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Inhibition and Killing of *Candida albicans* In Vitro by Five Imidazoles in Clinical Use

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Five imidazoles (clotrimazole, econazole, ketoconazole, miconazole, and tioconazole) in clinical use were compared for their ability to inhibit and kill *Candida albicans*. Eleven isolates were obtained from patients before therapy. By spectrophotometric determination of 50% growth inhibition, all isolates were inhibited at low concentrations, with clotrimazole slightly less active than the other four drugs. By the conventional MIC determination, tioconazole was more active than all of the others ($P < 0.01$) except clotrimazole. In killing (minimum fungicidal concentration [MFC] assay), tioconazole was the most active by several analyses. Studies of the kinetics of killing indicated that the drugs studied could kill under conditions used for the MFC determination and that tioconazole and ketoconazole could kill particularly rapidly. If the drug was washed from the cells before subculturing, concentrations above the MFC were required to kill, but tioconazole could produce a lethal lesion in all cells virtually instantaneously. These findings are pertinent to MFC and killing kinetics methodology and to the observation of drug persistence after topical application. The results differ from some previous in vitro comparisons made with different methods. They are relevant to conclusions about drug mechanisms based on their abilities to inhibit and to kill, and they underscore the need to study various assay methods and fungal species.

The development of new antifungal drugs is currently dominated by molecular modifications of the imidazole class (3, 5, 7. 11, 12, 14, 19). These modifications have resulted in several active compounds which have proceeded to clinical use in topical or systemic administration or both. Some in vitro comparisons of activity have been made with various organisms (2, 6, 14, 15, 21, 23). Conclusions about mechanisms of action have sometimes been related to the results (21), although it is unclear whether the differences seen would apply to different fungal species. In addition, comparisons have been directed largely to inhibition of growth, but little attention has been paid to killing of fungi. We have compared the inhibition and killing activities of five imidazoles currently in clinical use. The assay organism used was *Candida albicans*, a fungal pathogen of humans which can cause superficial or systemic disease.

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MATERIALS AND METHODS

Isolates. The *C. albicans* isolates used were recent clinical isolates obtained from patients before treatment and identified to species as described previously (20). The isolates were stored on agar slants at 4°C before use. Twelve were selected at random.

Susceptibility test methods. Spectrophotometric determination of the drug concentration that inhibited growth by 50% ($IC_{1/2}$) compared with growth of controls was performed as previously detailed (9). The tube dilution visual-endpoint MIC was determined by standard methods (9) with 10^3 cells of an overnight growth per ml as the inoculum, but readings were made after 48 h of incubation (when control growth was turbid). The method of preparation of the inoculum has been shown to reproducibly deliver inocula of ca. 10^3 CFU/ml. This was confirmed in these studies, particularly in verifying the starting inoculum in the time-killing studies. For example, in 19 tubes sampled, the inoculum was 875.8 ± 271.8 CFU/ml (mean ± standard deviation). The medium used was a synthetic medium well buffered near the physiological range, pH 7.2 to 7.4 (13). Twofold drug dilutions from 100 to 0.097 μg/ml were prepared.

The minimum fungicidal concentration (MFC) was determined with an adaptation of standard methods (8) by assaying the CFU on blood agar in a 0.05-ml aliquot of the 2-ml volume, obtained immediately after vortex mixing just after determining the MIC. The endpoint was ≤1 CFU in the aliquot (representing ≥98% killing of the inoculum).

The time-killing studies were performed by preparing identical tubes and using one at each time interval (5 min and 0.5, 1, 4, 8, 24, 48, and 72 h) as in the MFC method. In some studies, described below, the tube was centrifuged at 4°C (1,400 × *g*, 10 min), the pellet was suspended in fresh medium, the tube was vortex mixed, the centrifugation and washing procedure with medium were repeated, and a sample was assayed as in the MFC method.

Drugs. Clotrimazole, ketoconazole, and tioconazole were supplied as powders by Schering Corp., Bloomfield, N.J., Janssen Research and Development, New Brunswick, N.J., and Pfizer Inc., New York, N.Y., respectively. Each was dissolved in 99.9% methanol (MCB Manufacturing Chemists, Inc., Cincinnati, Ohio) to produce stock solutions of 10 mg/ml. Ketoconazole was supplied as the base, and the concentration was adjusted to 10 mg of active drug per ml (0.88 × weight of the base) (10). Miconazole and econazole were supplied as 10-mg/ml solutions in cremophor solubilizer by Janssen Research and Development and Cilag-Chemie AG, Schaffhausen, Switzerland, respectively. The stock solutions of the drugs were stored at 4°C and diluted in the medium on each experiment day. Neither the cremophor

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TABLE 1. $IC_{1/2}$, MIC, and MFC (micrograms per milliliter) of five imidazoles against *C. albicans*

Isolate	Clotrimazole			Ketoconazole			Miconazole			Tioconazole			Econazole		
	$IC_{1/2}$	MIC	MFC	$IC_{1/2}$	MIC	MFC	$IC_{1/2}$	MIC	MFC	$IC_{1/2}$	MIC	MFC	$IC_{1/2}$	MIC	MFC
6	≤0.097	0.39	>100	≤0.097	3.1	25	≤0.097	1.56	>100	≤0.097	0.19	12.5	≤0.097	50	>100
13	0.19	12.5	>100	≤0.097	25	>100	≤0.097	50	>100	≤0.097	6.25	25	≤0.097	>100	>100
26	≤0.097	3.1	>100	≤0.097	6.25	>100	≤0.097	6.25	>100	≤0.097	6.25	25	≤0.097	>100	>100
12	1.56	3.1	6.25	≤0.097	0.39	1.56	0.19	0.39	100	≤0.097	0.19	6.25	0.19	100	>100
28	0.19	12.5	100	≤0.097	6.25	100	≤0.097	50	>100	≤0.097	12.5	25	≤0.097	>100	>100
82–48	0.19	0.78	>100	≤0.097	1.56	50	≤0.097	>100	>100	≤0.097	≤0.097	25	≤0.097	>100	>100
82–5	0.19	3.1	12.5	≤0.097	6.25	6.25	≤0.097	50	100	≤0.097	1.56	6.25	≤0.097	100	>100
82–37	0.19	25	>100	≤0.097	12.5	100	≤0.097	100	>100	≤0.097	12.5	50	≤0.097	>100	>100
82–45	≤0.097	>100	>100	≤0.097	100	>100	≤0.097	>100	>100	≤0.097	25	50	≤0.097	>100	>100
82–49	≤0.097	12.5	>100	≤0.097	100	>100	≤0.097	>100	>100	≤0.097	25	50	≤0.097	100	>100
5	≤0.097	25	>100	≤0.097	25	>100	≤0.097	12.5	100	≤0.097	12.5	100	≤0.097	12.5	>100

alone (supplied by Janssen Research and Development) nor methanol in the concentration present at the highest drug concentration (100 μg/ml) used in these studies affected fungal growth as assayed spectrophotometrically.

Statistics. The data were analyzed by the rank sum test, Student *t* test, and two-tailed Fisher exact test (22), with significance taken to be $P < 0.05$. For determination of geometric mean concentrations, values of <0.097, 0.097, 0.19 . . . 50, 100, >100 μg/ml were assigned titers on a log base 2 scale of 1, 2, 3 . . . 11, 12, 13.

RESULTS

Partial inhibition. The susceptibility test results ($IC_{1/2}$, MIC, MFC) with the isolates are detailed in Table 1. One isolate did not grow sufficiently well to perform the tests.

By the spectrophotometric assay of partial inhibition, the $IC_{1/2}$ for all isolates was below the lowest concentration tested for tioconazole and ketoconazole. The $IC_{1/2}$ was similarly "off scale" for 10 isolates tested against miconazole and econazole. With clotrimazole the $IC_{1/2}$ was off scale for only five isolates, although the highest $IC_{1/2}$ for any isolate with any drug was only 1.56 μg/ml.

Visual endpoint of complete inhibition. With the MIC as the measure of susceptibility, tioconazole appeared to be superior to the other drugs. For six of the isolates, tioconazole had the lowest MIC of the five drugs, and it was tied for the lowest MIC for two of the remaining isolates. Clotrimazole had the lowest MIC for two of the isolates. By the rank sum test, therefore, tioconazole was significantly superior to all of the other drugs except clotrimazole, with $P < 0.01$. All of the drugs except miconazole were superior to econazole, with $P < 0.01$ (miconazole versus econazole, $P < 0.05$). Ketoconazole was significantly better than miconazole at this level of significance.

Another method of comparison is by the mean MICs (Table 2). Tioconazole had the lowest mean MIC (3 μg/ml). This tioconazole geometric mean MIC was significantly superior to that of miconazole or econazole by the *t* test ($P < 0.05$). No other comparisons were significant.

TABLE 2. Geometric mean MIC and MFC

Drug	Geometric mean (μg/ml)	
	MIC	MFC
Tioconazole	3.0	25.0
Clotrimazole	7.4	63.7
Ketoconazole	9.7	>100
Miconazole	27.3	>100
Econazole	>100	>100

Killing. Examination of the MFCs revealed a large discrepancy between tioconazole and the other drugs. Tioconazole killed all 11 isolates in the range of concentrations studied, and ketoconazole killed 6. Econazole did not kill any. By two-tailed Fisher exact analysis of whether the isolate was killed or not, tioconazole was significantly superior to ketoconazole ($P < 0.03$) and the other three drugs ($P < 0.00001$).

For 8 of the 11 isolates tioconazole had the lowest MFC, and it was tied for the lowest MFC for 2 of the remaining isolates. By the rank sum test, tioconazole was significantly superior to ketoconazole ($P < 0.05$) and to the other three drugs ($P < 0.01$). Ketoconazole was, as with the MIC comparison, superior to miconazole ($P < 0.05$).

Tioconazole also had the lowest mean MFC for the isolates, 25 μg/ml (Table 2). This is significantly superior by the *t* test to clotrimazole ($P < 0.02$) and to econazole or miconazole ($P < 0.001$). Clotrimazole and ketoconazole were also significantly superior to econazole ($P < 0.01$ and 0.05, respectively).

Time-killing studies. We examined the kinetics of killing as it occurs during the MFC test. We also wished to examine the effect of a concentration which is a multiple of the MFC, because in therapy, as a rule of thumb, clinicians attempt to have peak in vivo antimicrobial levels in tissue that exceed the minimal microbicidal concentration. We arbitrarily selected a concentration of four times the MFC.

From the preceding studies we hoped to select isolates against which the five drugs could be assayed. However, since econazole was not fungicidal in the range studied, we selected isolates 12 and 82-5, the two isolates against which all four other drugs exhibited a MFC. The results with isolate 12 are shown in Fig. 1. With clotrimazole, ketoconazole, and tioconazole, killing appeared to begin between 1 and 8 h of incubation and was completed (i.e., ≥98% killing is referred to as complete) after 8 to 24 h of incubation. With miconazole, these events were mildly delayed.

With isolate 82-5, the results are more striking (Fig. 1). In general, this isolate appeared to be killed more rapidly by imidazoles, though the final MFCs were identical to those for isolate 12 in three instances. Most striking is the magnitude of the initial decline, as it appears, particularly with tioconazole, ketoconazole, and miconazole, that a large fraction of the initial inoculum was killed at time zero. Moreover, with tioconazole no survivors were apparent after 30 min of incubation. These time zero data suggested to us the possibility that drug carry-over in the sample removed could account at least in part for these kinetics.

The results were even more pronounced at a concentration of four times the MFC (Fig. 2). Killing generally

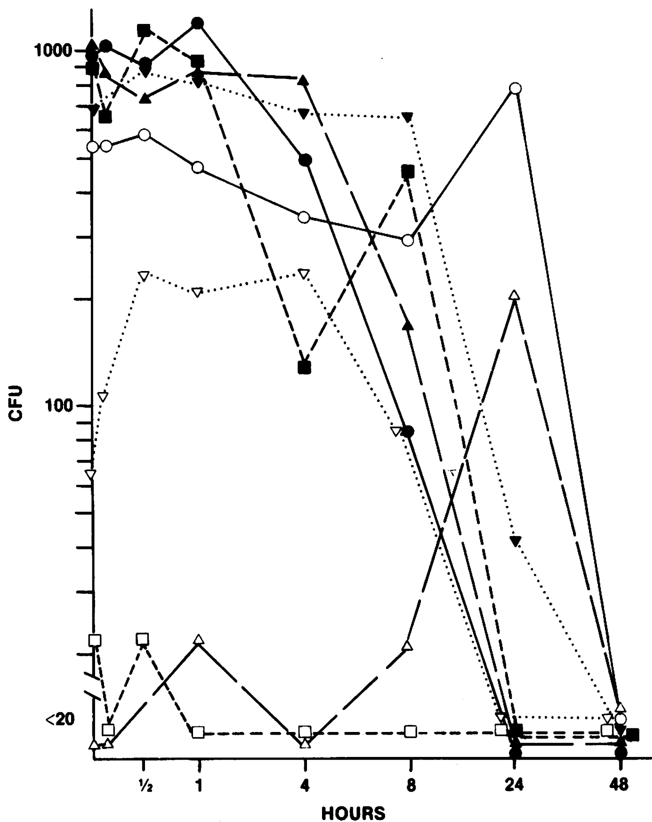

FIG. 1. Kinetics of fungal survival at the MFC (see Table 1) with two *C. albicans* isolates (isolate 12, solid symbols; isolate 82-5, open symbols). Symbols: ● and ○, clotrimazole; ▲ and △, ketoconazole; ▼ and ▽, miconazole; and ■ and □, tioconazole. Note that the time zero result shown is taken from a mixture in the presence of drug; the inoculum was prepared by spectrophotometric assay (9) to yield 1,000 cells per tube in the absence of drug and yielded 875.8 ± 271.8 CFU per tube.

proceeded more directly with both isolates at this concentration. There were no survivors after 30 min of incubation of isolate 12 with tioconazole. However, the most astonishing results were those with tioconazole and ketoconazole and isolate 82-5. The data suggested an instantaneous kill by both ketoconazole and tioconazole. This experiment underscored the possibility of drug carry-over. It was repeated, with ketoconazole and tioconazole at these concentrations and isolate 82-5, with identical results (data not shown).

In other studies with this method (data not shown), done at a concentration of four times the MIC with four isolates and all five drugs, the results were similar to the above. Only tioconazole and ketoconazole killed all four isolates at this concentration in the 48-h period. Tioconazole killed all four within 8 h; no other drug killed any within 8 h.

Time-killing studies with washing. We therefore repeated the preceding studies with ketoconazole and tioconazole and removed the residual drug in the sample taken by washing the isolate 82-5 cells (Fig. 3). In this study the initial inoculum in tubes without drug sampled at time zero was 353.4 ± 11.6 CFU/ml, lower than the 875.8 ± 271.8 CFU/ml used in the preceding studies. The former number was not significantly different from the 320 ± 53 CFU/ml enumerated when identical tubes without drug were centrifuged and the cells were washed, suspended, and sampled (the same procedure as that used to derive the time-killing curves in the presence of drug in Fig. 3). This indicated that the washing and centrifugation procedure does not affect the CFU present in the tube.

We found that with both drugs at their MFC as determined without washing (which produced apparent complete killing by 48 h) there was not complete killing even by 72 h. Noteworthy was the absence of the marked apparent killing at time zero that was noted with isolate 82-5 at the MFC of these drugs in the absence of washing (Fig. 1).

When the cells were washed, there was still a significant initial kill at time zero by ketoconazole at four times the MFC (Fig. 3) as determined without washing. However, when the isolates were washed this drug required >8 h for complete killing at this concentration, unlike the rapidity of killing seen previously (Fig. 2). With tioconazole at this concentration, there was complete killing of the inoculum at time zero, even when the drug was removed (Fig. 3). This was not different than the previous result (Fig. 2). It should be noted that the sampling, centrifugation, and washing procedures take up to 30 min, and therefore for some fraction of that time the cells are still exposed to drug. We can conclude that at this concentration ketoconazole induces in some of the cells and tioconazole induces in all the cells a lesion that is quickly lethal (<30 min) and irreversible.

DISCUSSION

We found in the spectrophotometric assay of $IC_{1/2}$ that all of the drugs were active, even compared with concentrations achieved in serum after systemic administration (4, 5, 7, 11, 12, 14, 19). Their activity was very similar, with clotrimazole

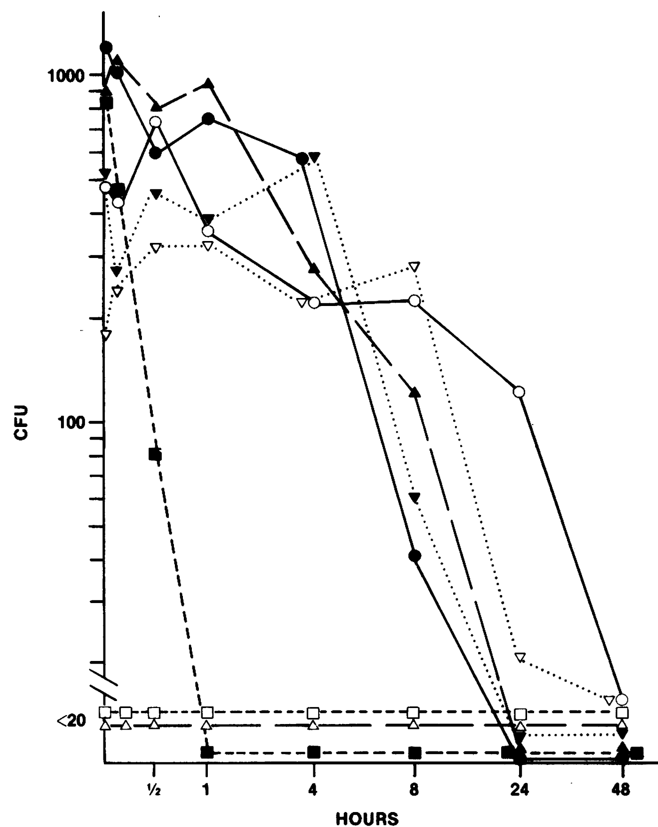

FIG. 2. Kinetics of fungal survival at four times the MFC. Isolates, drugs, and symbols are the same as in Fig. 1.

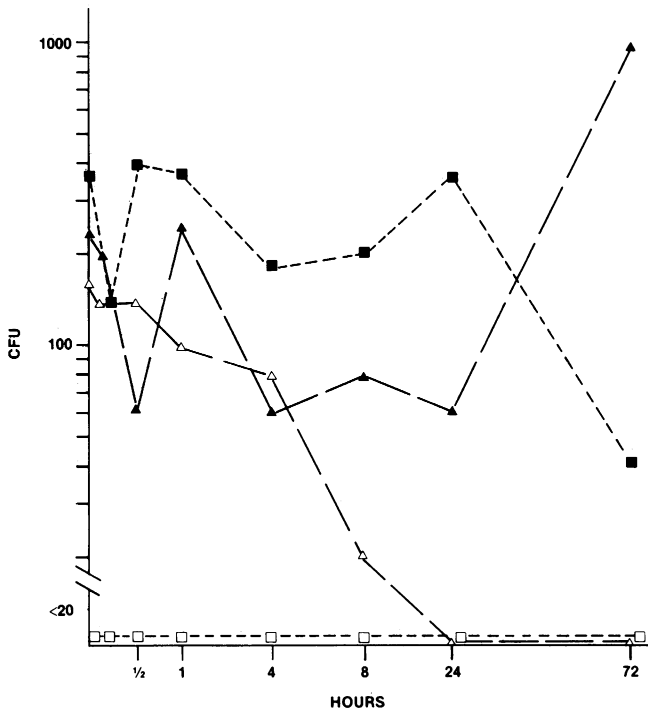

FIG. 3. Kinetics of isolate 82-5 survival at the MFC (closed symbols) and four times the MFC (open symbols), when the drug was removed from the aliquot sampled for survivors. Drug symbols are the same as in Fig. 1.

slightly less active. The differences became larger in the MIC determination, where tioconazole emerged in analyses as the most potent and econazole emerged as the least active. The MICs determined, with the possible exception of that of econazole, are achievable with topical administration. With the possible exception of tioconazole, for which data on human pharmacokinetics after systemic administration have not been presented, the MICs are generally below peak concentrations achieved after currently used systemic doses (4, 5, 7, 11, 12, 14, 19). Tioconazole was potent in comparisons of killing power (MFC). The MFCs, however, generally appear relevant only to topical use. If it is the case that these MFCs are relevant to other fungi, then imidazoles may be fungistatic in vivo in systemic mycoses, which is consistent with observations in animal models and in patients to date (3, 10–12, 19).

The time-killing studies revealed that killing occurred with the drugs studied, if the drugs were not removed on subculture. At high drug concentrations with one isolate, this effect was complete with tioconazole and ketoconazole even without incubation of the drug-fungus mixture before subculturing. However, if the fungi were exposed to the drug and then the drug was removed, even drugs apparently active in killing did not kill at their MFC. At higher drug concentrations, killing occurred even when the drug was removed. In one instance, we noted that tioconazole caused a lesion lethal to the whole inoculum virtually instantaneously. These data make it unlikely that the results described in this study as killing could be ascribed to clumping, since a reduction in CFU occurred even after the drug was washed away. No macroscopic clumping was seen in these studies.

These data suggest some conclusions. (i) Those who use MFC determination methods and killing kinetic studies with imidazoles must be alert to the factor of drug carry-over, as removing the drug gives markedly different results with these methods. (ii) When instantaneous killing is seen, a nonmetabolic mechanism of action is suggested, such as a direct effect on cell membranes (24). (iii) The demonstrated local persistence of these drugs after topical administration (1, 18) may, depending on the concentration, be an essential feature of their ability to completely eradicate the fungus. This is particularly true with regimens involving a single application.

In a comparison of potency of drugs, it may be more accurate to compare molar concentrations, although micrograms per milliliter were used here because pharmacological data are usually expressed in such terms. The molecular weights of clotrimazole, econazole, tioconazole, miconazole, and ketoconazole are 346.8, 381.7, 387.7, 416.1, and 531.4, respectively. These are very similar, with the lowest only 35% below the highest. The drug dilutions we used were twofold, and none of the conclusions presented would have been significantly affected if molar comparison had been used.

In work with *Saccharomyces cerevisiae*, Sud and Feingold indicated that clotrimazole and miconazole could kill, whereas ketoconazole could not (21). They postulated from this that ketoconazole lacks one antifungal mechanism (e.g., a direct membrane action). Our findings suggest that one should be cautious in generalizing these results to pathogens such as *C. albicans*. We note that ketoconazole had a lower MFC than did miconazole with 6 of the 11 isolates (and the MFCs were the same in 4), and ketoconazole had a lower MFC than did clotrimazole with 5 isolates (and the MFCs were the same in 6).

Beggs (2) also could not find differences between ketoconazole and miconazole, but with the one *C. albicans* isolate studied with his methods, neither drug could kill. Minagawa et al. (16) reported that ketoconazole could kill *C. albicans* (one isolate) and that this killing was optimal at pH 7 (the pH used in our studies). Van den Bossche et al. (23) reported that miconazole was more fungicidal with *C. albicans* than was clotrimazole. In our study, clotrimazole had the lower MFC with three isolates, and the MFCs were identical with seven isolates.

Although with different methods Odds (17) found lower mean MICs against *C. albicans* with these five drugs, the differences between them were also small, and tioconazole was slightly more active. Dixon et al. (6) found that miconazole was slightly more active and that ketoconazole was less active in MICs against *C. albicans* than we did. Our results are similar to those of Marriott et al. (15) and Jevons et al. (14), who found that tioconazole was more active than miconazole against *C. albicans*. Marriott et al. (15) noted, after studying one *C. albicans* isolate, that tioconazole was rapidly fungicidal above its MIC.

Published comparisons between these drugs and other agents in candidiasis, in animal models, and clinically, particularly in systemic disease, are badly needed.

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