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In Vitro Studies of Activities of the Antifungal Triazoles SCH56592 and Itraconazole against *Candida albicans*, *Cryptococcus neoformans*, and Other Pathogenic Yeasts

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We investigated the effects of various assay conditions on the activities of two triazole antifungal drugs, SCH56592 and itraconazole, against seven species of fungi by the broth macrodilution testing procedure proposed by the National Committee for Clinical Laboratory Standards (NCCLS). For both drugs, which are insoluble in water, the concentration and type of solubilizing agent produced differences in drug activity. Starting inoculum size differences from 10^2 to 10^5 yeast cells per ml resulted in approximately a fourfold effect on the MIC of both drugs, but other significant differences were not observed with variations in synthetic medium composition, pH, buffering reagent, or incubation temperature. Under standardized conditions of reference method M27-T with 1% polyethylene glycol as the solubilizing agent, median MICs of SCH56592 and itraconazole of 60 and 125 ng/ml, respectively, were demonstrated for 110 strains (12 to 23 strains for each of seven species). Broth microdilution results were typically severalfold higher than broth macrodilution results. We conclude that the NCCLS standard reference method can be applied without modification to the testing of SCH56592 and itraconazole, but particular attention to solubilizing the agents is critical to obtaining consistent results.

SCH56592, (−)-4-[4-[4-[4-[[(2*R-cis*)-5-(2,4-difluorophenyl)-tetahydro-5-(1H-1,2,4-triazol-1-ylmethyl)furan-3-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(*S*)-1-ethyl-2(*S*)-hydroxypropyl]-3H-1,2,4-triazole-3-one, is a novel analog of SCH51048 (3, 12, 26, 29) currently under development by Schering-Plough Research Institute for its antifungal potential. In preparation for clinical trials with this agent, it would be useful to know if the standard reference method for testing yeasts that was developed by consensus through the National Committee for Clinical Laboratory Standards (NCCLS) will be applicable to this agent (15, 16). In this report, we analyze the *in vitro* activity of SCH56592 by changing each of several test conditions that influence test results with other antifungal agents (2, 4, 9, 10, 13, 14, 25), using itraconazole for comparison. Based upon these studies, we propose conditions for standardized testing of SCH56592 and determine the susceptibilities of representative clinical isolates under those conditions.

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MATERIALS AND METHODS

Drugs. SCH56592 and itraconazole were provided as powders by their pharmaceutical sponsors (Schering-Plough Research Institute, Kenilworth, N.J., and Janssen Research Institute, Piscataway, N.J.) and were stored desiccated at room temperature until use. Except where specified otherwise in Results, a stock solution was prepared by dissolving antifungal powder in polyethylene glycol 400 (PEG 400; Aldrien Chemical Co. Inc., Milwaukee, Wis.) heated to 75°C in a water bath to a final concentration of 1.0 mg/ml, and the preparation was divided for storage at −70°C until needed.

Yeasts. A group of 10 yeast isolates (2 each of *Candida albicans*, *Candida tropicalis*, and *Cryptococcus neoformans*; 1 each of *Candida lusitaniae*, *Candida parapsilosis*, *Torulopsis glabrata*, and *Candida krusei*) were used for the initial studies. Susceptibilities to fluconazole for these isolates ranged from 0.125 to 32 μg/ml. In addition, 110 strains of yeasts were tested under a single set of standardized conditions. All told, there were 23 isolates of *C. albicans*, 12 *C. lusitaniae*, 15 *C. tropicalis*, 15 *C. parapsilosis*, 15 *T. glabrata*, 15 *C. krusei*, and 17 *C. neoformans* (2 of which were only used in the initial analysis of test conditions). Isolates were the generous gifts of M. A. Pfaller (69 genetically distinct strains from fungemic patients at the University of Iowa [20, 23]), Mahmoud Ghannoum (15 strains of *C. neoformans* [34]), J. R. Rex (11 strains recovered from fungemic patients during a clinical trial conducted by the NIAID-Mycoses Study Group [24]), A. Espinel-Ingroff (5 strains of *C. krusei*), and J. C. Pottage (1 strain of *C. krusei* from St. Johns Hospital, Detroit, Mich. [31]). The remaining 10 isolates were from the clinical laboratory of the Veterans Affairs Medical Center in Tucson, Ariz. When not in active use, isolates were stored in yeast nitrogen base (YNB; Difco Laboratories, Detroit, Mich.) with 2% (wt/vol) glucose–10% (vol/vol) glycerol at −70°C and were repeatedly subcultured on Sabouraud dextrose agar plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.) with overnight incubation at 37°C before testing.

Media and buffers. RPMI-1640 (Sigma Chemical Co., St. Louis, Mo.) constituted with 0.2% (wt/vol) glucose and buffered with morpholinopropanesulfonic acid (MOPS; Sigma Chemical Co.) at a final concentration of 0.165 M to a pH of 7.0 was used in most studies. Where specified in Results, YNB or antibiotic medium 3 (Difco) was substituted for RPMI-1640. In other studies, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; Sigma) or sodium phosphate was substituted for MOPS, and the pH was adjusted to between 3.0 and 7.0.

Susceptibility testing. Broth macrodilution susceptibility tests were performed in conformity with the NCCLS reference method M27-T (16) with conditions modified for specific experiments as described in the text. Briefly, twofold dilutions of the antifungal agents were prepared in medium to concentrations ranging from 1.9 to 1,000 ng/ml. Dilution procedures followed exactly the procedure described for M27-T. Yeast inocula were adjusted by spectrophotometer (19) to 0.5 to 2.5 × 10^3 yeast cells per ml. Yeast inoculum (0.9 ml) and diluted drug (0.1 ml) were mixed (final volume, 1.0 ml) in polystyrene tubes and incubated at 35°C without agitation for 48 h (72 h for *C. neoformans*). The MIC was defined as the lowest drug concentration which inhibited growth by 80% after 48 h of incubation (72 h for *C. neoformans*). In addition, a turbidimetric endpoint (50% inhibitory concentration [IC_{50}]) was determined during active growth as the lowest drug concentration that resulted in the following: $\%T > \%T_{control} + [0.5(100 - \%T_{control})]$, where %T is the percent transmission and $\%T_{control}$ is the turbidity in the drug-free control tube (9).

Broth microdilution MICs were determined by modifying the NCCLS procedure as outlined previously (16). Into each well of round-bottom 96-well microtitration plates (ICN Biomedical Inc., Horsham, Pa.) 100 μl of fungal suspension and 100 μl of drug solution was delivered and mixed with a pipette prior to

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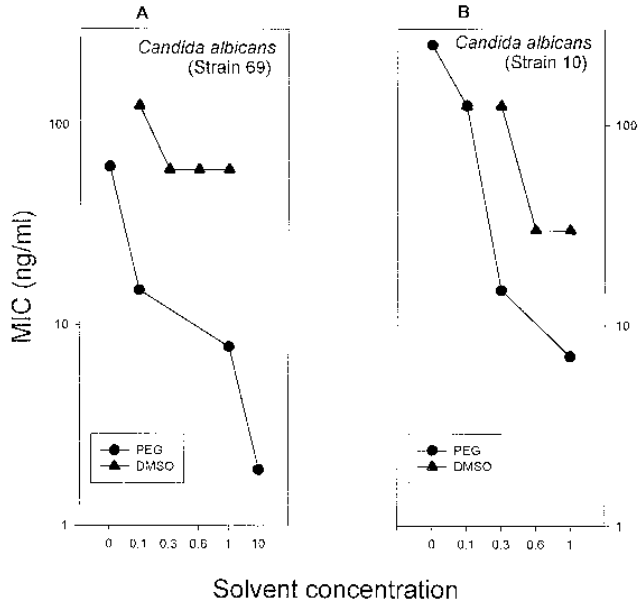

FIG. 1. The effect of solvent concentration (vol/vol) on MIC of SCH56592 for two strains of *C. albicans*.

incubation. MICs were read without agitation as the lowest drug concentration which prevented growth.

For both broth macrodilution and broth microdilution procedures, one strain of *C. albicans* was included with each experiment for internal quality control.

Statistical procedures. Significance of the effect of various different test conditions was tested by the Kruskal-Wallis nonparametric procedure as implemented by Systat (Systat, Inc., Evanston, Ill.).

RESULTS

Drug insolubility and the difference among solvents. Acidifying water to a pH of 1.5 with hydrochloric acid permitted both SCH56592 and itraconazole to be dissolved at a concentration of 1.0 mg/ml without solvent. However, at this concentration, raising the pH higher than 1.5 precipitated either drug. When SCH56592 was added to 100% dimethyl sulfoxide (DMSO) or PEG 400, diluted with unacidified water to a final solvent concentration of 1% (vol/vol), precipitates also occurred with concentrations of SCH56592 as low as 10 μg/ml. Acidification with 1% of either solvent resulted in disappearance of the precipitates, but precipitates reappeared after the pH was adjusted with sodium hydroxide above 4.0. Below 10 μg/ml, precipitates did not form as the pH was raised to 7.0, but the concentration may have been too low for visual inspection to be sensitive. Since drug solubility could be critical to *in vitro* activity, the effect of various concentrations of the two different solvents on the MIC was determined, and representative results for two of the five strains studied with SCH56592 are shown (Fig. 1). For both solvents, increasing the concentrations to 1% decreased the MIC for all strains. Studies of solvent alone over this range showed no effect on growth. Also, SCH56592 in PEG 400 was consistently more active than in DMSO at equivalent concentrations. Using PEG 400 at the higher concentrations resulted in further decreases in MICs. However, above 1.0% solvent concentrations, PEG 400 without antifungal agents had direct inhibitory effects on growth. At a 10% concentration, DMSO inhibited growth altogether, and an MIC could not be determined. Therefore, remaining studies utilized PEG 400 at a final concentration of 1.0%.

TABLE 1. Effect of various test conditions on susceptibility results

Variable	*P* value or MIC[a] (ng/ml)					
	SCH56592			Itraconazole		
	IC_{50}	Day 1	Day 2	IC_{50}	Day 1	Day 2
Inoculum	NS	NS	$P = 0.02$	NS	NS	$P = 0.04$
10^2	7.5	6.1	12.1	14.9	8.5	13.0
10^3	11.3	9.2	17.1	24.3	12.1	21.1
10^4	13.9	12.1	26.0	24.2	19.7	29.9
10^5	18.4	27.9	55.8	34.3	39.4	45.3
pH	NS	NS	NS	NS	NS	NS
3.0	21.1	24.2	39.4	8.6	19.7	29.9
4.0	14.9	19.7	36.8	10.6	17.1	36.8
5.0	17.1	17.1	48.5	22.6	24.3	59.7
6.0	17.1	21.1	52.0	19.7	27.9	64.0
7.0	12.1	14.9	29.9	10.6	17.1	42.2
Temperature	NS	NS	NS	NS	NS	NS
30°C	14.9	17.1	34.3	17.1	26.0	45.3
35°C	5.7	8.0	24.3	7.5	13.0	39.4
37°C	4.9	11.3	29.9	12.1	24.3	59.7
Medium	NS	NS	NS	NS	NS	NS
RPMI	10.6	24.3	36.8	17.1	39.4	45.3
YNB	6.5	21.1	42.2	9.8	32.0	48.5
AB-3[b]	7.0	59.7	104.0	5.7	84.4	78.8
Buffer	NS	NS	NS	NS	NS	NS
MOPS	9.2	19.7	34.3	11.3	24.3	39.4
HEPES	7.5	17.1	26.0	7.5	22.6	42.2
Phosphate	4.9	8.6	22.6	7.5	13.9	32.0

[a] Results are geometric means for 10 strains representing seven species of yeasts. The standard error of the mean for each result ranged from 0.60 to 0.99 ng/ml. *P* values are the result of Kruskal-Wallis tests of significance of differences between groups within each variable. NS, *P* value is greater than 0.05.

[b] AB-3, antibiotic medium 3.

Effects of changing other test conditions. Results with 10 strains from seven species were analyzed to identify other test conditions which influenced SCH56592 and itraconazole MICs (Table 1). For both drugs, second-day MIC results rose significantly with increasing inoculum size, representing approximately a fourfold change between starting concentrations of 10^2 and 10^5 cells per ml. With first-day MIC results, the same pattern was evident but differences did not reach statistical significance. IC_{50} results were not significantly influenced by inoculum, and neither MIC nor IC_{50} results were affected by changes in media pH, incubation temperature, medium composition, or buffer system.

Range and distribution of results under standard conditions. The above studies indicated that there was no need to modify the standard conditions recommended for testing other drugs by the NCCLS M-27T broth macrodilution reference procedure. Accordingly, 110 strains were tested by that method (Table 2). For both drugs, *C. neoformans* was the most susceptible species, while *C. krusei* and *T. glabrata* appeared to be the least susceptible species. The remaining four species were two- to fourfold more susceptible to SCH56592 than to itraconazole.

Broth microdilution results. The susceptibilities of the same 110 strains to SCH56592 and itraconazole were also tested by broth microdilution assay, and these results were compared to the reference standard results (Table 3). Both day 1 and day 2 results were on average two- to fourfold higher than the results obtained by the standard broth macrodilution procedure. Be-

TABLE 2. Susceptibility results determined under standardized conditions

Species	No. of strains	Result[a] (ng/ml) for:					
		SCH56592			Itraconazole		
		MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
C. neoformans	15	30	60	15–60	30	60	15–60
C. parapsilosis	15	30	60	15–60	125	250	60–250
C. lusitaniae	12	30	60	7–125	250	250	60–500
C. albicans	23	30	125	15–500	60	500	7–500
C. tropicalis	15	30	125	15–500	125	250	60–500
C. krusei	15	250	500	250–500	500	500	250–500
T. glabrata	15	1,000	>1,000	700–>1,000	1,000	1,000	15–>1,000

[a] MIC_{50} and MIC_{90}, result for 50 and 90% of strains tested, respectively.

cause of this systematic difference, fourfold agreement between both microdilution results and broth macrodilution results was 65 and 47% for day 1 results and 45 and 38% for day 2 results for SCH56592 and itraconazole, respectively.

DISCUSSION

Drugs that are insoluble at a physiologic pH may still be therapeutically useful. Itraconazole, for example, studied here in comparison to SCH56592, requires a solvent to keep it in suspension at neutral pH, and yet itraconazole clearly is effective as a treatment for a variety of fungal diseases (1, 5, 6, 11, 28, 32, 33). With SCH56592, solubility was achieved at concentrations as high as 1 mg/ml at a pH of 1.5, but precipitates formed at concentrations as low as 10 μg/ml even when SCH56592 was supplemented with 1% DMSO when the pH was raised above 4. Although solubility at lower concentrations of SCH56592 could not be reliably estimated visually, susceptibility testing demonstrated that changes in MICs at pH 7 occurred over a range of solvent concentrations (Fig. 1). Since direct effects of solvents were detected at concentrations above 1%, the lower MICs obtained above this solvent concentration may be the result of additive or synergistic effects between solvent and antifungal agents. Clearly, attention to solvent concentration is required to obtain reproducible results.

In surveying several other test conditions which have shown systematic effects on susceptibility results with other antifungal agents, we have found that only variations in inoculum size demonstrated small but statistically significant effects on MICs of both drugs (Table 1). Therefore, our studies support the testing of SCH56592 and itraconazole under the reference method's standard conditions without modification as recently outlined by the NCCLS (16).

TABLE 3. Agreement of broth microdilution with broth macrodilution results

Fold difference in results[a]	No. of strains each day			
	SCH56592		Itraconazole	
	Day 1	Day 2	Day 1	Day 2
Eightfold less	3	0	1	1
Fourfold less	2	2	0	0
Twofold less	12	3	4	0
No difference	22	10	16	8
Twofold more	37	37	32	34
Fourfold more	26	36	36	34
Eightfold more	8	18	19	24
16-fold more	0	3	1	7
>16-fold more	0	1	1	2

[a] Results are for microdilution.

Under standardized conditions, both drugs displayed nearly identical activities against *C. neoformans*, which was the most susceptible species studied (Table 2). For most other species SCH56592 appeared to be two- to fourfold more active than itraconazole. *C. krusei* was uniformly the most resistant species to both drugs. Although for *T. glabrata* both drugs usually had high MICs, MICs for occasional strains were similar to those for other more sensitive species. Induced resistance to azole antifungal drugs has been studied in detail in one strain of *T. glabrata* (30), raising the possibility that infections caused by these apparently sensitive strains *in vitro* might fail to respond to treatment. Further studies will be needed to address this point.

Although consensus has developed in support of the NCCLS reference standard M27-T, this method has several drawbacks as a practical procedure in clinical laboratories (8). It is for this reason that the broth microdilution procedure has been included as an alternative standard procedure. That the broth microdilution results are systematically higher and that day 1 readings show better agreement with broth macrodilution results than day 2 readings for SCH56592 and itraconazole is in keeping with previous comparisons of the two methods for other drugs (7, 18, 21, 22, 27). Furthermore, the broth microdilution MICs that we obtained are similar to those obtained by broth microdilution in another study (17). However, in contrast to studies with other drugs, agreement of broth micro- and macrodilution results was unacceptably low for the two drugs studied here. Only 65 and 47% of day 1 MICs agreed within a fourfold range with broth macrodilution MICs for SCH56592 and itraconazole, respectively. Pfaller et al. (22) studied differences in endpoint determination between visual inspection before and after agitation and found good agreement between readings. Direct comparisons to broth macrodilution results were not made in that study. However, when discrepancies occurred between readings made before and after agitation, those made before agitation were generally higher than readings made after agitation. Since broth microdilution MICs were determined without agitation in our study, it is possible that agitation could have systematically decreased broth microdilution MICs and thereby improved the correlation between broth and micro- and macrodilution results in our study.

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