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In Vitro Activity of D0870 Compared with Those of Other Azoles against Fluconazole-Resistant *Candida* spp.

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We compared the in vitro activity of a new triazole, D0870, with those of fluconazole, itraconazole, and ketoconazole against 41 clinical isolates of fluconazole-resistant *Candida* belonging to nine different species. The 50% inhibitory concentrations (IC_{50}s) were determined by a microdilution method with morpholinopropanesulfonic acid (MOPS)-buffered RPMI medium and an inoculum of $\approx 10^4$ yeasts per ml. After incubation for 48 h at 37°C the optical density at 550 nm was measured. The IC_{50} was the lowest drug concentration which reduced the optical density at 550 nm by ≥50% compared with that for a drug-free control. D0870 had significant activity against many of the isolates. Its activity was comparable to that of ketoconazole, slightly superior to that of itraconazole, and markedly superior to that of fluconazole against *Candida albicans*. Against *Candida glabrata*, *Candida krusei*, and *Candida inconspicua*, it had activity similar to those of itraconazole and ketoconazole but had activity superior to that of fluconazole. D0870 IC_{50}s for some isolates were increased. This may be due to cross-resistance mechanisms because the IC_{50}s of both itraconazole and ketoconazole for these isolates were often high. When IC_{50}s and IC_{80}s were compared there was a marked organism and drug variation. With *C. glabrata* much higher endpoints for itraconazole were observed when an IC_{80} endpoint was used. For *C. albicans* there was also a significant shift upward in endpoints for itraconazole and ketoconazole. Values were changed little when IC_{50} and IC_{80} endpoints of D0870 were compared. For 35 of 41 isolates tested the D0870 IC_{50} was less than the 2.5-mg/liter breakpoint threshold proposed previously. Therefore, D0870 may be a useful agent for the therapy of infections caused by fluconazole-resistant *Candida* spp.

Serious infections caused by yeasts, particularly *Candida albicans*, are an increasing problem because of factors such as intensive care practices, the use of indwelling catheters, human immunodeficiency virus infection, organ transplantation, and other immunosuppressive conditions. Such infections require prompt and appropriate therapy; however, the choice of drugs is limited and the first-choice drug for the treatment of systemic infections, amphotericin B, is associated with numerous toxic side effects. Imidazole and triazole antifungal drugs, such as itraconazole (ITZ) and ketoconazole (KTZ), have been developed and are among the most useful drugs for the treatment of human candidoses. The triazole drug fluconazole (FLU) has low toxicity, is well tolerated and absorbed, and has been used successfully in the treatment of both mucosal and invasive candidoses (9).

A disturbing consequence of the long-term use of FLU is the increase in the number of patients, particularly AIDS patients, failing treatment because of infection with FLU-resistant *Candida* spp. (1, 2, 4–6, 13, 15, 16, 20, 21, 26, 27, 29). Treatment failure may be attributable to the development of azole resistance, infection with a species intrinsically resistant to azoles, e.g. *Candida krusei* or *Candida glabrata*, or low concentrations of drug in serum, sometimes because of drug interactions (20). In almost all cases patients have had advanced human immunodeficiency virus infection. There are at least three mechanisms by which *C. albicans* develops resistance to azoles: resistance caused by decreased susceptibility to azoles of P-450, the target enzyme in ergosterol biosynthesis (11, 27), overproduction of the target fungal enzyme (10, 30), or decreased permeability of the yeast cells to the drug (12–14, 25). Because of this emergence of drug resistance and also because of the risks of toxicity and poor bioavailability associated with other antifungal agents, there is a need to develop new, safe, and effective antifungal drugs (8).

D0870 is a new bis-triazole with a broad range of antifungal activity (18, 22, 31). It is reported to have potency superior to those of other azoles both in vitro and in animal models. It has been documented that the pathogenic yeasts *C. albicans* and *Cryptococcus neoformans* are more susceptible to D0870 than other pathogenic yeasts. There were two main aims of the present study: (i) to assess the activity of D0870 against a range of yeasts with reduced susceptibilities to FLU and (ii) to compare the activity of D0870 with those of ITZ and KTZ against these yeasts.

MATERIALS AND METHODS

Antifungal agents. The four drugs were provided by the manufacturers as standard powders. D0870 was provided by the pharmaceutical sponsor (Zeneca Pharmaceuticals, Cheshire, England), FLU was provided by Pfizer Ltd. (Kent, England), and ITZ and KTZ were provided by Janssen Pharmaceutica (Beerse, Belgium). Stock solutions were prepared by dissolving each drug in dimethyl sulfoxide, adjusting the weight according to the potency of the drug to give a concentration of 1,280 mg/liter.

Cultures. Forty-one isolates of *Candida* spp. from our culture collection were tested. All were recent clinical isolates from a variety of patient types and infection sites. For all isolates FLU MICs were increased when the isolates were tested as part of a previous study investigating the incidence of antifungal resistance (16). The group comprised 15 *C. glabrata*, 14 *C. albicans*, 5 *C. krusei*, 2 *C. inconspicua*, 1 *C. humicola*, 1 *C. norvegensis*, 1 *C. guilliermondii*, 1 *C. famata*, and 1 *C. lambica* isolate. Isolates were identified by germ tube formation and the API 32C system (bioMérieux, Basingstoke, United Kingdom).

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Media and buffers. The medium used for susceptibility testing was RPMI 1640 (Sigma, Dorset, United Kingdom) buffered with morpholinopropanesulfonic acid (MOPS; Sigma) to a final molarity of 0.165 M and adjusted to pH 7.0 with 10 M NaOH. This medium contained L-glutamine and sodium bicarbonate and was stored at 4°C after filter sterilization.

Inoculum preparation. Yeast suspensions were prepared by suspending five colonies with a diameter of ≥1 mm from a 48-h Sabouraud plate in 5 ml of sterile water. The solutions were vortexed, and the turbidity of each suspension was measured with a spectrophotometer. The turbidity of the suspension was adjusted to an optical density at 550 nm (OD_{550}) of 0.125 (0.5 McFarland standard) by the addition of sterile water to give a suspension containing 10^6 CFU/ml. This suspension was then diluted 1:50 in RPMI 1640 medium to give the final inoculum density to be used in the assay (2×10^4 CFU/ml).

Susceptibility testing procedure. Sterile, flat-well microtiter plates (Alpha Laboratories, Hampshire, United Kingdom) were used for the tests. The standard 1,280-mg/liter solutions of the four drugs were diluted 1:20 in RPMI 1640 medium to yield a 64-mg/liter start solution. Doubling dilutions of these start solutions were made in RPMI 1640 medium in the wells to give a range of drug concentrations from 32 to 0.03 mg/liter; one well was left drug free to act as a control. Wells were inoculated with 100 μl of the culture suspension of 2×10^4 CFU/ml to give a final inoculum of 1×10^4 organisms per ml. A separate well was inoculated with 200 μl of RPMI 1640 medium to act as the medium sterility check. The plates were incubated in a moist chamber at 37°C for 48 h. Sabouraud agar and blood agar (Columbia agar base [Lab M, Bury, United Kingdom] plus 5% defibrinated horse blood [APP, West Midlands, United Kingdom]) plates were inoculated with 10 μl of each organism suspension to check the viable count and culture purity.

Reading of MIC endpoints. After incubation, the plates were shaken for 5 min to obtain a uniform suspension prior to reading. The turbidity of each well was determined at an OD_{550} by spectrophotometer. The IC_{50} and IC_{80} were the lowest drug concentrations which reduced the OD_{550} by 50 or 80%, respectively, compared with the OD_{550} of the drug-free control.

Reproducibility. In order to check the reproducibility of the method, the susceptibility testing procedure was repeated on 15 isolates with all four drugs.

Controls. Six quality control strains were selected to give a range of yeast species: *C. albicans* ATCC 90028, *C. albicans* ATCC 24433, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750, *C. krusei* ATCC 6258, and *Torulopsis glabrata* ATCC 90030. These strains were tested by the susceptibility procedure described above with all four drugs.

Statistical analysis. Differences between both drugs and species were tested by the Mann-Whitney U test, and $P < 0.05$ was taken as the critical level of significance.

Data presentation. IC_{50}s and IC_{80}s of all four drugs for the three main groups of yeasts are presented as box plots (3, 28).

RESULTS

All isolates showed good growth in the RPMI 1640 medium used in the study. The IC_{50} range and geometric mean for the groups of *Candida* spp. studied are given in Table 1. For the *C. albicans* isolates the D0870 and KTZ IC_{50}s were similar to each other, and the IC_{50}s of both drugs were significantly lower than those of ITZ ($P < 0.01$) and FLU ($P < 0.00001$). For three isolates the IC_{50}s of D0870 were raised to 4, 2, and 2 mg/liter, respectively. For two isolates the IC_{50}s of ITZ were high (IC_{50}s, >32 mg/liter), but for both isolates IC_{50}s of KTZ were 2 mg/liter. For the remaining isolate the D0870 IC_{50} was 2 mg/liter; the IC_{50}s of ITZ and KTZ were lower (0.5 and 0.25 mg/liter, respectively).

For the *C. glabrata* isolates the geometric mean IC_{50}s of ITZ and KTZ were slightly greater than those of D0870; however, the differences between IC_{50}s were not statistically significant ($P > 0.5$). The IC_{50}s of FLU were significantly greater ($P < 0.00001$) than those of D0870. D0870 IC_{50}s were high for two isolates (8 and 4 mg/liter, respectively). For these two isolates ITZ IC_{50}s were >32 mg/liter and KTZ IC_{50}s were 8 and 16 mg/liter, respectively.

Seven isolates of *C. krusei* and *C. inconspicua* were examined. All were highly resistant to FLU (IC_{50}s, ≥32 mg/liter). The IC_{50}s of D0870, ITZ, and KTZ were similar, no significant difference between them.

When comparing D0870 IC_{50} results there were significant differences between the *C. albicans* and the *C. glabrata* groups ($P < 0.01$) and between the *C. albicans* and the *C. krusei* groups ($P < 0.05$), with the IC_{50}s for *C. albicans* being lower,

TABLE 1. Susceptibility results for *Candida* spp.[a]

Organism	No. of isolates	D0870				FLU				ITZ				KTZ			
		IC_{50} (mg/liter)		IC_{80} (mg/liter)		IC_{50} (mg/liter)		IC_{80} (mg/liter)		IC_{50} (mg/liter)		IC_{80} (mg/liter)		IC_{50} (mg/liter)		IC_{80} (mg/liter)	
		R	GM	R	GM	R	GM	R	GM	R	GM	R	GM	R	GM	R	GM
C. albicans	14	<0.03–4	0.11	<0.03–32	0.38	4–>32	19.5	8–>32	33.65	0.06–>32	0.74	0.125–>32	3.81	<0.03–2	0.13	<0.03–32	4.45
C. glabrata	15	<0.03–8	0.91	<0.03–32	2.51	8–>32	30.5	16–>32	55.72	0.125–>32	1.2	0.5–>32	9.18	<0.03–16	1.04	0.06–32	1.91
C. krusei-C. inconspicua	7	0.25–4	0.82	0.25–4	1.1	32–>32	60.0	>32	>32	0.5–2	0.74	0.5–8	1.1	0.125–4	0.74	0.5–8	1.22
Other *Candida* spp.	5	<0.03–1	0.05	<0.03–1	0.09	2–>32	6.07	2–>32	9.18	<0.03–0.5	0.14	0.06–1	0.38	<0.03–0.5	0.03	<0.03–0.5	0.05

[a] R, range; GM, geometric mean. For the purpose of determining the geometric mean, ICs of >32 mg/liter were assumed to be 64 mg/liter and ICs of <0.03 mg/liter were assumed to be 0.015 mg/liter.

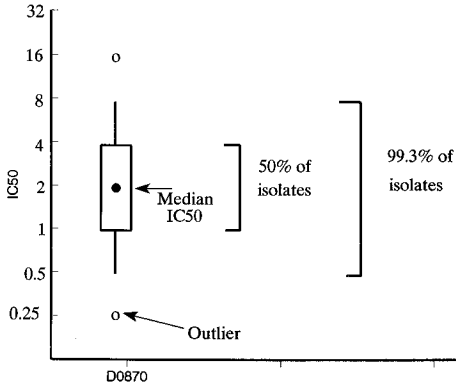

FIG. 1. Box plot (3, 28).

but there was no significant difference between the IC_{50}s for the *C. glabrata* and *C. krusei* groups.

The FLU IC_{50}s for the various other *Candida* spp. ranged from 2 to >32 mg/liter. The D0870, ITZ, and KTZ IC_{50}s were all very similar, ranging from <0.03 to 1 mg/liter.

Box plots have been used to summarize the activities of the four drugs against the three main groups of *Candida* spp. tested (Fig. 1 to 4). When IC_{50}s and IC_{80}s were compared there was marked organism and drug variation. For *C. albicans* there was a significant elevation in the endpoints of ITZ ($P < 0.02$) and KTZ ($P < 0.05$) when the IC_{80} was used as the endpoint. With *C. glabrata* isolates there was a marked increase in the endpoints for ITZ ($P < 0.005$). Changes in D0870 and KTZ were not so marked. For *C. krusei* and *C. inconspicua* isolates the use of different endpoint criteria had little effect on the susceptibilities of the isolates to all four drugs.

Reproducibility was checked by repeating the MIC determinations for 15 different isolates. For the IC_{50} of D0870 for 2 of the 15 isolates showed marked (greater than two wells) differences when IC_{50}s were determined on two separate occasions. All FLU results were within one well difference, and only one ITZ determination and two KTZ determinations were greater than two wells different.

The FLU IC_{50} results for the six control organisms are given in Table 2.

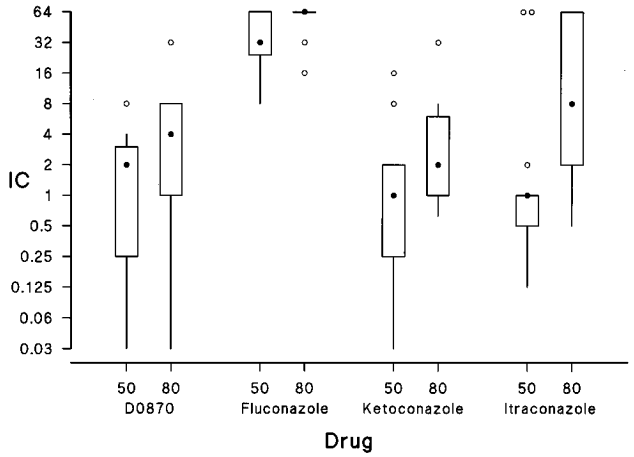

FIG. 3. Box plot comparison of 50 and 80% inhibition endpoints on the activities of D0870 and other azoles against *C. glabrata* isolates. IC, inhibitory concentration.

DISCUSSION

There is considerable debate concerning the correct method of performing fungal susceptibility testing. Factors which can influence tests include medium, buffering system, temperature of incubation, inoculum size, and endpoint determination (7, 24). In the present study we used a previously described method (17) similar to the method proposed by the National Committee for Clinical Laboratory Standards (NCCLS) (19). The differences between this method and the NCCLS method are (i) the size of the inoculum used, 1×10^4 in the present study compared with 0.5×10^3 to 2.5×10^3 by the NCCLS method; (ii) the endpoints, an 80% inhibition is used for the NCCLS method, whereas the method that we used has a 50% inhibition cutoff; and (iii) our method used microtiter plates instead of the broth macrodilution in tubes used by the NCCLS method. Although we used a higher inoculum than that recommended by NCCLS, which would tend to increase MICs, the use of a less stringent endpoint, i.e., the IC_{50} rather than the IC_{80}, reverses the trend toward high MICs. Our data on the NCCLS control organisms are in excellent agreement with

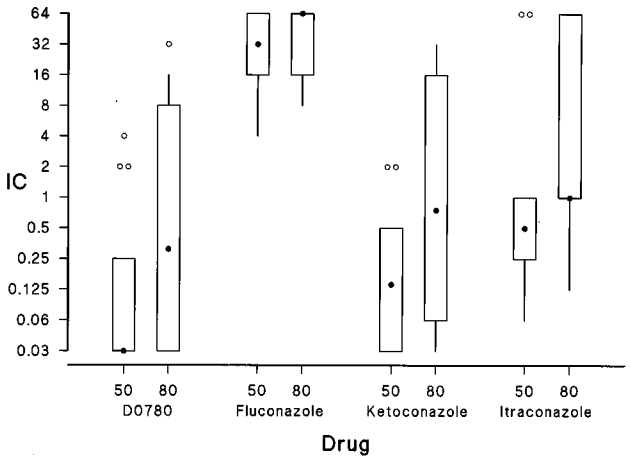

FIG. 2. Box plot comparison of 50 and 80% inhibition endpoints on the activities of D0870 and other azoles against *C. albicans* isolates. IC, inhibitory concentration.

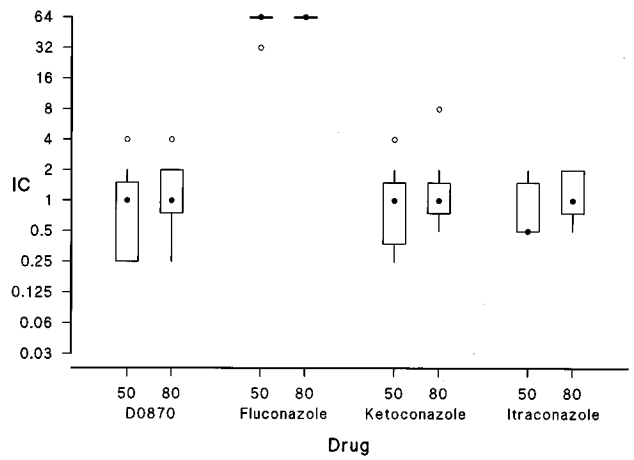

FIG. 4. Box plot comparison of 50 and 80% inhibition endpoints on the activities of D0870 and other azoles against *C. krusei* and *C. inconspicua* isolates. IC, inhibitory concentration.

TABLE 2. Susceptibility results for FLU against six NCCLS control organisms[a]

Quality control organism	IC_{50} (mg/liter)	IC_{80} (mg/liter)
C. albicans ATCC 90028	0.5	0.25–1
C. albicans ATCC 24433	0.5	0.25–1
C. parapsilosis ATCC	2	1–4
C. tropicalis ATCC 750	4	1–4
C. krusei ATCC 6258	>32	16–64
T. glabrata ATCC 90030	16	8–32

[a] IC_{50}s were determined in the present study, and IC_{80}s are from Pfaller et al. (23).

those of Pfaller et al. (23) for FLU. In addition, we found good reproducibility when susceptibility tests were repeated.

Isolates that were found to be FLU resistant as determined in a clinical laboratory setting (16) were selected for use in the study. The five other *Candida* spp. included in the study had intermediate susceptibilities to FLU, also when tested previously, and were included to give a wider picture of the in vitro activity of D0870.

When the IC_{80}s were compared with the IC_{50}s there was a tendency toward a higher degree of resistance, but this effect was dependent on the organism and the antifungal compound. We feel that under the conditions used in the present study the use of IC_{50} endpoints is more appropriate and is suitable for all species and all four drugs tested.

In the present study all four drugs were tested by the same method. The efficacy of D0870 was therefore compared with those of the established drugs ITZ and KTZ against 41 isolates of various *Candida* spp. with decreased in vitro susceptibility to FLU. Against this selected population of *Candida* isolates D0870 had activity virtually identical to that of KTZ against all groups of organisms tested. In addition, against *C. glabrata* and *C. inconspicua-C. krusei* both drugs had activity similar to that of ITZ. However, against *C. albicans* both KTZ and D0870 had activities superior to that of ITZ. All three drugs had activities markedly superior to that of FLU against the organisms tested. However, not all isolates were highly susceptible to D0870; we observed certain isolates for which D0870 IC_{50}s were high (>2 mg/liter). For some of these isolates IC_{50}s of both ITZ and KTZ were high. This suggests that cross resistance exists between these drugs, which has clear implications for susceptibility testing. It also implies that multiple mechanisms of azole resistance are likely to be operative in *Candida* spp.

D0870 had good activity against many isolates of FLU-resistant yeasts belonging to various species. Overall, its activity was comparable to that of KTZ and slightly superior to that of ITZ against *C. albicans*. These results suggest that D0870 may be a useful drug for the treatment of candidal infections because it is highly active against FLU-resistant yeasts. Its in vivo activity must be examined in clinical trials, and breakpoints for potentially susceptible and resistant groups must be devised on the basis of in vitro susceptibility tests results and clinical correlation.

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