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Characterization and Quantitation of the Pharmacodynamics of Fluconazole in a Neutropenic Murine Disseminated Candidiasis Infection Model

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We determined the pharmacodynamic parameter and the magnitude of that parameter that was predictive of the efficacy of fluconazole in the treatment of disseminated candidiasis. We used a neutropenic murine model of disseminated *Candida albicans* infection to characterize the time course of activity of fluconazole. Quantitation of colony counts in kidneys after 24 h of therapy with a wide range of doses and three dosing intervals was used to determine the dose required to achieve 50% of the maximal effect (ED_{50}). The ED_{50} was similar for each of the dosing intervals studied, supporting the area under the concentration-time curve (AUC) MIC ratio as the parameter that predicts the efficacy of fluconazole. Similar studies were performed with *C. albicans* strains for which fluconazole MICs are in the susceptible-dose-dependent range (MICs, 16 to 32 mg/liter). We found that the magnitude of the AUC/MIC ratio required to reach the ED_{50} was similar for all three organisms studied, ranging from 12 to 25. When the pharmacokinetics of fluconazole in humans are considered, these AUC/MIC ratios would support in vitro susceptibility breakpoints of 8 mg/liter for dosages of 200 mg/day and susceptibility breakpoints of 16 to 32 mg/liter for dosages of 400 to 800 mg/day.

The need for more potent and less toxic agents for treatment of *Candida* infections is the subject of ongoing study (12). However, until these agents are available, one approach to improving clinical efficacy is to optimize dosing of currently available drugs. The study of the time course of antimicrobial therapy or pharmacodynamics has enhanced the efficacies of several classes of antimicrobial agents (9, 11, 18). In addition, knowledge of pharmacodynamics has aided in predicting the most efficacious therapy for drug-resistant bacterial infections (2).

The time course of antimicrobial activity of a particular agent can be determined by two characteristics. The first is the effect of increasing drug concentrations on the rate and extent of organism killing. The second is the presence or absence of antimicrobial effects which persist after drug levels have fallen below the MIC. For example, characterization of the concentration-dependent killing by aminoglycosides and the presence of prolonged persistent effects or postantibiotic effects (PAEs) has provided the rationale for once-daily administration of these agents (3, 6, 18). This dosing schedule optimizes the concentration-dependent parameters that predict efficacy; the ratios of the peak concentration in serum to MIC and the area under the concentration-time curve (AUC) to the MIC (AUC/MIC). Similar studies of the beta-lactam class, however, have shown no enhancement of killing with increasing concentrations and short or no PAEs (9, 28, 29). Thus, continuous infusion or frequent dosing maximizes the time-dependent parameter that predicts efficacy or the time that levels in serum remain above the MIC for the infecting organisms (9, 29).

While these studies have been performed for most antibacterial classes, few studies have addressed similar issues about antifungal therapy. Both experimental infection models and clinical trials have found a modest correlation between in vitro susceptibility studies and in vivo outcomes; however, most have not attempted to correlate the activities of these agents with the pharmacokinetic/pharmacodynamic profiles of the individuals drugs (1, 10, 13, 20, 23, 30). In a recent publication by Louie et al. (17), fluconazole dose fractionation studies with an immunocompetent murine candidiasis model found that AUC/MIC was the pharmacodynamic parameter that best correlated with outcome.

This type of pharmacodynamic characterization in antifungal therapy should enable us to maximize dosing efficacy. In the current study we have characterized the pharmacodynamic parameter that is predictive of the efficacy of fluconazole in a neutropenic murine model of disseminated candidiasis over a wide range of doses and dosing intervals. Furthermore, we determined the magnitude of the pharmacodynamic parameter required to achieve efficacy in this model for several strains of *Candida albicans* with various susceptibilities to fluconazole in order to provide a framework for the rational development of in vivo breakpoints.

MATERIALS AND METHODS

Organisms. Three clinical isolates of *C. albicans* were used for these experiments. Each isolate was recovered from patients with invasive infections: one (strain K-1) from a patient with endophthalmitis and two (strains 98-17 and 98-234) from patients with esophageal candidiasis. The latter two organisms were obtained from the laboratory of A. W. Fothergill in San Antonio, Tex. The organisms were maintained, grown, subcultured, and quantified on Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, Mich.). The organisms were maintained at 4°C. Twenty-four hours prior to study, the organisms were subcultured at 35°C.

Antifungal agent. Fluconazole powder was provided by Pfizer Inc. (New York, N.Y.). The powder was stored at −70°C. All drug solutions were prepared on the day of study by dissolving the powder in sterile, pyrogen-free 0.9% saline.

In vitro susceptibility testing. The MICs for the organisms were determined by a broth microdilution modification of the M27-A method of the National Committee for Clinical Laboratory Standards (NCCLS) (19). The broth microdilution wells were read at both 24 and 48 hours. We did not observe differences in MICs between these time points for the organisms that we studied. Determinations

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were performed in duplicate on at least two separate occasions. Final results are expressed as the geometric means of these results.

Animals. Six-week-old specific-pathogen-free female ICR/Swiss mice (Harlan Sprague-Dawley, Madison, Wis.) weighing 23 to 27 g were used for all studies.

Infection model. The mice were rendered neutropenic (polymorphonuclear leukocyte count, $<100/mm^3$) by injecting cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, Ind.) intraperitoneally 4 days (150 mg/kg of body weight) and 1 day (100 mg/kg) before infection.

At 24 h prior to infection the organisms were subcultured on SDA. An inoculum was prepared by placing six fresh colonies into 5 ml of sterile pyrogen-free 0.9% saline which was warmed at 35°C.

Disseminated infection with *C. albicans* organisms was achieved by injection of 10^5 blastoconidia in 0.1 ml of saline via the lateral tail vein 2 h prior to drug therapy. At end of the study period the animals were killed by CO_2 asphyxiation. After killing of the animals, the kidneys of each mouse were immediately removed and placed in sterile iced 0.9% saline. The homogenate was then serially diluted 1:10, and aliquots were plated onto SDA for determination of viable fungal colony counts after incubation for 24 h at 35°C. Data for each time point represent the mean number of CFU per kidney for two animals.

Pharmacokinetics. Single-dose pharmacokinetics of fluconazole were determined in neutropenic infected ICR/Swiss mice following the administration of subcutaneous doses of 6.25, 25, and 100 mg/kg in a 0.2-ml volume. For each dose examined, groups of three mice were sampled one time by intracardiac puncture at 1- to 12-h intervals. Following collection, the blood was allowed to clot at 4°C. After clotting the samples were centrifuged (model MB; International Equipment Co.) at $10,000 \times g$ for 5 min, and serum was removed and stored at −70°C until processing. Serum drug concentrations were determined by gas chromatography, which was performed at Michael Rinaldi's Fungus Testing Laboratory, University of Texas at San Antonio (14). Pharmacokinetic constants including elimination half-life, AUC, and peak level were calculated by using a one-compartment model with first-order absorption and first-order elimination via nonlinear squares techniques (MINSQ; Micromath Inc., Salt Lake City, Utah).

Treatment protocols. (i) In vivo PAE. Two hours after infection with *C. albicans* K-1, the mice were treated with single subcutaneous doses of fluconazole (3.125 and 12.5 mg/kg). Groups of two treated and control mice were killed at each sampling time interval ranging from 1 to 12 h. Control growth was determined at five sampling times over 24 h. The treated groups were sampled nine times over 72 h. The kidneys were removed at each time point and were processed immediately for CFU determination as outlined above. The time following administration of the two doses studied that the levels of fluconazole in serum remain above the MIC for the organism (0.5 mg/liter) was calculated from the pharmacokinetic data. The PAE was calculated by determining the time that it took the counts for the controls to increase 1 $\log_{10}$ CFU/kidney (C) and subtracting this from the amount of time that it took organisms from the treated animals to grow 1 $\log_{10}$ CFU/kidneys (T) after levels in serum fell below the MIC for the organism: $PAE = T - C$ (5).

(ii) Pharmacodynamic parameter determination. Neutropenic mice were infected with *C. albicans* K-1 2 h prior to the start of fluconazole therapy. Groups of two mice were treated for 24 h with different fluconazole dosing regimens by using fourfold increasing total daily doses administered at 6-, 12-, and 24-h dosing intervals. The total dosages ranged from 0.78 to 200 mg/kg/day. Drug was administered subcutaneously in 0.2-ml volumes. The mice were killed after 24 h of therapy, and the kidneys were removed for CFU determination. Untreated control mice were killed just before treatment and after 24 h.

(iii) Pharmacodynamic parameter magnitude studies. Studies similar to those described above were performed with two *C. albicans* strains for which MICs were higher to determine if the magnitude of the parameter required to achieve efficacy would be similar for organisms independent of their in vitro susceptibility. The two clinical isolates chosen, 98-17 and 98-234, fell into the "intermediate" or "susceptible-dose-dependent" range on the basis of recent NCCLS interpretive guidelines (22). Dosing studies were designed as described above to vary the magnitude of the pharmacodynamic parameters. The total daily dose varied from 25 to 400 mg/kg and 6.25 to 400 mg/kg for treatment of animals with infections caused by 98-17 and 98-234, respectively. Groups of two mice were again used for each dosing regimen. Mice were treated for 24 h, after which they were killed and their kidneys were removed for subsequent CFU determination.

Pharmacodynamic extrapolation from other experimental infection studies. A MEDLINE search was performed to locate studies of deep *C. albicans* infections in which fluconazole was used as therapy. Studies were excluded (i) if no pharmacokinetic studies were performed and (ii) if no in vitro susceptibility studies were performed. The magnitude of the 24-h AUC/MIC which corresponded to the dose required to achieve 50% of the maximum effect (ED_{50}) and the dose required to produce 80% survival were calculated from the pharmacokinetic and susceptibility data.

Data analysis. A sigmoid dose-effect model was used to measure the in vivo potency of fluconazole. The model is derived from the Hill equation: $E = (E_{max} \times D^n)/(ED_{50} + D^n)$, where E is the observed effect (change in $\log_{10}$ CFU per kidney compared with that for untreated controls at 24 h), D is the cumulative 24-h dose, E_{max} is the maximum effect, ED_{50} is the dose required to achieve 50% of E_{max}, and n is the slope of the dose-effect relationship. The correlation between efficacy and each of the three parameters studied was determined by nonlinear least-squares multivariate regression analysis (Sigma Stat; Jandel Scientific Software, San Rafael, Calif.). The coefficient of determination (R^2) was used to estimate the percent variance in the change in $\log_{10}$ CFU per kidney over the treatment period for the different dosing regimens that could be attributed to each of the pharmacodynamic parameters.

The ED_{50}s for each of the dosing intervals (every 6, 12, and 24 h) were compared to determine which parameter would best be predictive of efficacy. If the ED_{50}s for the different regimens were similar, then the AUC/MIC was the parameter most important in the prediction of outcome. If the ED_{50} would increase significantly as the dosing interval was lengthened from every 6 through every 24 h, the duration of time that the levels in serum remained above the MIC was the parameter predictive of efficacy.

The ED_{50} was determined for the 12-h regimens for the standard strain and the two organisms for which the MICs were higher. The magnitude of the pharmacodynamic parameter predictive of the efficacy of fluconazole was then calculated for each of the three organisms studied to determine if a similar parameter magnitude was associated with efficacy as determined by these indices. The significance of differences between these values was determined by Mann-Whitney analysis.

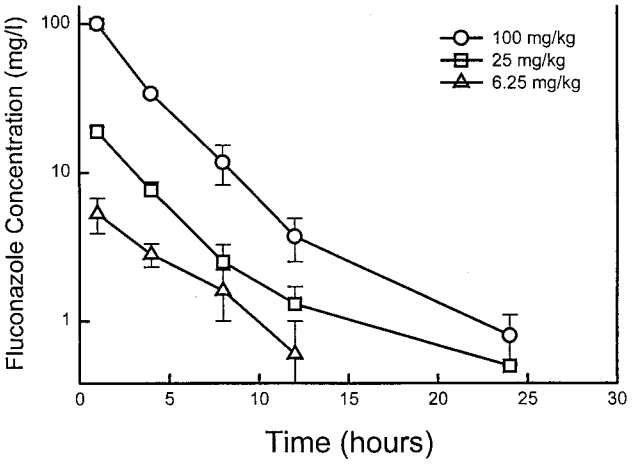

FIG. 1. Serum fluconazole concentrations after administration of doses of 100, 25, and 6.25 mg/kg in neutropenic infected mice. Each symbol represents the geometric mean ± standard deviation of the levels in the sera of three mice.

RESULTS

In vitro susceptibility testing. The MICs of fluconazole for the three *C. albicans* strains studied, strains K-1, 98-17, and 98-234, were 0.5, 16.0, and 32.0 mg/liter, respectively. On the basis of NCCLS interpretive guidelines, K-1 was susceptible (MIC, ≤8 mg/liter) to fluconazole, while the other organisms fell into the intermediate (MIC, 16 to 32 mg/liter) or susceptible-dose-dependent category (22). The resistance mechanisms responsible for decreased the fluconazole susceptibilities of the two organisms have not yet been determined.

Pharmacokinetics. The time course of serum fluconazole concentrations following the administration of subcutaneous doses of 6.25, 25, and 100 mg/kg are shown in Fig. 1. Total drug levels were used in all pharmacokinetic calculations due to the very low degree of protein binding (10 to 11%) (15). The inter- and intraday coefficients of determination ranged from 6.4 to 9.8 and 1.4 to 11.4%, respectively. The pharmacokinetic profile for fluconazole was linear, with the peak level and AUC increasing proportionally for each of the doses. Peak levels occurred within 1 h of administration and ranged from 5.2 to 100 mg/liter. The elimination half-life ranged from 2.8 to 3.6 h. The AUC, as determined by the trapezoidal rule, ranged from 24 to 461 mg · h/liter.

In vivo PAE. Following tail vein inoculation of 10^5 blastoconidia, growth of *C. albicans* organisms in the kidneys of untreated mice increased 2.6 ± 0.13 $\log_{10}$ CFU/kidneys over 24 h. Control growth of 1 $\log_{10}$ CFU/kidney in untreated mice

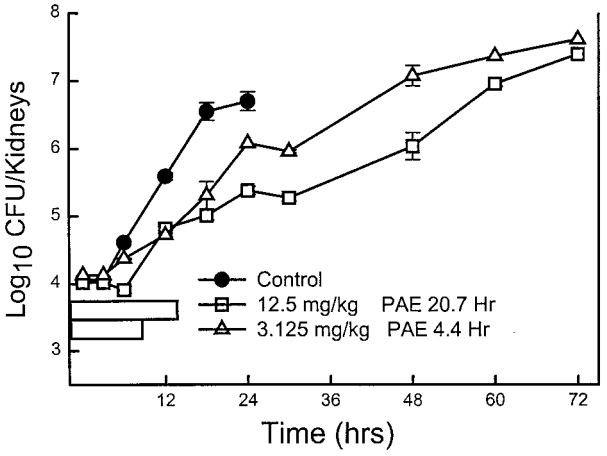

FIG. 2. In vivo PAE of fluconazole after administration of doses of 12.5 and 3.13 mg/kg against *C. albicans* K-1 in neutropenic mice. Each symbol represents the mean ± standard deviation for two mice. The widths of the bars reflect the duration of time that the serum fluconazole levels exceeded the MIC.

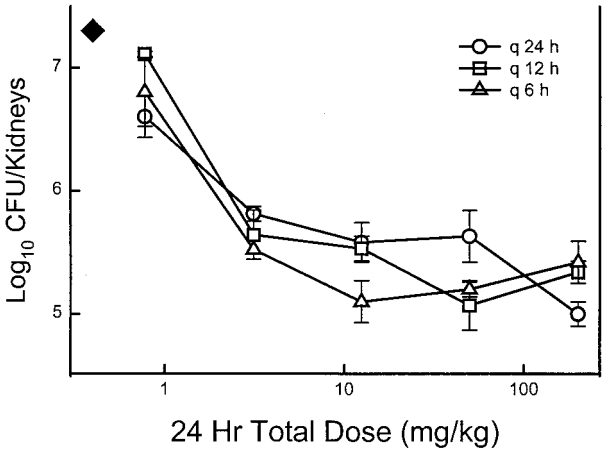

FIG. 3. Relationship between 24-h dose and $\log_{10}$ CFU per kidney for fluconazole administered at different dosing intervals in a neutropenic murine model of disseminated candidiasis. Each symbol represents data for two mice. The solid diamond symbol represents organism counts for untreated animals at 24 h. q 24 h, q 12 h, and q 6 h, dosing every 24, 12, and 6 h, respectively.

was achieved in 9.3 h. No drug carryover was observed in either treatment group. On the basis of the pharmacokinetics described above, the concentrations after administration of the two doses of fluconazole studied (3.125 and 12.5 mg/kg) would remain above the MIC for the *C. albicans* organism (0.5 mg/liter) for 9.6 and 14.3 h, respectively. Treatment with the two doses of fluconazole produced no reduction in colony counts compared with numbers at the start of therapy. Growth curves for the control group as well as those following administration of single doses of fluconazole are shown in Fig. 2. Treatment with both doses of fluconazole significantly flattened the *Candida* growth curves, suppressing net growth by 1 $\log_{10}$ CFU/kidney for 4 to 21 h after levels in serum had fallen below the MIC compared to the growth for the controls.

Pharmacodynamic parameter determination. At the start of therapy kidneys had 4.25 ± 0.31 $\log_{10}$ CFU/kidney. After the 24-h study period organisms grew to 2.98 ± 0.20 $\log_{10}$ CFU/kidney in untreated mice, and each of the control mice died. Drug carryover was not observed in any of the samples. Escalating doses of fluconazole produced no net killing from the number of organisms in control animals at the start of therapy but did result in a dose-dependent suppression of growth compared to the level of growth in the control, at 24 h, ranging from no effect to suppression to 2.33 $\log_{10}$ CFU/kidney. The highest total dose for each of the dosing intervals studied resulted in similar colony counts at 24 h, ranging from 4.9 ± 0.10 to 5.1 ± 0.10 $\log_{10}$ CFU/kidney.

The dose-response curves for each of the dosing intervals studied are shown in Fig. 3. There was a strong correlation among the datum points for each of the dosing intervals (R^2 = 89%). The ED_{50}s for each of the three dosing intervals were very similar, ranging from 1.5 ± 0.30 to 1.9 ± 0.37 mg/kg (P = 1.0). The concordance between these values as the dosing interval was lengthened supports the fact that AUC/MIC is the pharmacodynamic parameter that predicts the efficacy of fluconazole in this model. The AUC/MICs which corresponded to the ED_{50} for each of the dosing intervals were very comparable, ranging from 24 ± 1.0 to 25 ± 1.0 mg · h/liter (P = 1.0).

Correlation of magnitude of pharmacodynamic parameter with efficacy. The growth kinetics for the *C. albicans* strains for which MICs were higher, strains 98-17 and 98-234, were similar to those for K-1. At the start of fluconazole therapy the kidneys had between 3.8 ± 0.07 and 4.3 ± 0.10 $\log_{10}$ CFU. In control animals organisms grew to 2.9 ± 0.08 to 3.0 ± 0.01 $\log_{10}$ CFU/kidney during the 24-h study period. Again, even with the highest doses studied, maximal growth suppression still allowed growth of nearly 1 $\log_{10}$ CFU/kidney compared to the numbers at the start of therapy, affirming the static activity of this agent in neutropenic mice.

The relationship between AUC/MIC ratios and efficacy with the three strains for the 12-h dosing interval is displayed in Fig. 4. The effects were nearly identical for each corresponding AUC/MIC ratio. As shown in Table 1, the ED_{50} was observed when this ratio approached 12 to 24.

Pharmacodynamic parameter determinations from other animal model studies. The literature search identified only three publications that described studies of experimental *C. albicans* infection and that met the inclusion criteria. The others did not provide pharmacokinetic measurements to allow calculation of pharmacodynamic parameters. The observations of Louie et al. (17) for a nonneutropenic murine candidiasis model correlated the AUC/MIC ratio with outcome as measured by number of CFU per gram per kidney after 24 h of

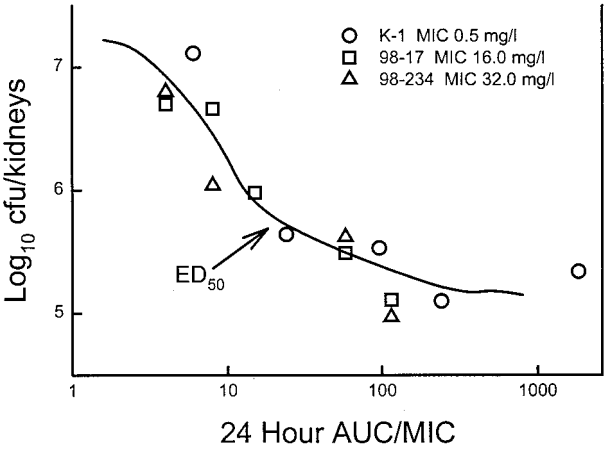

FIG. 4. Relationship between 24-h AUC/MIC ratio and $\log_{10}$ CFU per kidney for fluconazole against *C. albicans* organisms for which MICs varied. Each symbol represents data for two mice.

TABLE 1. Fluconazole ED_{50} in the treatment of disseminated *C. albicans* organisms with various in vitro susceptibilities

Organism	MIC (mg/liter)	ED_{50} (mg/kg)	AUC/MIC	R^2 (%)
C. albicans K-1	0.5	1.9	24	96
C. albicans 98-17	16	61	12	94
C. albicans 98-234	32	114	20	96

therapy with fluconazole. The ED_{50} in their studies was 4.56 mg/kg, which correlates with an AUC/MIC ratio of 44 mg · h/liter. In a similar murine infection model, van't Wout et al. (27) measured the *C. albicans* burden in the kidneys after the administration of fluconazole twice daily for 7 days. On the basis of their CFU determinations, we calculated an ED_{50} of 2.4 mg/kg. This dose correlates with an AUC of 11.2 mg · h/liter, which, when divided by the MIC for the *C. albicans* organism studied (0.8 mg/liter), gives an AUC/MIC magnitude of 14. The study by Rogers and Galgiani (24), which we reviewed, measured survival in a nonneutropenic rat model of disseminated candidiasis. After treatment with fluconazole twice daily for 21 days, the dosage which resulted in a survival rate of 80% was 0.5 mg/kg/day. On the basis of their pharmacokinetic and in vitro susceptibility data, this corresponds to an AUC/MIC ratio of 18.

DISCUSSION

Previous in vitro studies have characterized the fungistatic nature of fluconazole in time-kill studies (16). Similarly, our in vivo studies demonstrated a lack of killing with fluconazole in neutropenic animals.

Previous in vitro PAE studies with fluconazole have failed to demonstrate PAEs but did find significant sub-MIC effects (26). Our in vivo PAE studies, however, demonstrated significant persistent effects following fluconazole therapy. We feel that these prolonged in vivo PAEs are likely due to sub-MIC effects. Another possible explanation may include the potential difference between fluconazole kinetics in serum and urine, in which the pharmacologic effect is being measured. These persistent effects may also represent the time that it takes for ergosterol synthesis to fully recover and repair the fungal cell membrane.

The magnitude of the PAE observed with both doses of fluconazole suggests that this phenomenon likely plays a significant role in defining the pharmacodynamic parameter that predicts efficacy. We feel that this prolonged persistent effect helps explain why AUC/MIC is the parameter that is most important for describing the activity of this static drug. This pharmacodynamic pattern is similar to that seen with azithromycin, which demonstrates no concentration-dependent bacterial killing but which produces prolonged PAEs. The parameter that predicts the efficacy of this agent is also the AUC/MIC ratio (9).

Recent dose fractionation studies by Louie et al. (17) have suggested that AUC is the parameter most closely linked to outcome in an immune-competent murine candidiasis model. These determinations were made over a narrow dose range (3.5 to 5.5 mg/kg) and effect (0.33 to 0.54 $\log_{10}$ CFU/g). As opposed to Louie et al. (17), we used a neutropenic model, which allowed us to study a wider range of doses and effects and which is similar to the model used by Craig (9) to determine the pharmacodynamic characteristics of many classes of antibacterial agents. Our studies measured outcomes with doses that covered a 256-fold range and an effect that varied by nearly 3 $\log_{10}$ CFU/kidney. These experiments have shown that it is the total amount of drug administered and not the frequency of dosing which determines outcome, affirming that AUC/MIC is the pharmacokinetic or pharmacodynamic parameter that predicts efficacy.

The pharmacodynamic parameter AUC/MIC supports the once-daily administration of fluconazole. The utility of knowing which parameter is predictive of efficacy is therefore being able to determine the magnitude of that parameter that is predictive of efficacy (7). This knowledge allows one to study resistant organisms which are not encountered frequently enough in clinical trials to address important questions about appropriate dosing regimens and in vitro breakpoints. These types of observations have successfully been used most recently for determination of appropriate dosing regimens and breakpoints in the therapy of penicillin-resistant pneumococci (2, 8).

Our study with three organisms for which MICs varied 64-fold found that AUC/MIC ratios of 12 to 24 achieved a 50% maximal microbiologic effect. An AUC/MIC ratio of 12 to 24 roughly corresponds to maintenance of levels in serum at 1 × MIC once over the 24-h dosing period. The similarity between these observations suggests that one can use these magnitudes to predict the efficacy of a drug against organisms with a wide range of in vitro susceptibilities. From the limited amount of information that we were able to find from other studies, application of these pharmacodynamic principles suggests that these parameter magnitudes may be independent of the animal model, the presence or absence of neutrophils, or the specific *C. albicans* organism studied. In addition, similar results were seen when both CFU determinations and survival were used as endpoints. Whether these findings will be seen with other *Candida* species or other fungi is an important question for future study.

If one were to apply these parameter magnitudes to available fluconazole pharmacokinetics in humans, a dosage of 200 mg/day would reach AUC/MIC ratios of 15 to 20 for organisms for which MICs are 8 mg/liter (4). Similarly, a dosage of 400 mg/day would achieve this range of AUC/MIC ratios for organisms for which fluconazole MICs are 16 mg/liter. The pharmacokinetics of dose escalation to 800 mg/day, which is often recommended for those with severe illness, would attain these values for *Candida* organisms for which MICs are 32 mg/liter (10). These calculations have application to the recent NCCLS in vitro susceptibility breakpoint guidelines in the therapy of invasive *Candida* disease. Recommendations were based upon compilation of outcomes from a number of clinical trials that included 219 patients with oropharyngeal candidiasis and 97 patients with invasive or deep *Candida* infections.

Escalation of the fluconazole dosage to 400 to 800 mg/day would achieve an AUC/MIC ratio of 12 to 24 for organisms which were placed into the susceptible dose-dependent category (MICs, 16 to 32 mg/liter) (22). NCCLS review of clinical trials conducted by Pfizer found success rates that exceeded 90% with dose escalation (22). Other trials have shown significant rates of failure, regardless of the use of higher doses, for treatment of infections caused by organisms for which MICs are ≥64 mg/liter (21, 25).

These observations may serve as a model for the study of *C. albicans* organisms for which MICs are even higher as well as different *Candida* species. If studies with other organisms confirm our findings, those studies and the one described here may be useful for estimating dosing regimens for future clinical trials. In addition, the model described here could be used to identify the pharmacodynamic parameters that are predictive of efficacy for preclinical study of new antifungal agents in

order to establish appropriate preliminary dosing regimens for clinical trials.

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