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In vitro Susceptibility and Synergy Studies of *Aspergillus* Species to Conventional and New Agents

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In vitro susceptibility data using a macrodilution broth method on >100 isolates of Aspergillus *spp. are presented. For amphotericin B (Amp B)* (n = *105), 67% had minimum inhibitory concentrations (MICs)* ≤*2 μg/ml, and 90% had MICs* ≤*4 μg/ml; for 5-fluorocytosine [flucytosine (5FC)]* (n = *60), 35% had MICs* ≤*12.5 μg/ml; for miconazole (MCL)* (n = *18), 39% had MICs* ≤*5 μg/ml; for ketoconazole (KTZ) four (13%) of 32 isolates had an MIC* ≤*3.1 μg/ml; for itraconazole (ITZ)* (n = *88), 97% had MICs* ≤*6.3 μg/ml; and for saperconazole (SAP)* (n = *20), 90% had MICs* ≤*3.1 μg/ml. Of Amp B minimum fungicidal concentrations (MFCs)* (n = *25), 76% were* ≤*4 μg/ml; 5% of ketoconazole* (n = *20) and no flucytosine* (n = *38) MFCs were* ≤*25μg/ml; for itraconazole* (n = *60), 70% had MFCs* ≤*6.3 μg/ml, and for saperconazole* (n = *20), 75% had MFCs* ≤*3.1 μg/ml. Drug interaction studies were also performed. For Amp B and rifampin 36 (92%) of 39 showed synergy, for Amp B and flucytosine six (23%) of 26 showed synergy and another six (23%) showed antagonism; 13 (50%) were indifferent. In five Amp B–itraconazole combination studies, synergy and indifference were seen in two each and an additive effect was observed in one. The published literature on in vitro testing methodology and results for* Aspergillus *spp. is also reviewed, and recommendations for the clinical use of in vitro susceptibility testing are made.*

INTRODUCTION

Few reports have documented in vitro susceptibility results for *Aspergillus* spp. on more than approximately a dozen isolates. In comparing one report with another, difficulties arise because of the different methods used that have included agar dilution, microdilution broth, and macrodilution broth tests. In addition, there are many other differences between reports, in particular, inoculum size, medium used, duration of test, temperature of incubation, and end-point determination. Few reports have noted minimum fungicidal concentrations (MFCs), and only a few isolates have been previously tested in drug combination tests seeking synergy or antagonism.

Over the last 13 years, our laboratory has used the same methodology for in vitro testing of isolates of various *Aspergillus* species. We report these results here as the largest single data set tested by one method in one laboratory for this genus of fungi. Small portions of the data set have been previously published (Denning et al., 1989 and 1990b and c). We compare our methods and results with those previously published. Our data and these comparisons offer us a base that could be helpful for the systematic studies, methodologic comparisons, and in vivo–in vitro correlations that are presently needed.

METHODS

All isolates tested are clinical isolates derived from more than 30 different US hospitals. Testing was performed, with the exception of the saperconazole

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(SAP) studies, on fresh clinical isolates. Assignment to the genus *Aspergillus* relies on the recognition of a characteristic conidiophore with vesicle and sterigmata. Patient isolates were grown at 35°C on suitable media until conidia formed. Potato dextrose agar or, prior to 1986, sheep blood agar was used to induce conidiation if needed. Speciation was not available for most isolates. However, where species identification was performed, there appeared to be in no instance any differences in distribution of mean inhibitory concentrations (MICs) in that species from the whole. This summary statement of no differences between any species of speciated isolates and the total *Aspergillus* isolates is based on the following subgroups of either three or more isolates: 29 *Aspergillus fumigatus*, six *A. flavus*, six *A. terreus* tested against amphotericin B (Amp B); 16 *A. fumigatus* and three *A. terreus* against 5-fluorocytosine [flucytosine (5FC)]; seven *A. fumigatus* and four *A. niger* tested against ketoconazole (KTZ); and eight *A. fumigatus* tested against miconazole (MCL) (these four drug groups also included subgroups of less than three isolates of these four species and two other species; data not shown). No trends in susceptibility or resistance over the years of study were apparent.

Drug Preparation

Amphotericin B powder with deoxycholate (Squibb, Princeton, NJ) was suspended and diluted in sterile water and stored at 1.6 mg/ml in glass vials in the dark at −20°C. Flucytosine powder (Roche, Nutley, NJ) was solubilized by vortex mixing in sterile water at 2 mg/ml. Storage was in glass vials at −20°C. Rifampin (Ciba-Geigy, Lausanne, Switzerland) was solubilized in methanol at 10 mg/ml and stored in glass at −20°C. Miconazole (Janssen Pharmaceutica, Beerse, Belgium) was provided in a solubilizing agent (cremophor) at 10 mg/ml and kept at 4°C. Ketoconazole powder (Janssen Pharmaceutica), adjusted for potency, was diluted with 0.2 *N* hydrochloric acid to a concentration of 50 mg/ml. Further dilutions were made in sterile water to 2 mg/ml, and the stock was stored in sterile glass vials at −20°C. Itraconazole (ITZ) power (Janssen Pharmaceutica) and saperconazole powder (Ortho, Raritan, NJ) were solubilized in 50% acetone–50% 0.2 *N* HCl in a glass tube. To maximize solubility at this concentration, we suspended the drug preparation over hot water (64°C) for several hours and vortex mixed it repeatedly. ITZ and SAP stocks (10 mg/ml) were stored at room temperature and protected from the light.

Minimum Inhibitory Concentration and Minimum Fungicidal Concentration Test

To prepare the drug dilution series, we diluted each drug stock with a defined medium, namely, Yeast Nitrogen Base (YNB) broth unbuffered containing 0.5% glucose. A twofold dilution series of 1 ml of drug at each concentration tested was made in 5-ml plastic tubes. Incomplete solubility of ITZ and SAP at concentrations above 12.5 μg/ml was observed; end points above this value were therefore not determined. The range of drug concentrations used (in micrograms per milliliter) was intended to include achievable serum concentrations, and was, in most instances, 0.125–8 for Amp B, 0.78–100 for 5FC, 3.13–400 for rifampin, 0.39–50 for MCL and KTZ, and 0.39–25 for ITZ and SAP.

To prepare the inoculum, we pipetted 1 ml of YNB into a sterile tube. A sterile loop was dipped into the broth to wet it and then run over the conidiating *Aspergillus*. The conidia were delivered to the tube and counted using a hemacytometer. Conidia were diluted with YNB so that the inoculum contained 2000 conidia/ml.

Inoculum (1 ml) was delivered to each tube of the drug dilution series. The final conidia concentration was 1000 conidia/ml. Control tubes included conidia in YNB without drug. Tubes were incubated with loose caps at 35°C on a gyratory shaker at 30° to the horizontal (Figure 1) in ambient air for 24–48 hr at 140 rpm. When sufficient growth was found adhering to the sides of the control tubes, the MIC was read. All tubes containing drug were compared to controls. The concentration of drug in the first tube that showed no growth was the MIC. We placed 40–50 μl from each tube showing inhibition on an appropriately labeled segment of a sheep blood-agar plate after vigorously mixing each tube. Plates were incubated at 35°C for 48 hr. The concentration of drug yielding no growth or a single colony was designated the MFC. "Skip tubes" (tubes that exceeded this end point, containing higher drug concentrations than others that met the criterion) were occasionally encountered. In such instances, the first tube with a drug concentration higher than the "skip tube" whose plating results met the criterion of less than or equal to one colony was designated the MFC.

Drug Interaction Studies

These studies were performed in a similar manner as that described for the MIC tests (Harvey et al., 1980). Drug interaction was assessed in a "checkerboard" titration, in which tubes containing two drugs the usual volume of each drug concentration was halved and the concentration doubled. Thus, the final concentration of each was the same as that in the tubes containing one drug to which they were compared. Dilutions of drug were twofold.

The drug interaction was characterized by calculation of a fractional inhibitory concentration (FIC) index, in a manner similar to that described previously (Elion et al., 1954), and as previously applied

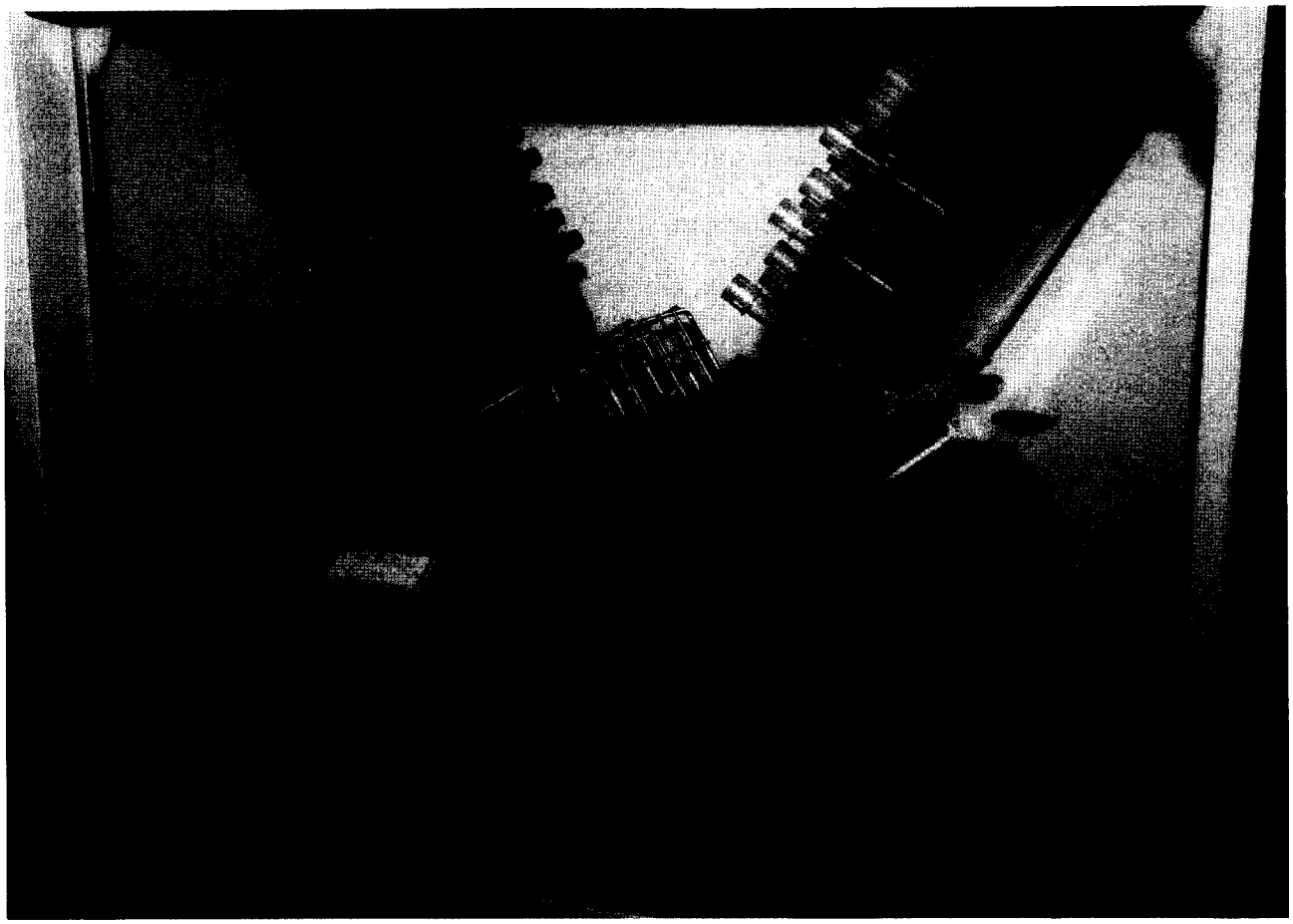

FIGURE 1 A photograph of the shaker used in these studies. This shaker occupies the bottom of a large incubator and rotates horizontally in a circular fashion. Racks containing 5-ml tubes for susceptibility or synergy studies are attached on either side.

to fungal susceptibility testing (Stevens and Vo, 1982). To calculate the FIC for a drug, we selected the tube with the lowest concentrations of both drugs inhibiting growth. The concentrations of the two drugs in that tube become the numerators of two fractions. The denominators are the MICs for each drug, respectively, and these fractions are the FICs. The FIC index equals the sum of the FICs for each drug. If it is not clear which tube to select (for instance, several tubes show synergy), then the FICs are calculated for each tube, and an FIC index is calculated for each; in this example, the lowest FIC index is selected. Where only antagonism occurs, the largest FIC index would be selected. By this formulation, an index of 1.0 represents an additive effect, >2.0 shows antagonism, and <1.0 shows synergy. "Indifference" included instances of the lack of effect of one drug on the presence of the other, rare complex interactions that contained elements of both synergy and antagonism patterns in the checkerboard, or instances where marked susceptibility to a drug resulted in the failure to determine a precise MIC, but the presence of another drug failed to antagonize that marked susceptibility. In the latter two instances, and instances where marked resistance to a drug resulted in a failure to determine a precise MIC, a precise FIC cannot be calculated.

RESULTS

MICs and MFCs

The arbitrary grouping of the results with these isolates relied on assumptions based on serum concentration data (Benson and Nahata, 1988) given in the Discussion section, although the relevance of tissue versus serum concentrations, and which concentrations of each are important, are subjects for future study.

We tested 105 *Aspergillus* isolates against Amp B: eight had MICs <1 μg/ml, 17 had MICs of 1 μg/ml, 45 had MICs of 2.0 μg/ml (cumulative ≤2 μg/ml = 67%), 25 (24%) had MICs of 4 μg/ml, and 10 (10%) had MICs >4 μg/ml. The MIC_{50} and MIC_{90} were 2

and 4 μg/ml, respectively. Twenty-five isolates had MFCs determined: three had an MFC of 1 μg/ml, seven (28%) had an MFC of 2 μg/ml, nine (36%) had an MFC of 4 μg/ml, and six (24%) had an MFC of ≥8 μg/ml.

In our laboratory, 60 isolates have been tested against 5FC, 21 (35%) of which had MICs ≤12.5 μg/ml. Two had MICs of 25 μg/ml, and 37 (62%) had MICs >25 μg/ml. The MIC_{50} and MIC_{90} for 5FC were both >25 μg/ml. All 38 isolates tested had MFCs >25 μg/ml.

We tested 18 isolates against MCL. Four (22%) had MICs ≤ 1.6 μg/ml. A total of seven isolates (39%) with MIC < 5 μg/ml were found. Another seven had an MIC of 6.3 μg/ml, and four (22%) had MICs of ≥ 10 μg/ml. The MIC_{50} and MIC_{90} for MCL were 6.3 and ≥10 μg/ml. Of eight isolates tested for MFC, one (13%) had an MFC of 3.13 μg/ml, and two (25%) had an MFC of 6.25 μg/ml. The other five (63%) had MFCs ≥10 μg/ml.

Thirty-two isolates were tested against KTZ in our laboratory, and resistance was the rule. Two isolates (5%) had an MIC of 1.6 μg/ml and two an MIC of 3.1 μg/ml. Another one had an MIC of 6.3 μg/ml, but the remaining 25 isolates (78%) had MICs ≥12.5 μg/ml. Both the MIC_{50} and MIC_{90} for KTZ exceeded 12.5 μg/ml. Of 20 isolates tested, 19 (95%) had MFCs of 25 μg/ml or more; the MFC of the other was 6.3 μg/ml.

We found that of 88 isolates, 85 (97%) had an MIC to ITZ of ≤6.3 μg/ml, and 69 (78%) had an MIC of ≤3.1 μg/ml. The MIC_{50} and MIC_{90} for ITZ were 3.1 and 6.3 μg/ml, respectively. ITZ was also fungicidal for most isolates. The MFC values on the 60 such isolates tested were 0.8 μg/ml (one, 1.6%); 1.6 μg/ml, 14 (23%); 3.1 μg/ml, 13 (22%); 6.3 μg/ml, 14 (23%); 12.5 μg/ml, 12 (20%); and >12.5 μg/ml, six (10%).

Twenty isolates were tested against SAP. Nine (45%) of 20 isolates had an MIC of 0.8 μg/ml, seven (35%) an MIC of 1.6 μg/ml, and two (10%) an MIC of 3.1 μg/ml [cumulative total, ≤3.1 μg/ml, 18 (90%) of 20]. Two isolates had MICs of >12.5 μg/ml. The same 20 isolates had MFCs determined. Six had MFCs of 0.8 μg/ml, seven of 1.6 μg/ml, and two of 3.1 μg/ml [cumulative total, 15 (75%) of 20]. All of the remaining five (25%) had MFCs >12.5 μg/ml.

Drug Interaction Studies

In our laboratory, 54 *Aspergillus* isolates were tested in combination studies for synergy or antagonism. These included 28 (52%) *A. fumigatus*, five (9%) *A. terreus*, and four (7%) *A. flavus* isolates; the remainder were unspeciated. Examination of the results with these four groups revealed no significant differences; therefore, for simplicity the results are combined.

Of 39 Amp B–rifampin studies, there were 36 (92%) instances of synergy and three (8%) of indifference (Table 1). This occurred despite uniform resistance to rifampin alone. One isolate had a rifampin MIC of 50, and all the others had MICs >100. Of 26 Amp B–5FC studies, there were six (23%) instances of synergy, one (4%) of an additive effect, 13 (50%) of indifference, and six (23%) of antagonism (Table 1). The Amp B–5FC drug interactions did not appear related to 5FC susceptibility or resistance. Half or more of the isolates in the synergy, antagonism, and indifference groups were resistant to 5FC (MIC >12.5 μg/ml). No prediction of synergy or otherwise was possible from knowledge of the 5FC MIC alone. In five Amp B–ITZ studies, two showed synergy, one an additive effect, and two indifference (Table 1). In two Amp B–KTZ studies, one showed synergy and one indifference. There were also one KTZ–rifampin and one ITZ–rifampin study; both showed indifference.

The marked superiority of Amp B–rifampin over Amp B–5FC combinations was also shown in other ways. There were 18 isolates that were tested in both combinations. In five (28%) instances, both combinations showed synergy, and in one (5%) both showed indifference. However, in one study, Amp B–rifampin showed synergy and Amp B–5FC showed an additive effect; in eight, Amp B–rifampin showed synergy and Amp B–5FC showed indifference; and, in three, Amp B–rifampin showed synergy and Amp B–5FC showed antagonism. The FIC index also reflects the degree of synergy, which, when present, was more profound in Amp B–rifampin interactions than in Amp B–5FC. In 25 (86%) of 29 instances where an FIC index could be determined with sufficient precision, the index was ≤0.33 for Amp B–rifampin; in contrast, in four of four instances of Amp B–5FC synergy, the FIC index was ≥0.5. Antagonism, when present for Amp B–5FC, could be profound; in two of four instances the FIC index was ≥5.0.

Reproducibility

Reproducibility was addressed by repeat testing of 10 susceptible isolates at different times. Five were tested twice against ITZ for MICs. An identical result was obtained on repeat testing with one isolate; with three isolates a twofold (one tube) difference in results was seen and with the fifth isolate a fourfold (two tubes) difference was seen. Two isolates were tested against ITZ three times for MICs. In both instances, MIC results were within a fourfold range for the three tests (1.5–6.3 μg/ml). Using SAP, one isolate was tested four times and two isolates twice. In all instances, the repeat assay was identical or

TABLE 1 In vitro Combinations of Antifungal Agents Against *Aspergillus* spp.

		Rifampin	KTZ	MCL	ITZ
	5FC				S(11), I(9)[a]
Amp B	S(7), I(13), A(6)[g] I(29), S(2)[b] I(7)[c] S(7)[d]	S(36), I(3)[g] S(31)[b]	S(1), I(1)[g] I(31)[b] A(8)[e,h] I(3)[f]	S(1), I(1), A(1)[f]	S(3), I(2)[g]

S, synergistic/additive (number of isolates tested); I, indifferent; and A, antagonistic. KTZ, ketoconazole; MCL, miconazole; and ITZ, itraconazole.
[a]Kerkering and Espinel-Ingroff, 1987.
[b]Hughes et al., 1984.
[c]Lauer et al., 1978.
[d]Fields et al., 1974.
[e]Schaffner and Frick, 1985.
[f]Odds et al., 1984.
[g]Present study.
[h]Antagonism of killing, not inhibition.

differed by one twofold dilution with the original result for both MICs and MFCs.

DISCUSSION

Future, prospective studies will likely wish to use contemporaneous testing of all isolates, interlaboratory studies of result reproducibility, speciation of all isolates, correlation of species and results, testing of all drugs against the same isolates, and a uniform dilution series as part of a systematic evaluation. Variables such as temperature, inoculum size, shaking, media, and so on, could then be addressed. This discussion is focused on a comparison of our results with the published data. However, as each investigator has used different methodology, comparison is not straightforward. We have, therefore, divided the discussion into two major sections, the first dealing with methodologic issues, the second with a direct comparison of results for each drug. A great deal of the detail has been condensed into nine tables and we do not reiterate this detail here.

Methodologic Considerations

Minimum Inhibitory Concentration Testing

Three principal methods have been used to determine MIC values for *Aspergillus* spp.: macrodilution broth, microdilution broth, and agar dilution tests (Tables 2 and 3). Many methodologic variations were used however and these are described below. Eleven different basic media have been used in these tests. Several variations in additives including serum, pH, and buffering capacity have been employed. In our tests, we used Yeast Nitrogen Broth supplemented with glucose as have six other investigators (Tables 2 and 3), although most are supplemented with both L-asparagine and glucose. Others have added buffers to YNB in addition to altering the pH from the original of 5.4. Drugs whose results are pH dependent will give different results in buffered and unbuffered media. The acidic pH of our unbuffered medium could, for example, have contributed to apparent relative resistance with azoles. Defined media are likely to yield more reproducible results as interactions with complex organic macromolecules are minimized. Incubation temperatures varied widely from 22°C to 37°C. No studies have addressed the influence of temperature on susceptibility results of *Aspergillus* spp. We believe that the use of physiologic temperatures (for humans) of 35°–37°C are more appropriate than lower temperatures, but this variable is one among many that will need to be examined in in vitro–in vivo correlations. Incubation periods also varied widely, from 14 to 336 hr, most being in the 24- to 48-hr range. The stability of the antifungal compounds tested during MIC tests was not verified in most reports. Our method appears to differ from most other broth tests in that our tests were continually shaken at a 30° angle to the horizontal during incubation (Figure 1). This enhances growth, possibly by increasing oxygenation of the medium, but also may deposit material at or above the meniscus out of contact with the test drug—this would not influence growth in the medium which usually determines the MIC cutoff. However, for a minority of isolates, growth was observed primarily at the meniscus, and in this situation the MIC might be altered by shaking. The MFC results (see below) might also be altered by shaking.

The inoculum used has varied greatly in various reports from 4×10^1 to 1×10^7 conidia.

TABLE 2 Broth Dilution Method Variables for Testing *Aspergillus* spp. Susceptibility to Antifungal Agents

Variable	Method A	B	C	D	E	F	G	H	I	J
Assay system (ml)	Macro (2)	Macro (1)	Macro (1)	Macro (2.05)	Macro (1)	Macro (5)	Micro (0.1)	Micro (?)	Micro (0.2)	Micro (?)
Medium	YNB and 0.5% glucose	SAAMF	MEM[c]	YNB[b], AM3	RHB[a]	MEM, BHI, Sab ± 10% serum	YNB[b,c], MEM[c]	CCM	AM3	YNB[b]
pH	5.4	7.4	7.3	5.4, 7.0	6.8	7.4, 7.3, 5.6, 4.0	7.5, 6.5	6.6	7.0	5.4
Temp (°C)	35	35	37	30	30	25	37	30	30	37
Duration (hr)	36–48	24	14–17	47	48	336	24	24	18–72	48
Shaking	Yes	No	?No	?	?	?	No	No	No	No
Inoculum (ml)	10^3	10^4	2 mg of ATP biomass	10^5	40	[d]	10^6	10^7	10^5	2×10^4
MIC end-point determination	Visual, no growth	Visual, no growth	RIF	Visual, no growth	Visual, no growth	? 90% inhibition	Microscopy, ≤10 conidia	Photometer, comparison with formalin-treated inoculum	Mirror reader, no growth	Visual, no growth
MFC test, % volume plated	2.0–2.5	10	ND	?	ND	NR	100	ND	ND	?
MFC test duration	48–72	24–72	ND	?	ND	336	24	ND	ND	48
MFC test end point	Visual, ≤1 colony	Visual, no growth	ND	Visual, 3 colonies	ND	Visual, no growth	Visual, no growth	ND	ND	Visual, no growth
Reference	This study	Hoeprich and Merry, 1984	Odds et al., 1980, 1982, 1984, and 1989	Kerkering and Espinel-Ingroff, 1987; Shadomy et al., 1985	Odds et al., 1980	Van Cutsem et al., 1983, 1988, 1989	Hughes et al., 1984	Dupont and Drouhet, 1987	Bezjak, 1985	Steer et al., 1972

SAAMF, synthetic amino acid medium/fungi; AM3, antibiotic medium 3; RHB, Rowley–Huber broth; Sab, Sabouraud dextrose broth; MEM, minimal essential medium; BHI, brain–heart infusion broth; CCM, casitone complex broth medium; RIF, relative inhibition factor (see text); ND, not done; NR, not relevant (see text); Macro, macrodilution broth test; and Micro, microdilutoin broth test.

[a]Contains detergent (for example, Tween).

[b]YNB supplemented with L-asparagine and dextrose.

[c]Buffered.

[d]A 2-mm block of agar containing spores and hyphae.

TABLE 3 Agar Dilution Method Variables Used in Susceptibility Tests of *Aspergillus* spp.

	Method							
Variable	Z	Y	X	W	V	U	T	S
Medium	YMA, Sab	YNB[a]	YMA	YNB and 0.5% glucose	CYE	Kimmig and glycerol 0.5%	YNB[a]	BHI
pH	6.2, 5.6	5.4	6.2	5.4	6.6	?	5.4	7.3
Temp (°C)	30	30	37	30	30	30	30, 37	22
Duration (hr)	Variable	48	48	24	72–144	48	24–72	48
Method of inoculation	Replica inoculator	Pipetted in saline	?	Steer replicator onto filters on agar surface[c]	Pipetted in saline	Steer replicator	Pipetted in saline	Pipetted in saline[b]
Inoculum (ml)	?	10^4–10^5	?	?	10^5–10^6	10^5	?	?
MIC end-point determination	Visual, no growth	Visual, no growth on central portion of agar slant	Visual, no growth	Visual, no growth	Visual, no growth	Visual, no growth on central portion of agar slant	Visual, no growth	Visual, no growth
Reference	Shadomy and Paxton, 1976	Lauer et al., 1978	Scholer, 1970	Wagner et al., 1975	Dixon et al., 1978	Espinel-Ingroff et al., 1984	Shadomy, 1969	Brandsberg and French, 1972

YMA, yeast morphology agar; CYE, casein yeast extract glucose; and other abbreviations as per Table 2.
[a]YNB supplemented with L-asparagine and dextrose.
[b]Containing Tween.
[c]Semisolid agar system; MFC also determined by removal of filter and culture in YNB broth.

Higher inocula were used in the microdilution broth tests than in the macrodilution broth tests. Most inocula were in the 10^4–10^6 range; again our method differs from most in using the lower inoculum of 10^3 conidia. This inoculum size was used because with yeasts it correlated most closely with an inoculum-independent method (Galgiani and Stevens, 1976). Our own studies indicate some variation in the viability of the inocula, a problem which is reduced but not eliminated by the use of freshly conidiating cultures. One study using a microdilution broth assay system has addressed the question of possible differences in susceptibility results using conidia or hyphae as the inoculum (Bezjak, 1985). No differences were found. However, differences may exist. For example, immune responses to conidia have been shown to vary depending on whether the conidia are "activated" or "swollen" (two expressions for the same event) (Schaffner et al., 1982). Entry of an antifungal drug into the fungal cell may be the final critical step for some drugs in effecting any inhibitory or cidal effect. It is not known whether resting or swollen conidia will take up antifungal drugs and, if so, which drugs. These issues are worthy of study in order to clarify some of the uncertainties, and conclusions may vary for different antifungal drugs.

End-point determination varied with the basic method employed. In the macrodilution broth tests, a visual end point was used (most investigators are aware of the subjectivity inherent in such an end point). This problem is compounded in the ITZ and SAP tests at concentrations of drug >12.5 μg/ml because of visible drug precipitate. We examined interobserver variation in reading end point on MIC tests for SAP on six isolates. Complete agreement between two observers was obtained in reading four of the tests. Differences arose only in interpretation of a possible single colony in a single tube of the other two tests. All the results for all drugs except SAP reported in this article were read by one of four observers, and the vast majority (>90%) by only one

of two observers. All the SAP tests were read by one observer. We believe, therefore, that, in our hands, the subjectivity of a visual end point has been reduced to the minimum possible, but reproducibility of results in different laboratories may be worse, and reproducibility between laboratories remains to be studied.

The microdilution tests were usually read microscopically or photometrically, each of which may have their own problems of interpretation. A visual end point for agar dilution of no growth is potentially more clear cut, however, two investigators define the *end point* as that of "no growth on central portion of the slant." This introduces the potential for subjectivity as to how far the central portion of the agar extends. As the whole slant should have an equal concentration of antibiotic throughout, the reason for this definition appears arbitrary and is unclear.

Minimum Fungicidal Concentration Testing

Few investigators have attempted to determine MFCs. Only three state the volume subcultured from an MIC test. We subculture 2.0%–2.5% of our original volume and define the MFC as one colony or less; this represents a killing of ≥97.5%. Hoeprich and Merry (1984) subcultured 10% of a tenfold higher inoculum and defined the end point as no growth, therefore, representing a killing ≥99.9%. Hughes et al. in a microtiter test subcultured 100% of the volume of an even greater inoculum. As they defined the end point as no growth (not surprisingly, they found inconsistency with any other definition), their definition represents a killing of ≥99.9999% (Hughes et al., 1984). Clearly, these definitions are widely different. In a single experiment using four isolates tested against SAP, we previously examined some of these issues by subculturing several different volumes from the same assay tube. There was good agreement between end points at ≥97.5%, ≥98%, and ≥99% killing. However, when larger volumes were subcultured representing ≥99.5% and ≥99.9% end points, small numbers of colonies were cultured from almost all tubes. This could reflect the fact that as our tests are shaken during incubation, a few conidia will adhere to the tube at or above the meniscus (there is always a little evaporation of the medium over a 36- to 48-hr test) out of reach of the test drug. When the tube is vortex mixed prior to plating for the MFC, the conidia may be carried down into the medium and subcultured. By using a lower cutoff as we do, a killing of ≥97.5% of inoculum will likely prevent these few conidia producing an artifically elevated MFC result in our system. For bactericidal tests, these and many other issues pertaining to methodologic variables have been addressed in detail (Taylor et al., 1983), but not as yet for molds.

Susceptibility Results

Activity of Amphotericin B

Our data from tests on 105 isolates suggest considerable interisolate variation, from <0.5 to 8 μg/ml Amp B in MIC and 1 to >8 μg/ml in MFC values. Considerable variation was seen in some other studies (Odds et al., 1984; Kerkering and Espinel-Ingroff, 1987; Shadomy et al., 1985; Van Cutsem and Janssen, 1988; Bezjak, 1985), but not in others (Hughes et al., 1984; Dupont and Drouhet, 1987; Lauer et al., 1978; Scholer, 1970; Brandsberg and French, 1972) (Table 4). There are considerable phenotypic differences between different *Aspergillus* isolates, and recently *A. fumigatus* isolates have been shown to be distinguishable by DNA restriction fragment-length polymorphisms (RFLP) (Denning et al., 1990a). Although it is unlikely that particular DNA types will be associated with a particular susceptibility pattern (and variation of susceptibility within a given DNA type is likely because of differences in gene expression), variation in RFLP pattern and many phenotypic characteristics among isolates suggests that other differences may exist between isolates as for example, susceptibility to a given antifungal agent. For this reason, we believe differences in susceptibility among *Aspergillus* isolates are to be expected. However, the relationship between an in vitro MIC and in vivo performance of Amp B is far from clear. Although peak serum concentrations of Amp B in humans after intravenous infusion may exceed 2.0 μg/ml, binding to blood constituents exceeds 99% and tissue concentrations vary considerably over time. In two patients, tissue concentrations of Amp B (as measured in both ethanol and aqueous extracts) far exceeded the MIC of the infecting isolate, yet disseminated aspergillosis was found at autopsy in both (Christiansen et al., 1985). The method used to determine the MIC in that study (method 1 but cultured at 34°C and with a visible end point) yielded low MICs, 0.2 μg/ml. This dichotomous result suggests that this particular MIC determination did not predict therapeutic outcome for Amp B. (This issue is discussed more extensively later.)

Activity of Flucytosine

In general, 5FC has limited activity against *Aspergillus* spp. by all methods. Desirable serum concentrations of 5FC are generally recommended to be 20–100 μg/ml. Based on this assumption, most laboratories utilize 12.5 μg/ml as the breakpoint for susceptibility with yeasts. By such a criterion, some methods suggest all isolates are resistant (Odds et al., 1984; Shadomy, 1969); by others, all or almost all are susceptible (Shadomy et al., 1985; Scholer, 1970; Lauer et al., 1978; Wagner et al., 1975; Dixon et al., 1978); and, in most, the majority are resistant

TABLE 4 In vitro Susceptibility of *Aspergillus* spp. to Amphotericin B (μg/ml)

No. of Isolates	Method	MIC_{50}	MIC_{90}	MIC Range	MFC_{50}	MFC_{90}	MFC Range	References
105[a,b,c,d]	A	2	4	<1–>4	4[h]	8[h]	1–>8[h]	This study
8[a,b]	C	36[e]	NR	26–49[e]	ND	ND	ND	Odds et al., 1984
18[a,b]	D	0.6	>10	0.2–>10	1.25	>10	0.3–>10	Kerkering and Espinel-Ingroff, 1987
NR[a,b,c,d]	D	NR	NR	0.1–8	NR	NR	6.3–>100	Shadomy et al., 1985
NR[a,b,c,d]	F	NR	NR	0.1–10[g]	ND	ND	ND	Van Cutsem and Janssen, 1988
17[a]	G	1.6	3.1	1.6–3.1	3.13	6.25	1.6–6.3	Hughes et al., 1984
9[b]	G	3.1	3.1	3.1	ND	ND	ND	Hughes et al., 1984
5[c]	G	NR	NR	1.6–3.1	ND	ND	ND	Hughes et al., 1984
16[a]	H	<0.1	0.4	<0.1–0.8	ND	ND	ND	Dupont and Drouhet, 1987
6[a,b]	I	NR	NR	0.3–5[f]	ND	ND	ND	Bezjak, 1985
7[a]	Y	1	2	0.5–2	ND	ND	ND	Lauer et al., 1978
6[c]	Y	1	1	1	ND	ND	ND	Lauer et al., 1978
11	X	NR	NR	0.2–0.4	ND	ND	ND	Scholer, 1970
21	S	0.3	NR	0.1–0.6	ND	ND	ND	Brandsberg and French, 1972

Abbreviations as per Tables 1 and 2.
[a]*Aspergillus fumigatus*.
[b]*A. flavus*
[c]*A. niger*
[d]Other *Aspergillus* spp.
[e]Relative inhibition factors.
[f]Results depended on test conditions, see text.
[g]Range for only >90% isolates tested.
[h]25 isolates.

(Kerkering and Espinel-Ingroff, 1987; Lauer et al., 1978; Steer et al., 1972; Scholer, 1970), including our own (Table 5). No fungicidal activity was demonstrated by any study. A small number of patients have been successfully treated with 5FC monotherapy and some activity is demonstrable in animal models (reviewed in Denning and Stevens, 1990). However, in the treated patients, in vitro results were not given, thus preventing any attempt at correlation of in vivo and in vitro activity.

Activity of Miconazole

MCL was the first azole licensed for systemic use. Peak serum concentrations may be transiently >7.5 μg/ml. By such criterion, some activity is demonstrable in vitro against a minority of isolates in our hands. Others have found both susceptible and resistant isolates (Odds et al., 1980 and 1984; Shadomy et al., 1976 and 1985; Van Cutsem and Janssen, 1988), and one study found all isolates to be resistant (Dixon et al., 1978) (Table 6). MCL has some activity in vivo (reviewed in Denning and Stevens, 1990). However, as newer azoles emerge, some with considerably greater activity against *Aspergillus* spp., we doubt that further work to optimize in vitro testing is warranted for MCL.

Activity of Ketoconazole

There was a wide divergence in the degree of activity of KTZ against *Aspergillus* spp. found in various studies. Doses currently licensed in the USA (up to 400 mg/day) produce peak serum concentrations approximately at the 6.25 μg/ml drug dilution used in vitro. Some studies thus found most or all isolates to be susceptible (Hoeprich and Merry, 1984; Dupont and Drouhet, 1987), whereas others found that species susceptibility varied, some species being susceptible, whereas others (especially *A. fumigatus*) were less so (Odds et al., 1980; Espinel-Ingroff et al., 1984). Some investigators, including ourselves, found the majority of isolates to be resistant (Odds et al., 1984; Van Cutsem, 1983; Van Cutsem and Janssen, 1988), and some found a wide range of results (Shadomy et al., 1985) (Table 7). Essentially, we found no fungicidal activity at achievable serum concentrations of KTZ. This contrasts with another study where, although lack of fungicidal activity was true with *A.*

TABLE 5 In vitro Susceptibility of *Aspergillus* spp. to Flucytosine (μg/ml)

No. of Isolates	Method	MIC_{50}	MIC_{90}	MIC Range	MFC_{50}	MFC_{90}	MFC Range	References
60[a,b,c,d]	A	>25	>25	≤12.5–>25	>25[e]	>25[e]	>25[e]	This study
8[a,b]	C	92[f]	NR	79–100[f]	ND	ND	ND	Odds et al., 1984
20[a,b]	C	>10	>10	1.3–>10	>10	>10	10–>10	Kerkering and Espinel-Ingroff, 1987
NR	D	NR	NR	0.2–1.6	NR	NR	>100	Shadomy et al., 1985
7	K	125	>500	0.48–>500	>500	>500	125–>500	Steer et al., 1972
7[a]	Y	>128	>128	0.25–>128	ND	ND	ND	Lauer et al., 1978
5[c]	Y	0.5	1	0.5–1	ND	ND	ND	Lauer et al., 1978
11[a]	X	NR	NR	0.2–0.8	ND	ND	ND	Scholer, 1970
5[a]	W	0.5	>128	0.5–>128	>128	>128	>128	Wagner et al., 1975
2[c]	W	—	—	0.1–0.5	>128	>128	>128	Dixon et al., 1978
6[a]	T	50	>100	50–>100	ND	ND	ND	Shadomy, 1969

Abbreviations as per Tables 1 and 2.
[a,b,c,d]As per notes *a*, *b*, *c*, and *d* in Table 4.
[e]38 isolates.
[f]Relative inhibition factors.

fumigatus, it was not for other *Aspergillus* spp. (Hoeprich and Merry, 1984). As human and animal studies have indicated little to no activity of KTZ against invasive aspergillosis (reviewed in Denning and Stevens, 1990), we are inclined to regard methods that yield a high proportion of susceptible results as unlikely to provide a useful in vitro–clinical correlation. However, as KTZ is not an agent of choice for invasive aspergillosis and may antagonize amphotericin B in some circumstances (Schaffner and Frick, 1985), further work to clarify these issues is probably unnecessary.

Activity of Itraconazole

Itraconazole, a new triazole, is remarkable for its activity against *Aspergillus* spp. when given orally to patients (Denning et al., 1989). The majority of isolates are susceptible to ITZ in vitro in our and others' hands (Odds et al., 1984; Kerkering and Espinel-Ingroff, 1987; Van Cutsem and Janssen, 1988; Du-

TABLE 6 In vitro Susceptibility of *Aspergillus* spp. to Miconazole (μg/ml)

No. of Isolates	Method	MIC_{50}	MIC_{90}	MIC Range	MFC_{50}	MFC_{90}	MFC Range	References
18[a,b,c,d]	A	6.3	≥10	>1.6–≥10	≥10[e]	≥10[e]	3.13–≥10[e]	This study
8	C	67[f]	NR	54–81[f]	ND	ND	ND	Odds et al., 1984
NR[a,b,c,d]	D	NR	NR	0.4–>100	NR	NR	0.8–>100	Shadomy et al., 1985
25[a]	E	3	8	0.5–16	ND	ND	ND	Odds et al., 1980
5[c]	E	2	4	1–4	ND	ND	ND	Odds et al., 1980
4[b,d]	E	1	1	1	ND	ND	ND	Odds et al., 1980
NR[a,b,c,d]	F	NR	NR	1–10	ND	ND	ND	Van Cutsem and Janssen, 1988
15[a,b,c]	Z	4	4	1–>4	ND	ND	ND	Shadomy and Paxton, 1976
14[a]	V	8	16	8–16	ND	ND	ND	Dixon et al., 1978

Abbreviations as per Tables 1 and 2.
[a,b,c,d]As per notes *a*, *b*, *c*, and *d* in Table 4.
[e]Eight isolates.
[f]Relative inhibition factors.

TABLE 7 In vitro Susceptibility of *Aspergillus* spp. to Ketoconazole (μg/ml)

No. of Isolates	Method	MIC_{50}	MIC_{90}	MIC Range	MFC_{50}	MFC_{90}	MFC Range	References
32[a,b,c,d]	A	≥12.5	≥12.5	1.6–≥12.5	≥25[e]	≥25[e]	6.25–≥25[e]	This study
10[a]	B	<5.3	<5.3	2.7–5.3	>42.5	>42.5	>42.5	Hoeprich and Merry, 1984
10[b]	B	<1.3	<1.3	NR	5.3	21.2	NR	Hoeprich and Merry, 1984
10[c]	B	≤0.7	1.3	<0.7–>1.3	1.3	5.3	1.3–>5.3	Hoeprich and Merry, 1984
8[a,b]	C	58[f]	NR	51–69[f]	ND	ND	ND	Odds et al., 1984
NR[a,b,c,d]	F	NR	NR	0.1–100	NR	NR	NR	Shadomy et al., 1985
25[a]	E	8	16	1–16	ND	ND	ND	Odds et al., 1980
5[c]	E	2	4	1–4	ND	ND	ND	Odds et al., 1980
4[b,d]	E	1	1	1	ND	ND	ND	Odds et al., 1980
NR[a,b,c,d]	F	NR	NR	10–100	NR	NR	NR	Van Cutsem and Janssen, 1988; Van Cutsem, 1983
16[a]	H	0.78	12.5	<0.1–12.5	ND	ND	ND	Dupont and Drouhet,1987
10[a]	U	4	8	4–16	ND	ND	ND	Espinel-Ingroff et al., 1984
9[b]	U	0.5	1	0.3–1	ND	ND	ND	Espinel-Ingroff et al., 1984

Abbreviations as per Tables 1 and 2.
[a,b,c,d]As per notes *a*, *b*, *c*, and *d* in Table 4.
[e]20 isolates.
[f]Relative inhibition factors.

TABLE 8 In vitro Susceptibility of *Aspergillus* spp. to Itraconazole (μg/ml)

No. of Isolates	Method	MIC_{50}	MIC_{90}	MIC Range	MFC_{50}	MFC_{90}	MFC Range	References
85	A	3.1	6.3	0.4–>25	6.3[e]	12.5[e]	0.8–>25[e]	This study
8[a,b]	C	25[f]	NR	19–30[f]	ND	ND	ND	Odds et al., 1984
10[a]	D	1.3	2.5	0.3–2.5	5	>10	0.3–>10	Kerkering and Espinel-Ingroff, 1987
10[b]	D	0.6	1.25	<0.3–1.3	1.3	5	0.3–5	Kerkering and Espinel-Ingroff, 1987
136[a,b,c,d]	F	0.1	1	0.01–100	NR	NR	NR	Van Cutsem and Janssen, 1988
16[a]	H	0.1	0.2	0.1–0.4	ND	ND	ND	Dupont and Drouhet, 1987
10[a]	U	0.06	0.1	0.1–2	ND	ND	ND	Espinel-Ingroff et al., 1984
9[b]	U	0.06	0.1	0.06–0.1	ND	ND	ND	Espinel-Ingroff et al., 1984

Abbreviations as per Tables 1 and 2.
[a,b,c,d]As per notes *a*, *b*, *c*, and *d* in Table 4.
[e]60 isolates.
[f]Relative inhibition factors.

pont and Drouhet, 1987; Espinel-Ingroff et al., 1984) (Table 8). Inhibitory concentrations are lower than fungicidal concentrations. Approximately 60% of isolates are killed at or below achievable serum concentrations of ITZ (Denning et al., 1989), and some tissue concentrations are higher than serum concentrations. One group found *A. flavus* isolates to be more susceptible than those of *A. fumigatus* (Kerkering and Espinel-Ingroff, 1987); we did not find this to be the case.

Activity of Saperconazole

Our data and all the studies published so far indicate a high degree of activity of SAP against *Aspergillus* spp. (Odds, 1989; Van Cutsem et al., 1989) (Table 9). We found two (10%) of 20 isolates to have high MICs and six (30%) of 20 to have high MFCs. At the time of this writing, serum concentrations of SAP in humans are not published. In vivo correlations have not been done by Janssen Pharmaceutica, although they have generated a large body of in vivo data indicating considerable activity (Van Cutsem et al., 1989).

Antifungal Combinations

Very few studies have examined antifungal combinations in vitro or in vivo. The most powerful studies are those of animal models, but they have of necessity one significant drawback: few *Aspergillus* isolates can be tested. In addition, murine aspergillosis in the laboratory may not be a good model for human disease in the immunocompromised host. In vitro work, however, can more easily cope with a large number of isolates, but it is not yet clear what the important methodologic variables are. Table 1 displays the results of six published studies combined with our own. In these studies, 79 isolates of *A. fumigatus, A. flavus,* and *A. niger* were studied. Our own experience contributes an additional 54 isolates.

Essentially, the following conclusions can be drawn (overlooking for the moment the methodologic differences that are not discussed further here): Amp B and 5FC are variably synergistic, additive (Fields et al., 1974; Hughes et al., 1984; our data), indifferent (Lauer et al., 1978; our data), or antagonistic in vitro (our data). Antagonism has not been demonstrated in vivo (reviewed in Denning and Stevens, 1990). Amp B and rifampin are usually synergistic in vitro (Hughes et al., 1984; our data), occasionally additive or indifferent (our data), and never antagonistic (our data). Using a single isolate, synergism was demonstrated in vivo (Arroyo et al., 1977). Amp B and KTZ are usually indifferent (Hughes et al.,1984; Odds, 1982) or antagonistic in vitro (Schaffner and Frick, 1985). In one experiment, antagonism was demonstrated only in killing and was most pronounced with pretreatment of conidia with KTZ prior to addition of Amp B (Schaffner and Frick, 1985). We found one isolate to be synergistic and one to be indifferent. KTZ and Amp B are antagonistic or indifferent in vivo (reviewed in Denning and Stevens, 1990). Amp B and MCL together were synergistic, indifferent, and antagonistic in vitro with one isolate each (Odds, 1982). Similarly, ITZ and Amp B were either synergistic, additive, or indifferent in vitro (our data). Combinations of ITZ and 5FC in vitro revealed synergism in five of 10 *A. fumigatus* strains and three of 10 *A. flavus* strains that was independent of the susceptibility to 5FC (Kerkering and Espinel-Ingroff, 1987). However, the other 50% of *A. fumigatus* isolates showed possible antagonism of MFCs but not MICs (Kerkering and Espinel-Ingroff, 1987).

Perspective on Future Directions of Research

The key to clarifying the appropriate method to evaluate any given in vitro susceptibility testing method is whether the result predicts what happens in vivo. Unfortunately, this evaluation is far from straightforward. First, patients who develop *Aspergillus* infections usually have an immune deficit that may be profound and may vary over time. This is a major compounding factor in evaluating the efficacy of any antifungal agent and to delineate clearly different

TABLE 9 In vitro Susceptibility of *Aspergillus* spp. to Saperconazole (μg/ml)

No. of Isolates	Method	MIC_{50}	MIC_{90}	MIC Range	MFC_{50}	MFC_{90}	MFC Range	References
20[a,b,d]	A	1.5	3.1	0.8–>12.5	1.5	>12.5	0.8–>12.5	This study
8[a,b]	C	14[e]	NR	6–18[e]	ND	ND	ND	Odds, 1989
221[a]	F	0.1	1	0.1–1	ND	ND	ND	Van Cutsem et al., 1989
15[b]	F	0.1	1	0.1–1	ND	ND	ND	Van Cutsem et al., 1989
19[c]	F	1	1	0.1–10	ND	ND	ND	Van Cutsem et al., 1989
14[d]	F	0.01	0.1	0.01–1	ND	ND	ND	Van Cutsem et al., 1989

Abbreviations as per Tables 1 and 2.
[a,b,c,d]As per notes *a, b, c,* and *d* in Table 4.
[e]Relative inhibition factors.

responses according to different in vitro susceptibility results may only be possible in specific host groups. Second, infection of different organ systems may respond differently to antifungal agents for reasons of tissue penetration, blood supply, and local immune factors (to name three examples); thus difficulties of correlating in vivo and in vitro results are greatly compounded. Cerebral aspergillosis is a case in point—among patients in whom therapy was evaluable, only eight survivors are recorded in the literature (Denning and Stevens, 1990). Together, these two issues make the comparative evaluation of in vitro testing results for *Aspergillus* spp. problematic.

For these and other reasons, investigators have turned to animal model studies to clarify some of these issues. However, none have done comparative studies using multiple isolates with different susceptibility results to evaluate a range of in vitro methods. Some combination studies have been done in vivo which are reviewed elsewhere (Denning and Stevens, 1990), using a total of 16 isolates. In most of these studies, in vitro data are not given and, where they are, methodology differs in each report. Clearly, work along these lines is warranted, in addition to standardization of in vitro methods.

It may be that a particular method for one drug that yields reproducible results that correlate with in vivo outcome will fail to perform adequately for another drug. Our hope is that an optimal testing method with one lipophilic azole will be useful for other similar compounds, for example, fluconazole and SCH 39304, although to date in vitro testing with fluconazole has been problematic.

We are of the opinion that in vitro testing can yield clinically useful information. For example, a 5FC–Amp B combination study showing antagonism would deter us from using this combination. An isolate resistant to MCL from a patient unable to take Amp B and oral medication would not encourage us that MCL would be the therapy of choice but that experimental treatment might be more appropriate. A low MFC to ITZ might encourage us to discontinue therapy earlier in an immunocompromised patient if toxicity that is due likely to ITZ were observed during therapy. Of course, these examples are as yet unfounded on a solid scientific basis but they serve as examples for how reliable in vitro results could guide therapy. The scientific basis needed awaits in vitro–in vivo correlations.

Bacterial susceptibility testing has a major role to play in the successful treatment of bacterial infection. We believe the same will be true for fungal susceptibility testing and fungal infection in years to come. Correlations are beginning to emerge in yeast infections (Powderly et al., 1988; Galgiani, 1990), and we hope it will not be too many years before the same will be true of mold infections and, in particular, *Aspergillus* infections.

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