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In vitro activity of cloconazole, sulconazole, butoconazole, isoconazole, fenticonazole, and five other antifungal agents against clinical isolates of *Candida albicans* and *Candida* spp.

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Abstract

The *in vitro* activity of several new imidazoles, cloconazole, sulconazole, butoconazole, isoconazole and fenticonazole, were compared with those of amphothericin B, flucytosine, and three azoles: econazole, miconazole and ketoconazole against isolates of pathogenic *Candida*. A total of 186 clinical isolates of 10 species of the genus *Candida* and two culture collection strains were tested by an agar-dilution technique. Isoconazole was the most active azole, followed by butoconazole and sulconazole. Differences between some of the species in their susceptibility to the antifungal agents were noted. Sulconazole and cloconazole had the highest activity *in vitro* against 106 isolates of *C. albicans*. Butoconazole and isoconazole were also very active against isolates of *C. albicans*, and were the most active azole compounds against 80 isolates of *Candida* spp.

Introduction

The incidence of clinically significant diseases caused by opportunistic yeasts, particularly *Candida albicans* and related species, has increased in recent years, especially in compromised and immunosuppresed patients [1–4]. Only a limited number of effective antifungal agents are presently available, including the polyene antibiotic amphotericin B, the synthetic antimetabolite flucytosine, and the synthetic imidazoles [5, 6]. Furthermore, resistance of yeasts to these antifungal drugs has been described [7, 8], together with various side-effects associated with their use [9, 10]. Thus, a number of new synthetic antifungal agents have been developed for clinical use in both topical and systemic administration [11–14].

Azole derivatives are prominant in the development of new antifungal agents. Cloconazole, sulconazole, butoconazole, isoconazole, and fenticonazole are new N-substituted (mono) imidazoles developed by Shionogi & Co, Ltd, Osaka, Japan, Syntex Inc, Palo Alto, USA, Janssen Pharmaceutica, Beerse, Belgium, and Recordati SpA, Milano, Italy, respectively. At present limited data are available regarding the antifungal properties of these imidazoles.

In this report we present our findings of the *in*

vitro activity of cloconazole, sulconazole, butoconazole, isoconazole, and fenticonazole compared to that of amphotericin B, flucytosine, and three azoles: econazole, miconazole, and ketoconazole against a great number of clinical isolates of *Candida albicans* and other *Candida* species.

Materials and methods

Yeast strains. A total of 186 pathogenic *Candida* strains isolated from clinical specimens were tested, including 106 *C. albicans*, 24 *C. parapsilosis*, 20 *C. guilliermondii*, 19 *C. tropicalis*, 9 *C. krusei*, 3 *C. pseudotropicalis*, 2 *C. pseudointermedia*, 1 *C. mogii*, 1 *C. viswanathii*, and 1 *C. zeylanoides*. These isolates were identified previously (J.M. Hernández Molina, PhD thesis, Universidad de Sevilla, Sevilla, 1989) by conventional methods and procedures as described elsewhere [15, 16]. Two culture collection strains, *S. cerevisiae* ATCC 36375 and *C. pseudotropicalis* ATCC 28838, were used as reference strains. All cultures were grown on Glucose-Peptone-Yeast extract agar slants [17], and maintained at 4 °C.

Antifungal drugs. Ten drugs were studied: amphotericin B (E.R. Squibb & Sons, Princeton, NJ), 5-fluorocytosine (Hoffmann-LaRoche Inc, Basel, Switzerland), econazole, miconazole, ketoconazole, isoconazole (Janssen Pharmaceutica), cloconazole (Shionogi & Co, Ltd), sulconazole, butoconazole (Syntex Inc), and fenticonazole (Recordati SpA), all supplied as pure powders. Because of their varying solubilities the compounds were dissolved in different solvents to produce stock solutions of 10 mg of active drug per ml. Amphotericin B, econazole, miconazole, ketoconazole, isoconazole, butoconazole and fenticonazole were dissolved in 100% dimethylsulfoxide, flucytosine in distilled water and cloconazole and sulconazole in absolute ethanol. Stock solutions were sterilized by filtration through 0.22-μm-pore membrane filters (Millipore Corp, Bedford, MA), and kept at 4 °C for no longer than a week.

Antifungal susceptibility testing medium. The medium used for all the groups of compounds was Yeast Morphology Agar (YMA) (Difco Laboratories, Detroit, MI), adjusted at pH 7.0, by a 0.01 M sterile phosphate buffer, as described elsewhere [18]. Serial doubling dilutions of each drug were added to a melted medium culture, maintained at 50 °C in a water bath, to obtain a final range of concentrations between 0.03 to 64 μg/ml. These mixtures were mixed and poured into 9-cm diameter petri plates and allowed to harden. The maximun concentration of dimethyl sulfoxide or ethanol when finally diluted was 2%; in no case did these amounts of solvents inhibit the growth of any test organism. Two drug-free plates of buffered YMA were added to each set of antifungal agent concentrations. The plates were used within a week of their preparation.

Preparation of inoculum. Inocula for *in vitro* susceptibility testing were prepared from 24–48-h cultures on Sabouraud dextrose agar (Difco). Cells of well-isolated colonies were washed with sterile distilled water and suspended in a sterile saline solution to obtain a density of approximately a 0.5 MacFarlad standard. These cell suspensions were adusted to about 10^6 CFU/ml by hemacytometer counting and viability analysis [6].

Performance of the susceptibility test. An agar dilution replicate plate method was employed for antifungal susceptibility testing [6, 18]. All tests were performed at least in duplicate. Before use, each set of plates was dried at 37 °C for 30 min; they were then inoculated with spots of the yeast suspensions using a Steers replicator, so that each spot contained about 10^3 CFU/ml. One of the drug-free control plates was inoculated with the

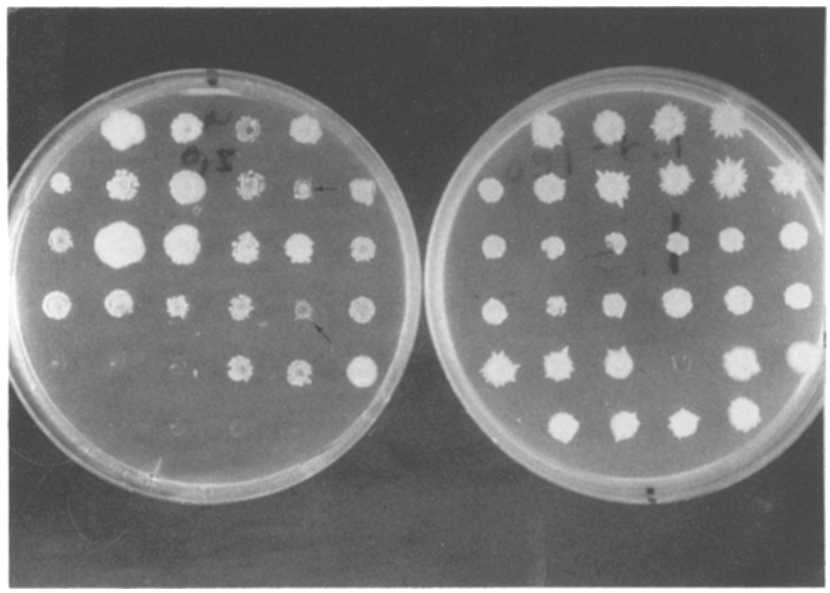

Fig. 1. Test plates illustrating the sharp endpoints obtained with this method; the arrows show the 'ghost phenomenon' sometimes seen in inhibitory concentrations of azole with *Candida* spp.

corresponding test microorganisms at the beginning and the other at the end of the set. The reference strains with known minimal inhibitory concentrations (MIC's) were used in each set of experiments as controls of drug activity and intertest reproducibility. The inoculated plates were incubated at 35 °C for 24 h, or until the control plates showed clearly visible growth, examinations being made at 24-h intervals. After incubation, the resulting MIC was determined as the lowest concentration of antifungal agent which prevented visible growth, with the control plates as reference; a little 'ghost growth', which is sometimes seen in inhibitory concentrations of azole with *Candida* spp., is not counted as growth. To define antifungal resistance, those strains requiring MIC's > 2 μg/ml of amphotericin B, and >16 μg/ml of flucytosine were regarded as resistant [19, 20]. As for the imidazoles are concerned, in accordance with other authors [11, 21], we have chosen >32 μg/ml as being a suitable, albeit arbitrary, value to represent resistance.

Results

Typical test plates illustrating the sharp endpoints obtained with this method are shown in Fig. 1. The arrows show the 'ghost phenomenon' sometimes seen in inhibitory concentrations of azole with *Candida* spp. Summaries of MIC statistics (range, mode, 50% MIC [MIC_{50}], and 90% MIC [MIC_{90}]) of the antifungal agents tested against the 188 *Candida* strains used in the present study are given in Table 1. The MIC's for each individual strain were the same when the strains were tested in different experiments. For 186 *Candida* spp. isolates (not selected for clinical failure of antifungal therapy), MIC's for cloconazole and fenticonazole ranged between 0.12–32 μg/ml. For isoconazole, sulconazole and butoconazole, MIC's for 186 strains were between 0.12–8 μg/ml, approximately, four fold lower than those for cloconazole and fenticonazole. With the MIC_{90} as the measure of antifungal activity, isoconazole appeared to be superior to the other imidazoles, 100% of the strains were inhibited

Table 1. In vitro inhibitory activity of Amphothericin B, Flucytosine, Econazole, Miconazole, Ketocanazole, Cloconazole,

Species	Strains	Amphothericin B				Flucytosine			
		MIC_{50}	MIC_{90}	Mode	Range	MIC_{50}	MIC_{90}	Mode	Range
C. albicans	106	0.50[a]	0.50	0.50	0.12–0.50	0.12	32.00	0.12	<0.03–>64.00
C. parapsilosis	24	0.50	1.00	0.50	0.25–2.00	<0.03	0.06	<0.03	<0.03–0.12
C. tropicalis	19	0.50	1.00	0.50	0.50–1.00	0.12	0.12	0.12	0.03–0.12
C. krusei	9	1.00	2.00	1.00	1.00–2.00	0.25	0.25	0.25	0.25
C. guilliermondii	20	0.25	1.00	0.25	0.12–1.00	0.03	0.12	0.12	<0.03–32.00
C. mogii	1				1.00				<0.03
C. pseudotropicalis	3				0.50				<0.03
C. pseudointermedia	2				1.00				0.50
C. viswanathii	1				0.50				<0.03
C. zeylanoides	1				1.00				0.06
S. cerevisiae[b]	1				0.12				0.06
C. pseudotropicalis[b]	1				0.25				0.06
Species	Strains	Cloconazole				Sulconazole			
		MIC_{50}	MIC_{90}	Mode	Range	MIC_{50}	MIC_{90}	Mode	Range
C. albicans	106	1[a]	2	2	0.12–8.00	0.50	1	1.00	0.12–8.00
C. parapsilosis	24	4	4	4	0.50–8.00	0.25	2	0.25	0.12–8.00
C. tropicalis	19	8	8	8	0.50–8.00	2.00	4	2.00	0.25–8.00
C. krusei	9	8	16	8	8.00–16.00	4.00	8	4.00	4.00–8.00
C. guilliermondi	20	8	8	8	2.00–32.00	2.00	2	2.00	0.50–2.00
C. mogii	1				4.00				2.00
C. pseudotropicalis	3				0.12–0.50				0.12–0.25
C. pseudointermedia	2				2.00				1.00
C. viswanathii	1				4.00				0.25
C. zeylanoides	1				4.00				0.50
C. cerevisiae[b]	1				0.12				0.12
C. pseudotropicalis[b]	1				0.12				0.12

[a]MIC's in μg/ml.
[b]Reference strains.

with 4 μg/ml of isoconazol. Sulconazole and butoconazole were superior to econazole, miconazole and ketoconazole and about 90% of the strains were inhibited with 4 μg/ml of sulconazole and butoconazole. Of the ten drugs studied, amphothericin B was the most active at the lower concentrations as more than 95% of the strains were inhibited by 1 μg/ml of this agent.

When data for individual species of *Candida* were analyzed, sulconazole and cloconazole were the most active imidazoles against 106 isolates of *C. albicans*, 97% of strains being susceptible to 2 μg of these agents per ml, but were among the least active against *C. parapsilosis* and *C. krusei*. Butoconazole and isoconazole were active against *C. albicans*, and were the most active azole compounds against 80 isolates of *Candida* species. The activities of fenticonazole, econazole and miconazole were generally of the same magnitude against *C. albicans* and *Candida* spp. Ketoconazole was the least active drug against *C. albicans* with a range of 0.12–>64 μg/ml and a MIC_{90} of 64 μg/ml; similarly, little *in vitro* activity was observed with the isolates of *C. tropicalis* and *C. krusei*. Amphothericin B was the most effective of all the antifungal agents against *C. albicans*, whereas flucytosine was the most active against *Candida* spp.; *C. albicans* was least susceptible, 90% of the strains were inhibited by 16 μg of flucytosine per ml, and >64 μg/ml was required for the inhibition of all strains.

Of the 186 *Candida* strains isolated, resistance to amphothericin B was not noted. One (4%) strain of *C. parapsilosis* and two (20%) strains of

Sulconazole, Butocanazole, Isocanazole, and Fenticonazole on 186 isolates of *Candida* species and 2 culture collection strains.

Econazole				Miconazole				Ketoconazole			
MIC_{50}	MIC_{90}	Mode	Range	MIC_{50}	MIC_{90}	Mode	Range	MIC_{50}	MIC_{90}	Mode	Range
4.00	16	8.00	0.25–32.00	2.00	16.00	8.00	0.25–32.00	16.00	64.00	8.00	0.12–>64.00
0.50	4	0.50	0.25–4.00	0.25	0.50	0.25	0.12–0.50	0.25	0.50	0.25	0.06–1.00
4.00	8	4.00	0.50–16.00	4.00	8.00	4.00	0.50–16.00	1.00	8.00	0.50	0.25–16.00
8.00	8	8.00	4.00–8.00	2.00	4.00	2.00	2.00–4.00	8.00	32.00	8.00	1.00–32.00
4.00	4	4.00	0.50–4.00	1.00	1.00	1.00	0.25–1.00	1.00	1.00	1.00	0.50–4.00
			4.00				1.00				0.50
			0.25–0.50				0.12–0.25				1.00–0.50
			0.25				0.12–0.25				0.50
			0.50				0.25				0.12
			4.00				0.50				1.00
			0.25				0.25				0.25
			0.25				0.12				0.12
Butoconazole				Isoconazole				Fenticonazole			
MIC_{50}	MIC_{90}	Mode	Range	MIC_{50}	MIC_{90}	Mode	Range	MIC_{50}	MIC_{90}	Mode	Range
1.00	8.00	1.00	0.12–8.00	1.00	4.00	4.00	0.12–4.00	8.00	8.00	8.00	0.25–32.00
0.12	0.25	0.12	0.12–1.00	0.12	0.25	0.12	0.12–0.25	0.50	0.50	0.50	0.25–1.00
0.12	1.00	0.12	0.12–1.00	0.50	2.00	0.50	0.12–2.00	4.00	8.00	4.00	0.50–16.00
0.25	0.50	0.25	0.25–0.50	2.00	2.00	2.00	1.00–2.00	16.00	16.00	16.00	4.00–16.00
0.12	0.12	0.12	0.12–0.25	0.50	0.50	0.50	0.12–1.00	4.00	4.00	4.00	2.00–8.00
			0.25				0.25				2.00
			0.12				0.12				0.50
			0.12				0.12				0.50
			0.12				0.12				0.50
			0.25				0.25				4.00
			0.12				0.12				0.50
			0.12				0.12				0.50

C. krusei were found to be relatively resistant (MIC value of 2 μg/ml). Twelve isolates of *Candida* were resistant to 5-fluorocytosine, one (5%) strain of *C. guilliermondii* and eleven (10%) strains of *C. albicans* had MIC values of ≥32 μg/ml. The majority of the isolates were susceptible to the imidazoles, according to the upper concentration chosen by us to define resistance (>32 μg/ml); only 25 (24%) strains of *C. albicans* showed any resistance to ketoconazole.

Discussion

The *in vitro* susceptibility data presented here show that the new azoles, isoconazole, cloconazole, sulconazole, butoconazole, and fenticonazole are more highly active against the pathogenic yeasts tested than econazole, miconazole or ketoconazole. When the data for individual species of *Candida* are compared in terms of MIC values, amphothericin B is the most effective drug against strains of *C. albicans*, followed by sulconazole, cloconazole, and isoconazole in that order. On the other hand, all *Candida* spp. isolates were more susceptible to flucytosine, butoconazole, and isoconazole than to other antifungal agents; only one strain of *C. guilliermondii* had a MIC for 5-fluorocytosine of 32 μg/ml. MIC responses for *S. cerevisiae* ATCC 36375 and *C. pseudotropicalis* ATCC 28838 never exceeded 0.50 μg/ml with any of the ten drugs studied (range, 0.06–0.50 μg/ml).

Although studies by various authors describe drug resistance by clinical yeast isolates [22–25], we encountered few resistant *Candida* isolates during our study. All *Candida* species have a symmetrical distribution of susceptibilities (uni-

modal) to amphothericin B and the majority of imidazoles. Susceptibility to ketoconazole and 5-fluorocytosine is more complex; ketonazole and flucytosine revealed bimodal responses in tests with *C. albicans*. Few strains were more resistant to either drug than the others; there are clearly two populations of *C. albicans*. Our results are similar to those of Hamilton–Miller [26], Espinel–Ingroff et al. [27] and Hussain Qadri et al. [28], who found *in vitro* bimodal patterns of susceptibility or resistance on the part of isolates of *Candida* to ketoconazole and flucytosine. We also observed that the yeasts that we found to be resistant to ketoconazole or 5-fluorocytosine did not show any cross-resistance to the other drugs.

Otherwise, the MIC's with amphothericin B and flucytosine were generally well-defined, and development of resistant colonies was not observed. MIC's of imidazoles were not always well-defined, since there was more of a gradual decrease in growth with increasing azoles rather than a marked transition at any one concentration. This might be put down to the anticellular effects produced by these antifungal agents; the major mechanisms of action of the imidazoles remain unclear. Comparative studies in our laboratory have shown the concentration of the agar to be an important factor in eliminating trailing endpoints, which are characteristic of both liquid and solid agar media used for estimating the MIC's of the azoles. Other important factors are the adjustment of the pH, the use of a standardized inoculum, and the relapse time before the results are read, this last point being particularly crucial.

Our *in vitro* data suggest therefore that the new imidazoles, cloconazole, sulconazole, butoconazole, isoconazole, and fenticonazole may be promising antifungal agents against *Candida* isolates, but further *in vivo* tests with these drugs in candidiasis in animals and humans are needed.

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References

1. Marrie TJ, Cooper JH, Costerton JW. Ultrastructure of *Candida parapsilosis* endocarditis. Infect Immun 1984; 45: 390–398.
2. Morgan MA, Wilkowske CJ, Roberts GD. *Candida pseudotropicalis* fungemia and invasive disease in an immunocompromised patient. J Clin Microbiol 1984; 20: 1006–1007.
3. Odds FC, Palacio A, Cuadra J, Sanchez J. Disseminated *Candida* infection syndrome in heroin addicts. J Med Microbiol 1987; 23: 275–277.
4. Reinhardt JF, Ruane PJ, Walker LJ, George WL. Intravenous catheter-associated fungemia due to *Candida rugosa*. J Clin Microbiol 1985; 22: 1056–1057.
5. Graybill JR. Antifungal agents of the 1980's. Antimicrob News 1988; 5: 45–51.
6. McGinnis MR, Rinaldi MG. Antifungal drugs: mechanisms of action drug resistance, susceptibility testing, and assays of activity in biological fluids. In: Lorian VMD, ed. Antibiotics in laboratory medicine, 2nd ed. Baltimore: Williams & Wilkins, 1986: 223–281.
7. Dick JD, Rosengard BR, Merz WG, Stuart RK, Hutchins GM, Saral R. Fatal disseminated candidiasis due to amphothericin B-resistant *Candida guilliermondii*. Ann Intern Med 1985; 102: 67–68.
8. Guinet R, Chanas J, Goullier A, Bonnefoy G, Ambroise-Thomas P. Fatal septicemia due to amphothericin B-resistant *Candida lusitaniae*. J Clin Microbiol 1983; 18: 443–444.
9. Douglas JB, Healy JK. 1969. Nephrotoxic effects of amphothericin B including renal tubular acidosis. Am J Med 1969; 46: 154–162.
10. Kauffman CA, Frame PT. Bone marrow toxicity associated with 5-fluorocytosine therapy. Antimicrob Agents Chemother 1977; 11: 244–247.
11. Gordon MA, Lapa EW, Passero PG. Improved method for azole antifungal susceptibility testing. J Clin Microbiol 1988; 26: 1874–1877.
12. Humphrey MJ, Jevons S, Tarbit MH. Pharmacokinetic of UK-49, 858, a metabolically stable triazole antifungal drug, in animals and humans. Antimicrob Agents Chemother 1985; 28: 648–653.
13. Lefler E, Stevens DA. New azole compounds: vibunazole (Bay n7133) and Bay 19139, compared with ketoconazole in the therapy of systemic candidosis and in pharmacokinetic studies, in mice. J Antimicrob Chemother 1985; 29: 660–662.
14. Rogers TE, Galgiani JN. Activity of fluconazole and ketoconazole against *Candida albicans* in vitro and in vivo. Antimicrob Agents Chemother 1986; 30: 418–422.

15. Cooper BH, Silva-Hutner M. Yeasts of Medical importance. In: Lennette EH, Balows A, Hausler WJ, Shadomy JH, eds. Manual of clinical microbiology, 4th ed. Washington: American Society for Microbiology, 1985: 526–541.
16. Kreger van Rij NLW. The yeasts, a taxonomic study. New York: Elsevier, 1984.
17. Arx JA von, Schipper MAA. The CBS fungus collection. In: Perlman D, ed. Advances in applied microbiology. London: Academic Press, 1978: 215–236.
18. Shadomy S, Espinel-Ingroff A, Cartwright R. Laboratory studies with antifungical agents: susceptibility test and bioassays. In: Lennette EH, Balows A, Hausler WJ, Shadomy HJ, eds. Manual of clinical microbiology, 4th ed. Washington: American Society for Microbiology, 1985: 991–999.
19. Dick JD, Merz WG, Saral R. Incidence of polyene-resistant yeasts recovered from clinical specimens. Antimicrob Agents Chemother 1980; 18: 158–163.
20. Working group of the British Society for Mycopathology. Laboratory methods for flucytosine (5-fluorocytosine). J Antimicrob Chemother 1984; 14: 1–8.
21. Guinet R, Nerson D, De Closets F, Dupouy-Camet J, Kures L, Marjollet M, Poirot JL, Ros A, Texier-Maugein J, Volle PJ. Collaborative evaluation in seven laboratories of a standardized micromethod for yeast susceptibility testing. J Clin Microbiol 1988; 26: 2307–2312.
22. Iwata K. Drug resistance in human pathogenic fungi. In: Iwata K, Den Bossche H van, eds. In vitro and in vivo evaluation of antifungal agents. Amsterdam: Elsevier, 1986: 65–86.
23. Johnson EM, Richard MD, Warnock DN. *In vitro* resistance to imidazole antifungals in *Candida albicans*. J Antimicrob Chemother 1984; 13: 547–558.
24. Riley JF, Wilson RG, Barrett-Bee J. Azole resistance in *Candida albicans*. Sabouraudia 1984; 22: 53–63.
25. Whelan WL, Kerridge D. Decreased activity of UMP pyrophosphorylase associated with resistance to 5-fluorocytosine in *Candida albicans*. Antimicrob Agents Chemother 1984; 26: 570–574.
26. Hamilton-Miller JM. A comparative in vitro study of amphotericin B, clotrimazole and 5-fluorocytosine against clinically isolated yeasts. Sabouraudia 1972; 10: 276–283.
27. Espinel-Ingroff A, Shadomy S, Gebhart RJ. In vitro studies with R 51,211. Antimicrob Agents Chemother 1984; 26: 5–9.
28. Hussain Qadri SMH, Flournoy DJ, Qadri SGM, Ramirez EG. Susceptibility of clinical isolates of yeasts to antifungal agents. Mycopathologia 1986; 95: 183–187.

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