \text{MIC}$ ($P < 0.05$), but the difference between 2.5 and $5 \times \text{MIC}$

was not significant. At 48 h, the number of viable yeast was significantly less than for the untreated control at all voriconazole concentrations except $0.1 \times \text{MIC}$. There was inhibition at 0.5 and $1 \times \text{MIC}$, but there was fungicidal activity only at 2.5 and $5 \times \text{MIC}$.

Fig. 2 shows regression curves fitted to the observed (experimental) data shown in Fig. 1, as well as the observed data.

Fig. 2. Fitted regression curves and observed (experimental) data showing intracellular killing of *Candida* by voriconazole in MDMs. The geometric means are shown of four strains per species and three assays per strain, expressed as a percentage of the c.f.u. ml⁻¹ at 0 h, at 24 and 48 h for *C. glabrata* (A, B), *C. krusei* (C, D) and *C. parapsilosis* (E, F) in MDMs. Solid lines, fitted regression curves; ●, observed (experimental) data.

The fitted values, specific for assay hours 24 and 48, were calculated using the equation defined in Methods. The r^2 values ranged from 0.983 to 0.996, indicating very close fit of the regression curves to the observed (experimental) data (Draper & Smith, 1981b). Comparison of the fitted regression curves for the three *Candida* species at 48 h shows that in the absence of voriconazole, intracellular killing continued for *C. krusei* but not for *C. glabrata* or *C. parapsilosis* (Table 1, Fig. 2). Addition of voriconazole resulted in a dramatic decrease in the number of viable intracellular *C. parapsilosis* and, to a lesser extent, of *C. glabrata* and *C. krusei*. For *C. parapsilosis*, maximum intracellular fungicidal activity was reached at 4–5 $\times$ MIC of voriconazole, and for *C. glabrata* and *C. krusei*, at 3.5–5 $\times$ MIC of voriconazole. For *C. krusei*, the addition of voriconazole enhanced intracellular killing, although the lowest percentage viable count attained was not as low as for *C. parapsilosis* (Table 1, Fig. 2).

DISCUSSION

Intracellular survival and ability to grow intracellularly in the absence and presence of voriconazole varied markedly among the three yeast species evaluated. *C. krusei* did not grow intracellularly, even in untreated controls. In contrast, during the same period, the number of viable *C. glabrata* increased slightly, while the number of viable *C. parapsilosis* increased considerably more (Table 1, Fig. 2). Our data from previous studies with *C. albicans* shows that *C. albicans* grows more rapidly in MDMs than these three species, especially during the first 24 h, but that at 48 h, the number of c.f.u. ml⁻¹ is similar to that of *C. parapsilosis* (Baltch *et al.*, 2001, 2005). Voriconazole (5 $\times$ MIC) had significant fungicidal activity against all three species, with the greatest intracellular fungicidal activity against *C. parapsilosis*, the most rapidly growing of the three species tested. When treated with voriconazole at 5 $\times$ MIC, the number of viable *C. parapsilosis* at 48 h was only 5 % of that at 0 h, compared to 13 % for *C. krusei* and 28 % for *C. glabrata*. Thus, among these *Candida* species, a relatively high (or low) viable count in the absence of voriconazole did not imply a relatively high (or low) viable count in the presence of the most effective concentration of voriconazole.

In this study, the availability of abundant data for each species (four strains, three separate experiments, each experimental condition done in duplicate in each experiment) allowed the development of regression curves showing the relationship between voriconazole concentration (expressed as a multiple of the MIC) and intracellular killing over time. To our knowledge, this is the first application of this type of mathematical description of time-kill data for *Candida* species. Such analyses can be useful, because they demonstrate the level of intracellular killing at any voriconazole concentration, specific for time, and suggest the maximum level of killing possible for each *Candida* species. Since maximum intracellular fungicidal activity against all 12 strains of the three *Candida* species studied

was reached at approximately 3.5–5 $\times$ MIC, this could represent a threshold of activity, and increasing the extracellular voriconazole level beyond the threshold may have limited usefulness.

Although up to 95 % killing of intracellular yeast was achieved with voriconazole, the regression curves clearly demonstrate that in our *in vitro* model, complete eradication of intracellular yeast is not likely to be achieved using voriconazole alone. Use of a triazole along with an appropriate cytokine (Baltch *et al.*, 2001) and/or another antifungal drug with a different mode of action may be useful to enhance the killing of intracellular yeast. The potential utility of combination therapy against *Candida* infections has recently been reviewed (Mukherjee *et al.*, 2005), and is supported by reports of efficacy *in vitro* against *C. albicans* (Ghannoum *et al.*, 1995) and *in vivo* against fluconazole-resistant *C. krusei* and *C. glabrata* (Girmenia *et al.*, 2003).

In conclusion, our data show that intracellular growth and survival of different *Candida* species in the absence or presence of voriconazole vary markedly. The activity of voriconazole depends on the concentration of the drug and the time of exposure. For the 12 strains of the three *Candida* species studied, the regression curves demonstrate the level of intracellular killing by MDMs, with or without voriconazole, at any drug concentration, and show that the maximum intracellular anticandidal activity of voriconazole was reached at 3.5–5 $\times$ MIC.

ACKNOWLEDGEMENTS

This work was supported by Pfizer Laboratories and in part by the Veterans Affairs Research Service. The authors thank Dr Vishnu Chaturvedi, Wadsworth Center, New York State Department of Health, Albany, NY, and Dr Michael Pfaller, University of Iowa, Iowa City, IA, for providing the *Candida* strains used in this study.

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