MYCOLOGY

Effect of fluconazole on agar invasion by *Candida albicans*

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Subinhibitory concentrations of azoles are known to inhibit hyphal branching of *Candida albicans* in liquid media. This study showed that subinhibitory concentrations of fluconazole also inhibit agar invasion by *C. albicans* in YPD solid medium. Agar invasion was markedly inhibited in two *C. albicans* strains that were resistant to fluconazole. This suggests that the degree to which fluconazole inhibits agar invasion by *C. albicans* hyphae could serve as a marker of susceptibility to azoles.

Introduction

Subinhibitory concentrations of azoles are known to inhibit the formation of hyphae of *Candida albicans* in liquid media [1–3]. Hyphal formation appears to be a major factor in the virulence of *C. albicans* [4]. To investigate this further, the present study examined the effect of fluconazole on the inhibition of invasion by *C. albicans* hyphae in agar.

Materials and methods

Growth media

Standard culture medium was prepared as described previously [5]. YPD plates (yeast extract 'Difco' 2%; Bactopeptone 'Difco' 4%; glucose 2%; Bacto-agar 'Difco' 2%; tryptophan 0.003%) were allowed to cool, polymerise and dry for 2 days at room temperature before use. YPD media containing different concentrations of fluconazole (Pfizer, New York, USA) were made by mixing the appropriate amount of a stock solution (5 g/L in water) with the molten agar to obtain final concentrations of 2, 4 and 64 mg/L. The

YPD and YPD + fluconazole plates were kept in a cold room for up to 2 weeks.

C. albicans isolates

Fifteen clinical isolates of *C. albicans* were investigated. Their susceptibility to fluconazole was based on the MIC determined by the NCCLS-based broth microdilution method in RPMI liquid medium [6]

Growth conditions and agar invasion assay

C. albicans strains taken from a single colony on YPD were streaked out with a flat toothpick to form single colonies (typically *c.* 50 colonies/plate) on YPD plates. Care was taken to avoid scratching the agar surface. The cultures were incubated at 37°C for 24 h. Each YPD plate was then replica-plated with a replica block and velveteen pads on to YPD plates containing different concentrations of fluconazole in the following order: YPD; YPD + fluconazole 2 mg/L; YPD + fluconazole 4 mg/L; YPD + fluconazole 64 mg/L. The degree of inhibition of agar invasion in the presence of fluconazole was determined further in a subset of isolates by exposing the organisms to a gradient of concentrations as follows: 20 µl of a fresh overnight culture of each *C. albicans* isolated in YPD liquid medium grown with constant shaking at 37°C (*c.* 10⁵ cells) were point inoculated with a micropipette on to YPD plates in previously marked areas, equally distant from a paper disk containing 300 µg of fluconazole (achieved by applying 60 µl of fluconazole 5 g/L stock solution on to the disk). All plates were incubated at 37°C and the extent of invasion was scored after 2

Received 7 Dec. 1999; revised version received 2 May 2000; accepted 17 May 2000.

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days. Invasion was determined as follows: cells that had not invaded the agar were washed away by rubbing the plate with a gloved finger while rinsing under running water; cells that had invaded the agar remained as macroscopically visible microcolonies on the surface of the washed plate. Invasiveness was confirmed by microscopical examination of the remaining cells (under a Zeiss WL light microscope with a bright light field, $\times 10$ magnification) and determination that the plane of focus was within the agar. The invasiveness of the different *C. albicans* strains was evaluated by estimating the percentage of colonies (typically 50 colonies from one streak) in which a significant number of cells remained in the agar after washing. For the purpose of this study, five different classes of invasiveness were defined: +++, >75% of colonies with macroscopically discernible cells in the agar; ++, >50–75%; +, >25–50%; $\pm$, 5–25%; and –, <5%. The designation $\pm$ was used as the end-point to define no significant invasion. All experiments were done in duplicate at two different times.

Photography

Representative plates were photographed with a 35-mm Nikon F3 camera and Kodak film after incubation for 2 days at 37°C. A stream of water was then used to rinse all cells from the agar surface. These plates were allowed to dry for 10 min and photographed again. Photographs were taken also with a Nikon inverted microscope ($\times 10$ magnification) to assess the degree of agar invasion.

Results

Inhibition of agar invasion by subinhibitory concentrations of fluconazole

The invasive growth of *C. albicans* strains Y-64345 and Y-65544 was assessed in YPD agar and YPD + fluconazole 4 mg/L (Fig. 1–3). As shown in Fig. 1, inhibition of agar invasion occurred in the presence of a concentration of fluconazole in YPD that did not affect growth. For all 15 *C. albicans* strains used, the inhibition of agar invasion was seen in the presence of concentrations of fluconazole that did not affect growth on YPD agar (Table 1). The degree of inhibition of agar invasion paralleled the increasing concentrations of fluconazole (Table 1, Fig. 2). The *C. albicans* strains tested showed strain-dependant differences in invasion; however, fluconazole inhibited agar invasion by all strains tested (Table 1) [7].

Inhibition of agar invasion: correlation with the NCCLS-based MIC

As summarised in Table 1, the 13 *C. albicans* isolates shown to be sensitive to fluconazole by the NCCLS-based method had a low level of agar invasion in the presence of low concentrations of fluconazole. Corre-

Fig. 1. Inhibition of agar invasion by *C. albicans*. Invasive growth of *C. albicans* isolates Y-64345 (right) and Y-65544 (left) is shown on plates containing fluconazole 4 mg/L before (a) and after (b) the cells were washed off the agar surface. (c) The growth (after the cells were washed off) of the same *C. albicans* isolates in plain YPD is shown for comparison.

spondingly, in the two *C. albicans* isolates shown to be fluconazole-resistant by the NCCLS-based method, agar invasion was inhibited by higher concentrations of fluconazole.

Discussion

This study demonstrated that fluconazole, when used at concentrations that do not affect growth, inhibits agar

Fig. 2. Inhibition of agar invasion by fluconazole is shown to be inversely related to the fluconazole gradient. *C. albicans* isolate Y-64345 was point inoculated on a YPD plate with a fluconazole 300- μ g disk. The plate was photographed before (a) and after (b) washing cells off the agar surface.

invasion in *C. albicans* isolates. A rich medium (YPD) was chosen to study the effects of fluconazole on invasion of *C. albicans* because invasive growth has already been characterised in YPD for *Saccharomyces cerevisiae*, a genetically tractable yeast closely related to *C. albicans* [8]. The most likely explanation for the inhibition of invasive *C. albicans* growth by subinhibitory concentrations of fluconazole is that, even at low concentrations, this drug alters the sterols in fungal membranes [9] and, thus, indirectly affects hyphal growth and invasion. Previous studies have shown that a subinhibitory concentration of azoles affects the development of *C. albicans* hyphae in liquid media [1, 3, 10], but until now the effects of such drugs had not been evaluated in agar. These findings give further insight into additional, growth-independent but subtle effects of azoles on *C. albicans*.

As a correlation between the level of sensitivity or resistance of *C. albicans* to azoles and its ability to form hyphae in liquid media was reported recently [10], the present study also reports the preliminary

correlation of the invasion assay results with the results of the standard NCCLS-based broth microdilution technique. In the present study, the degree of inhibition of agar invasion by fluconazole seemed to correlate with the results obtained with the NCCLS-based method; the fluconazole-resistant *C. albicans* isolates appeared to invade the agar surface more actively in the presence of the drug. Preliminary observations suggest that the concept of inhibition of agar invasion by subinhibitory concentrations of fluconazole may lead to additional standardised methods that both correlate with and complement the NCCLS-based method for detecting fluconazole-resistant *C. albicans* strains. As this approach allows simultaneous testing of multiple colonies of each *C. albicans* strain, it might also have the theoretical advantage of detecting heterogeneity in a *C. albicans* population in terms of azole resistance. Future studies on a larger number of isolates will determine the utility of the principle described here and define the optimal conditions and media for screening clinically relevant early azole resistance in *C. albicans*.

Fig. 3. Photomicrographs of agar invasion by *C. albicans* isolate Y-64345 (Nikon inverted microscope; $\times 10$ magnification) after cells were washed off the agar surface. **(a)** Growth in YPD shows hyphae invading the agar at different angles and planes. **(b)** Growth in YPD + fluconazole 4 mg/L shows a complete lack of invasion.

Table 1. Inhibition of agar invasion by *C. albicans* with varying concentrations of fluconazole

<i>C. albicans</i> strain no.	Fluconazole MIC (mg/L)	Agar invasion in YPD plates* at fluconazole concentration (mg/L)		
		0	2	4
Y-65544 [†]	0.25	+++	+++	—
Y-2335 [†]	0.25	+++	++	±
Y-2337 [†]	0.25	++	+	±
Y-2334 [†]	0.5	+	±	—
Y-2338 [†]	0.5	++	+	±
Y-2348 [‡]	0.5	++	+	±
Y-2329 [†]	0.5	+++	+	±
Y-2327 [†]	1.0	+	±	—
Y-64345 [†]	2.0	++	+	—
Y-2330 [†]	4.0	+	±	—
Y-2349 [‡]	4.0	+++	++	±
Y-2328 [†]	8 (DD)	+++	+	±
Y-2350 [‡]	10 (DD)	++	+	±
Y-2351 [‡]	>64 (R)	++	++	++
Y-2352 [‡]	>64 (R)	++	++	++

*See *Methods*.

Agar invasion inhibitory concentration of fluconazole is depicted in bold type.

R, resistant; DD, dose-dependent resistance (according to NCCLS criteria [7]).

[†]Blood isolates, from immunocompromised patients at M.D. Anderson Cancer Center.

[‡]Clinical isolates, gift from Dr M. Rinaldi, Fungus Testing Laboratory, San Antonio, TX, USA.

This work was supported by the National Cancer Institute's Cancer Center Support Grant (CA16672) to the University of Texas M. D. Anderson Cancer Center. We thank Dr Michael Rinaldi for providing *C. albicans* strains, Jim Lemoine for assistance in photography and Denise Barientos for secretarial support.

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