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In Vitro Drug Interaction Modeling of Combinations of Azoles with Terbinafine against Clinical *Scedosporium prolificans* Isolates

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The in vitro interaction between terbinafine and the azoles voriconazole, miconazole, and itraconazole against five clinical *Scedosporium prolificans* isolates after 48 and 72 h of incubation was tested by a microdilution checkerboard (eight-by-twelve) technique. The antifungal effects of the drugs alone and in combination on the fungal biomass as well as on the metabolic activity of fungi were measured using a spectrophotometric method and two colorimetric methods, based on the lowest drug concentrations showed 75 and 50% growth inhibition (MIC-1 and MIC-2, respectively). The nature and the intensity of the interactions were assessed using a nonparametric approach (fractional inhibitory concentration [FIC] index model) and a fully parametric response surface approach (Greco model) of the Loewe additivity (LA) no-interaction theory as well as a nonparametric (Prichard model) and a semiparametric response surface approaches of the Bliss independence (BI) no-interaction theory. Statistically significant synergy was found between each of the three azoles and terbinafine in all cases, although with different intensities. A 27- to 64-fold and 16- to 90-fold reduction of the geometric mean of the azole and terbinafine MICs, respectively, was observed when they were combined, resulting in FIC indices of <1 to 0.02. Using the MIC-1 higher levels of synergy were obtained, , which were more consistent between the two incubation periods than using the MIC-2. The strongest synergy among the azoles was found with miconazole using the BI-based models and with voriconazole using the LA-based models. The synergistic effects both on fungal growth and metabolic activity were more potent after 72 h of incubation. Fully parametric approaches in combination with the modified colorimetric method might prove useful for testing the in vitro interaction of antifungal drugs against filamentous fungi.

Scedosporium prolificans is a filamentous fungus which causes various types of human infections, ranging from asymptomatic colonizations and localized infections in immunocompetent individuals to disseminated infections, usually in immunocompromised patients (7, 18). The mortality rate of invasive infections is high (92%) despite antifungal chemotherapy with amphotericin B, miconazole, fluconazole, or itraconazole (18). However, the effectiveness of antifungal chemotherapy is uncertain since any success reported in the literature has been associated with additional intervention such as surgery, immunomodulation, or recovery of host defense. Furthermore, in vitro susceptibility tests indicate a multiresistant fungus due to the very low growth-inhibitory activities of conventional and new antifungal drugs (9, 11, 27). Therefore, new approaches for treating these infections are warranted. Promising results are coming from the field of in vitro combination of antifungal drugs against fungi (3, 35, 41), especially after the recent findings of synergistic interaction between itraconazole and terbinafine against different fungi, including *S. prolificans* (31, 32). Despite the fact that these two drugs were inactive alone, the in vitro combination of them at concentrations achievable in serum succeeded in inhibiting the fungal growth.

However, assessing the nature and intensity of drug interactions is still a debated area of chemotherapy. The observed in vitro interaction of two or more agents is dependent both on the methodology which is used to generate the data as well as on the way the results are analyzed, resulting in variable as well as controversial conclusions (6, 20, 35).

Despite the considerable progress in the in vitro antifungal susceptibility testing of filamentous fungi with single drugs, there are several problems regarding the choice of the proper testing method, reading system, MIC endpoint, and incubation period (10, 15, 34, 39). Different results might be obtained by choosing different combinations of these parameters. This is even more complicated when two drugs are combined in vitro. Spectrophotometric and colorimetric methods have been described which quantify more precisely the fungal growth and are able to detect small changes in metabolic activity of fungi, respectively (26, 30). Based on these methods, the effects of antifungal drugs either on the fungal biomass or on metabolic status of fungi can be measured.

However, even when the same methodology is used for testing the in vitro susceptibilities to drug combinations variable conclusions might be inferred depending on the way data are analyzed. Many models and approaches have been described for the assessment of in vitro drug interactions, and extensive reviews have summarized them (5, 6, 20, 38, 43). The assumption of no interaction has a central position in these debates, since synergy and antagonism are defined as departures from this. Thus, when the observed effect is more or less than the effect predicted from the no-interaction theory, synergy or antagonism, respectively, is claimed. Among the various no interaction theories, the Loewe additivity (LA) and Bliss independence (BI) theories are the two major competitor candidates for reference models (20). The LA theory is based on the

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idea that a drug cannot interact with itself, while the BI theory is based on the idea that two drugs act independently with the probabilistic sense of independence. Based on these concepts, various models have been described based on both parametric and nonparametric approaches of these two reference theories. These include estimates of the intensity of interaction and summary results incorporating statistically significance levels.

Given that a synergistic interaction might have great impact on approaches for controlling infections caused by *S. prolificans* in clinical practice (19, 24), the unbiased assessment of in vitro interaction of antifungal drugs is crucial. In this study, the interaction between three azoles—voriconazole, miconazole, and itraconazole—and an allylamine, terbinafine, was tested in vitro against five clinical isolates of *S. prolificans*. In order to reduce biases derived from factors related to the method of antifungal susceptibility testing as well as to theoretical and mathematical models for defining synergism, the in vitro interactions of antifungal drugs were determined by recently described spectrophotometric and colorimetric (26) NCCLS-based (33) methods, after 48 and 72 h of incubation based on two MIC endpoints and assessed using two parametric and two nonparametric models based on the LA and BI theories, described in the literature.

MATERIALS AND METHODS

Isolates. Five *S. prolificans* strains were used in this study, namely, AZN7898 and AZN7918 isolated from blood; AZN7901 and AZN7902 isolated from sputum; and a reference strain, AZN7906 (NCPF7108). The isolates were stored in 50% glycerol in water at −70°C. *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used as quality controls.

Antifungal drugs. Three antifungal drugs belonging to the azoles—namely, miconazole (molecular weight [MW], 479.15) (Janssen Research Foundation, Beerse, Belgium), itraconazole (MW, 705.64) (Janssen Research Foundation), and voriconazole (MW, 349.32) (Pfizer Central Research, Sandwich, United Kingdom)—and the allylamine terbinafine (MW, 291.44) (Novartis, Basel, Switzerland) were used in this study. Miconazole and voriconazole were dissolved in dimethyl sulfoxide (Merck, Darmstadt, Germany) at final concentrations of 25,600 and 6,400 mg/liter, respectively. Terbinafine was dissolved in dimethyl sulfoxide with 5% Tween 80 (Merck) and itraconazole in 0.1 M HCl–100% acetone (1:1) (Merck) at final concentrations of 25,600 and 12,800 mg/liter, respectively.

Medium. The medium used was liquid RPMI 1640 (with L-glutamine, without sodium bicarbonate) (GIBCO BRL, Life Technologies, Woerden, The Netherlands) supplemented with 0.165 M MOPS (morpholinepropanesulfonic acid) buffer (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The pH of the medium was adjusted to 7.0 ± 0.1 at 22°C.

Inoculum preparation. Each isolate had been grown for 7 days on Sabouraud dextrose agar with 0.5% chloramphenicol at room temperature and was then subcultured on the same medium for a further 5 to 7 days at 37°C. Conidia were collected with a swab and suspended in sterile water. After the heavy particles were allowed to settle, the turbidity of the supernatants was measured spectrophotometrically at 530 nm with a Spectronic 20D device (Milton Roy, Rochester, N.Y.), and the transmittance was adjusted to 68 to 70% by diluting. Each suspension was diluted 1:50 in RPMI 1640 to obtain two times the final inoculum size, ranging from 0.5×10^4 to 5×10^4 CFU/ml. Inoculum size was verified by plating 100 μl of serial dilutions of each inoculum onto a Sabouraud dextrose agar plate and incubation until growth became visible.

For the quality controls, the transmittance of blastoconidium suspensions obtained from 1- to 2 day-old colonies was adjusted to 75 to 77% at 530 nm. The suspensions were diluted 1/1,000, and a final inoculum ranging from 0.5×10^3 to 2.5×10^3 CFU/ml was used.

Susceptibility testing method. The in vitro interaction between the three azoles and the allylamine terbinafine was studied using a two-dimensional (eight-by-twelve) checkerboard microdilution technique in sterile, 96-well flat-bottom microtitration plates as described below. Each isolate was tested four times on different days. In order to choose the appropriate range of drug concentrations for the combination studies, the MICs of the individual drugs were determined in an exploratory study for each strain.

(i) Microtitration plates set up. For the combination studies, each drug was first serially diluted 2-fold in the corresponding solvents and then 100-fold in the medium according to the dilution scheme of the NCCLS for water-insoluble drugs in order to obtain four times the final concentration. A 50-μl aliquot of each drug concentration of the azole was added to columns 1 to11, and then a 50-μl aliquot of each concentration of terbinafine was added to rows A to G. In the wells of column 12, 50 μl of the medium containing 1% of the azole solvent was added, and in wells of row H, 50 μl of the medium containing 1% terbinafine solvent was added. Thus, row H and column 12 contained alone the azole and terbinafine, respectively, and the well at the intersection of row H and column 12 (well H12) was the drug-free well that served as the growth control. The final concentrations after the addition of 100 μl of inoculum ranged from 64 to 0.06 μg/ml for miconazole, 32 to 0.03 μg/ml for itraconazole, 16 to 0.015 μg/ml for voriconazole, and 64 to 1 μg/ml for terbinafine. The microtitration plates were kept at −70°C until the day of the experiments.

(ii) Incubation and reading method. On the day of the test, microtitration plates were thawed and each well was inoculated with 100 μl of the inoculum suspension. After agitation for 15 s, the microtitration plates were incubated for 72 h at 37°C. The growth in each well was quantified by using three methods: a spectrophotometric method after 48 and 72 h of incubation (referred to herein as SP48 and SP72, respectively), in which the optical density (OD) at 405 nm of each well was measured; a colorimetric method utilizing the dye 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) after 72 h of incubation (COL72), in which the conversion of the dye MTT to colored formazan derivative after 3 h of exposure by metabolic active fungi was measured at 550 nm; and a modification of the latter colorimetric method after 48 and 72 h of incubation (referred to herein as mCOL48 and mCOL72), in which the dye MTT was added to the inoculum and therefore fungi were exposed to the dye for 48 and 72 h as described previously (26). The percentage of growth in each well was calculated as the OD of each well/OD of the drug-free well after subtracting the background OD obtained from microorganism-free microtiter plates processes in the same manner as the inoculated plates. Thus, growth- and metabolism-inhibitory effects of the drugs alone and in combination were estimated based on the results of spectrophotometric and colorimetric methods, respectively.

Drug interaction modeling. In order to assess the nature of the in vitro interactions between the three azoles and terbinafine against each *S. prolificans* strain, the data obtained using the three reading methods, as described above, were analyzed using four different models. The models were parametric and nonparametric approaches of the following two no-interaction theories: the LA and the BI theories. In the LA-based models, concentrations of the drugs, alone or in combination, that produce the same effect, are compared, while in the BI-based models the estimates of the combined effect based on the effect of the individual drugs were compared with these obtained by the experiment.

(i) LA-based models. The LA theory is described by the following equation: $1 = d_A/D_A + d_B/D_B$, where d_A and d_B are the concentrations of the drugs A and B in the combination which elicit a certain effect and D_A and D_B are the iso-effective concentrations of the drugs A and B when acting alone. The nonparametric approach is based on the fractional inhibitory concentration (FIC) index model expressed as $\Sigma FIC = FIC_A + FIC_B = C_A^{comb}/MIC_A^{alone} + C_B^{comb}/MIC_B^{alone}$, where MIC_A^{alone} and MIC_B^{alone} are the MICs of the drugs A and B when acting alone and C_A^{comb} and C_B^{comb} are concentrations of the drugs A and B at the iso-effective combinations, respectively (22). Among all ΣFICs calculated for each data set, the FIC index was determined as the ΣFIC_{min} (the lowest ΣFIC) when the ΣFIC_{max} (the highest ΣFIC) was smaller than 4; otherwise, the FIC index was determined as the ΣFIC_{max} (22) (Fig. 1A, panel i). Two MIC endpoints were used for the evaluation of each data set. These were the MIC-1 and MIC-2 and were defined as the lowest drug concentration showing 25 and 50% of growth compared to the growth control, respectively. Based on these endpoints, two types of FIC indices were determined, FICi-1 and FICi-2, respectively. Off-scale MICs were converted to the next-highest or -lowest doubling concentration. Finally, the median and the range of FIC indices of the replicates were determined for both the MIC-1 and MIC-2. When the FIC indices in all four replicates were smaller than 1, significant synergy was claimed; when they were higher than 1, significant antagonism was claimed; and in all other cases additivity or indifference was concluded. In addition isobolograms were plotted in order to visualize the departure from additivity (Fig. 1A, panel ii).

The fully parametric concentration effect response surface approach described by Greco et al. (21) was used based on the following equation:

A. (i)

Terbinafine (mg/l) \ Voriconazole (mg/l)	16	8	4	2	1	0.5	0.25	0.12	0.06	0.03	0.015	0
64	0%	2%	3%	11%	10%	14%	10%	7%	8%	11%	15%	36%
32	3%	7%	8%	15%	21%	22%	21%	24%	24%	29%	27%	**43%**
16	0%	9%	9%	11%	17%	17%	23%	26%	29%	30%	41%	52%
8	3%	5%	13%	16%	18%	22%	29%	31%	31%	37%	49%	59%
4	0%	5%	9%	14%	21%	32%	36%	38%	37%	**40%**	51%	67%
2	0%	13%	22%	24%	35%	48%	54%	51%	51%	55%	60%	91%
1	3%	20%	24%	36%	49%	65%	74%	70%	62%	66%	72%	107%
0	5%	25%	**40%**	60%	68%	96%	111%	93%	100%	101%	103%	100%

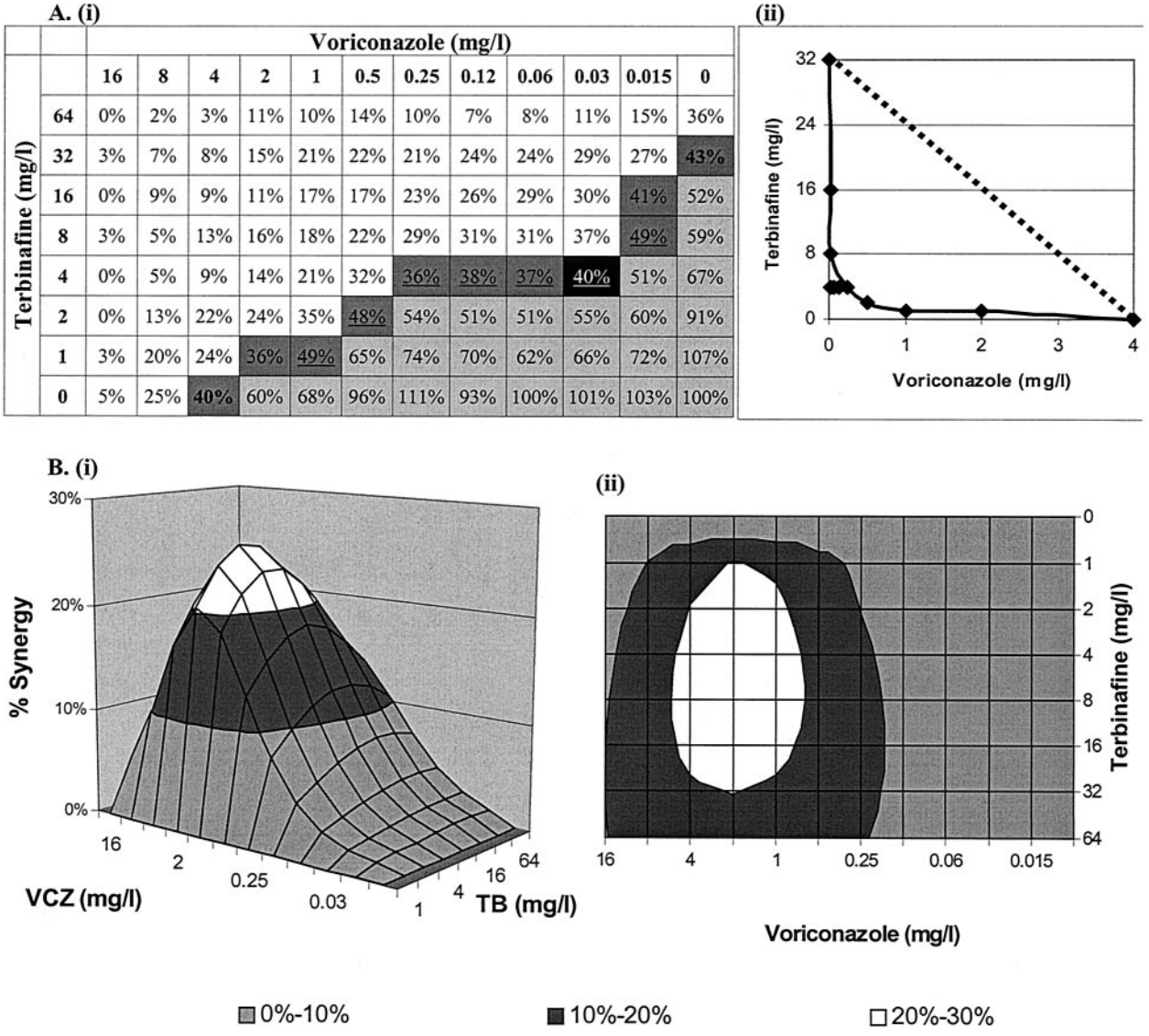

FIG. 1. Assessment of the in vitro interaction between voriconazole (VCZ) and terbinafine (TB) against a clinical *S. prolificans* strain (AZN87898) based on the LA-based models using the SP48 data. (Ai) Checkerboard showing the percentage of growth for each combination, combinations with more than 50% growth (light grey area), the MIC-2 of voriconazole and terbinafine (percentages in boldface type) as well as the iso-effective combinations based on which the ΣFIC-2 indices were calculated (dark grey area), combinations with ΣFIC-2 indices lower than 0.5 (underlined percentages) and the combination with the lowest ΣFIC-2 index (0.20), corresponding to the FICi-2 (percentage shown in black cell). (Aii) Corresponding isobologram with the additivity line (dashed line). (B) The 3-D (i) and contour (ii) plots of the percent synergy calculated with the Greco model with the following fitted parameters (means ± 95% CI): $IC_{50,TB} = 5.33 \pm 0.57$, $IC_{50,VCZ} = 2.63 \pm 0.31$, $m_{TB} = -0.51 \pm 0.04$, $m_{VCZ} = -1.1 \pm 0.11$, $\alpha = 13.9 \pm 1.6$.

$$1 = \frac{D_A}{IC_{50,A}\left(\frac{E}{E_{max}-E}\right)^{1/m_A}} + \frac{D_B}{IC_{50,B}\left(\frac{E}{E_{max}-E}\right)^{1/m_B}} + \alpha \frac{D_A D_B}{IC_{50,A} IC_{50,B}\left(\frac{E}{E_{max}-E}\right)^{0.5(1/m_A+1/m_B)}}$$

where E is the OD (dependent variable) at the drug concentrations D_A and D_B (independent variables); E_{max} is the maximal OD observed in the drug-free control; $IC_{50,A}$ and $IC_{50,B}$ are the 50% inhibitory concentrations (IC_{50}s)of drugs A and B, respectively, producing 50% of the E_{max}; m_A and m_B are the slopes of the concentration-effect curves (Hill coefficient) for the drugs A and B, respectively; and α is the interaction parameter which describes the nature of the interaction. This model was fitted directly to experimental data (the average OD among the replicates for all concentrations of the two drugs alone or in combination) with a nonweighted, nonlinear regression analysis using the MODLAB program (MEDIMATICS, Maastricht, The Netherlands). Goodness-of-fit criteria included the 95% confidence interval (CI) of the fitted parameters, the R^2, the sum of the squares, correlation and covariance matrices, and the residual plots.

When the parameter α was positive and its 95% CI did not include 0, statistically significant (SS) synergy was claimed, while when α was negative and its 95% CI did not include 0, SS antagonism was claimed. In any other case the LA was concluded. The additivity surface was simulated by fixing all parameters of the Greco model to values obtained after the model fitted to experimental data, except α, which was fixed at 0. The fitted experimental surface calculated by the Greco model was then subtracted from the additivity surface calculated as de-

scribed above. The percentage above (synergistic combinations) or below (antagonistic interactions) the additivity surface was plotted in a three-dimensional (3-D) graph, and a contour plot was constructed in order to determine the range of drug concentrations producing synergistic effects (Fig. 1B).

(ii) BI-based models. The BI theory is described by the equation $I_i = I_A + I_B - I_A \times I_B$ where I_i is the predicted percentage of inhibition of the theoretical noninteractive combination of the drugs A and B and I_A, I_B are the experimental percentages of inhibition of each drug acting alone, respectively. Since $I = 1 - E$, where E is the percentage of growth, by substituting into the former equation, the following equation is derived: $E_i = E_A \times E_B$, where E_i is the predicted percentage of growth of the theoretical noninteractive combination of the drugs A and B, respectively, and E_A and E_B are the experimental percentages of growth of each drug acting alone, respectively. Interaction is described by the difference (ΔE) between the predicted and measured percentages of growth with drugs at various concentrations. Because of the nature of interaction testing using microtiter plates with twofold dilution of either drug, this results in a ΔE for each drug combination. A 3-D plot with the ΔE depicted on the z axis results in a surface plot. In the nonparametric response surface approach described by Prichard et al. (36, 37), the E_A and E_B are obtained directly from the experimental data, while in the semiparametric concentration effect response surface approach (14, 20), these values are derived from fitting the E_{max} model to the concentration-effect curves of each drug alone. Thus, for the latter approach E_A and E_B are obtained by the following equation: $E_{A,B} = E_{max} \times (D/IC_{50})^m/[1 + (D/IC_{50})^m]$, where E, D, E_{max}, IC_{50}, and m are the same parameters for drug A and B as described above. The parameters of the model were obtained by a nonweighted nonlinear regression analysis using the GraphPad (San Diego, Calif.) Prism software. Data were normalized by using the percentages, and the maximum and the minimum of the E_{max} model, corresponding with 100 and 0%, respectively, were kept constant. The fit of the model was interpreted using the run test and the R^2 values. After the E_{max} model was fitted to the data, the parameters generated were used to calculate the no-interaction surface for each replicate separately.

For each combination of the two drugs in each of the four independent experiments, the observed percent growth obtained from the experimental data was subtracted from the predicted percentage, calculated as described above for each model. When the average difference was positive and its 95% CI among the four replicates did not include 0, SS synergy was claimed; when the difference was negative and its 95% CI did not include 0, SS antagonism was claimed. In any other case BI was concluded. The values thus obtained for each combination were used to construct a 3-D plot. Peaks above and below the 0 plane indicate synergistic and antagonistic combinations, respectively, while the 0 plane indicates the absence of SS interaction (Fig. 2). The contour plots were also constructed in order to visualize the drug concentrations producing an interaction.

Since the plot only shows the interactions for each separate combination of the concentrations, a value is needed to summarize the interaction surface. This was achieved by calculating the sum percentage of all SS synergistic (ΣSYN) and antagonistic (ΣANT) interactions. Interactions with <100% of SS interactions were considered weak, those with 100 to 200% of SS interactions were considered moderate, and those with >200% of SS interactions were considered strong, as was found previously (Meletiadis et al., submitted for publication). In addition the numbers of SS synergistic and antagonistic combinations among the 77 combinations tested was calculated for each strain.

RESULTS

Interaction between voriconazole and terbinafine. The in vitro antifungal effects of voriconazole and terbinafine were tested alone and in combination at concentrations of 0.015 to 16 mg/liter and 1 to 64 mg/liter, respectively. Using the SP72 data, 75% growth inhibition was achieved for all strains at concentrations of voriconazole of 4 to 16 mg/liter and concentrations of terbinafine of more than 64 mg/liter. When the two drugs were combined, the geometric mean (GM) of the MIC-1 decreased up to 27- and 20-fold, respectively, based on FIC-1 indices. The MIC-1 of voriconazole and terbinafine were then shifted down to 0.03 and 2 mg/liter, respectively (Table 1).

(i) LA-based models. (a) Nonparametric approach. The median FIC indices for each strain regarding the growth inhibitory effects (SP methods) ranged from <0.75 to 0.02, indicating synergy. This was significant in most of the cases (Table 2). However, the degree of synergy as well as the significance level was dependent on the MIC endpoint and incubation period used, with reading of the MIC-1 after 48 h of incubation showing stronger synergy. Based on the metabolism-inhibitory effects observed, strong synergy was found with the mCOL72 data. More-variable results were obtained with the COL method since FIC indices ranged from 0.02 to 1.00 and discrepancies between FICi-1 and FICi-2 were found.

(b) Parametric approach. The interaction parameters α of the fully parametric Greco model were positive, and the 95% CI did not overlap 0, indicating SS synergy in all cases (Table 2). The interaction parameter α ranged from 2.7 to 42 for all strains and methods except for one strain for which high α values were obtained.

(ii) BI-based models. When the interaction was determined based on BI, SS synergy ($P < 0.05$) was found using both nonparametric and semiparametric models. In most of the cases more than 200% and up to 1,582% of synergy was found, indicating strong synergy (Table 2). Overall lower levels of synergy were found with the COL72 data (median ΣSYN, <165%). The SP methods resulted in higher levels of synergy after 48 h of incubation (median, ΣSYN >195%), as did mCOL methods after 72 h of incubation (median ΣSYN, >268%). Antagonistic effects were not observed, although the SP72 and mCOL48 data indicated some weak antagonistic combinations.

Interaction between miconazole and terbinafine. The in vitro antifungal effects of miconazole and terbinafine were tested alone and in combination at concentrations of 0.06 to 64 mg/liter and 1 to 64 mg/liter, respectively. Using the SP72 data, 75% growth inhibition was achieved for all strains with miconazole at concentrations higher than 8 mg/liter and terbinafine at concentrations higher than 64 mg/liter. When the two drugs were combined, the GM of the MIC-1 decreased 54- to 90-fold for both drugs, respectively, based on FIC-1 indices. The MIC-1 of miconazole and terbinafine were then shifted down to 0.125 and 1 mg/liter, respectively (Table 1).

(i) LA-based models. (a) Nonparametric approach. The median FIC indices for each strain regarding both the growth- and metabolism-inhibitory effects were between 0.03 and 0.38, indicating synergy, which was significant in all cases (Table 3). The degree of synergy between 48 and 72 h was more consistent using the MIC-1 rather than the MIC-2 endpoint; for the latter endpoint weaker synergy was found after 48 h than after 72 h of incubation. This was even more pronounced with the mCOL method. The MIC-2 of the drugs in combination were lower than that of the lowest drug concentration according to the COL72 data. Thus, the calculation of the exact FIC indices for many replicates was impossible.

(b) Parametric approach. The interaction parameters α of the fully parametric Greco model were positive, and the 95% CI did not overlap 0, indicating SS synergy for each strain-method-incubation period, however, with different degrees of synergy (Table 3). The interaction parameter α ranged from 10 to 9.5×10^3, indicating very strong synergy, with higher values obtained with the SP72 and mCOL72 data.

(ii) BI-based models. SS synergy ($P < 0.05$) was also found based on the semiparametric and nonparametric approach of BI since synergy levels up to 1,751% were found (Table 3).

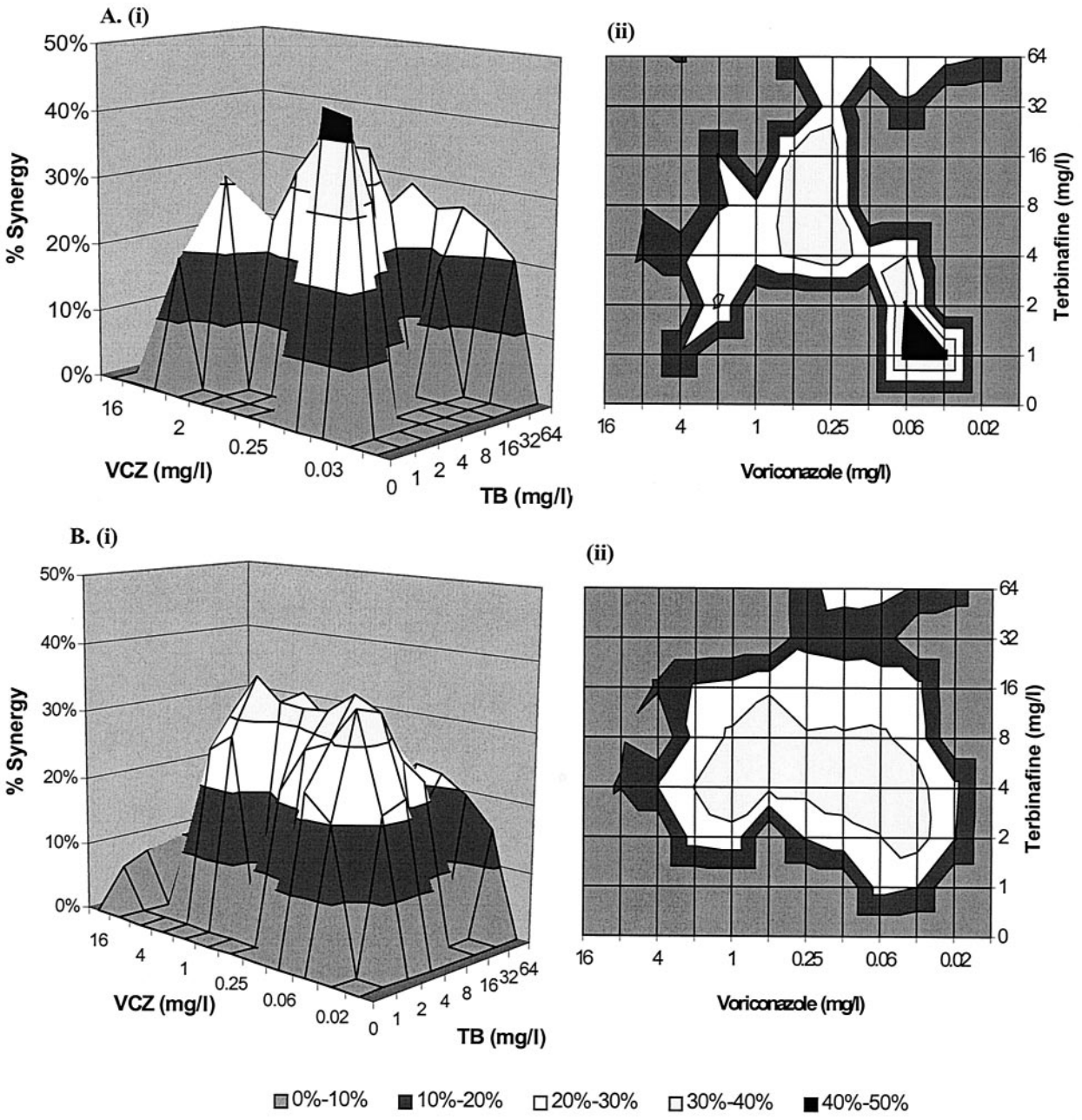

FIG. 2. Assessment of the in vitro interaction between voriconazole (VCZ) and terbinafine (TB) against a clinical *S. prolificans* strain (AZN7898) based on BI-based models using the SP48 data. (A) The 3-D (i) and contour (ii) plots of the percent synergy calculated with the nonparametric approach, which resulted in 700% synergy. (B) The 3-D (i) and contour (ii) plots of the percent synergy calculated with the semiparametric approach, which resulted in 1,118% synergy with the following fitted parameters (means ± 95% CI) of the E_{max} model: $IC_{50,TB} = 19.9 \pm 6.7$, $m_{TB} = -0.76 \pm 0.21$, $IC_{50,VCZ} = 2.8 \pm 0.7$, $m_{VCZ} = -1.3 \pm 0.37$.

Higher levels of synergy were found with the semiparametric approach than with the nonparametric approach based on the growth-inhibitory activities. Among the methods used, mCOL72 and mCOL48 data generated the highest levels of synergy (median ΣSYN, >526%), followed by the SP48 and SP72 data (median ΣSYN, <284%). Between the two incubation periods, stronger synergistic interactions were found after 48 h based on growth-inhibitory effects and after 72 h based on metabolism-inhibitory effects. By contrast the COL72 data resulted in weak SS synergistic interactions, with three strains in the semiparametric approach showing no SS synergistic interactions. Antagonistic effects were not observed, although some weak antagonistic combinations were observed, particularly with the semiparametric approach.

Interaction between itraconazole and terbinafine. The in vitro antifungal effects of itraconazole and terbinafine were tested alone and in combination at concentrations of 0.5 to 32 mg/liter and 0.06 to 64 mg/liter, respectively. Using the SP72

TABLE 1. Susceptibilities of *S. prolificans* strains against various azoles alone and in combination with terbinafine[a]

Combination and strain	GM MIC-1 (range)			
	Drug alone		Drugs in combination	
	Azole	TB	Azole	TB
VCZ and TB				
7898	9.51 (8–16)	>64 (>64)	0.71 (0.13–2)	9.51 (2–32)
7901	6.73 (4–8)	>64 (>64)	0.25 (0.03–1)	6.73 (4–16)
7902	6.73 (4–8)	>64 (>64)	0.5 (0.13–1)	8 (8)
7906	8 (8)	>64 (>64)	1 (0.5–2)	9.51 (8–16)
7918	9.51 (8–16)	>64 (>64)	1.19 (0.5–2)	11.31 (2–32)
MCZ and TB				
7898	64 (32–>64)	>64 (>64)	1.19 (0.25–4)	2.83 (2–4)
7901	>64 (>64)	>64 (>64)	2.83 (2–4)	2 (1–4)
7902	45.25 (8–>64)	>64 (>64)	2 (0.13–8)	2 (2)
7906	>64 (>64)	>64 (>64)	2.83 (2–4)	1.41 (1–4)
7918	26.91 (8–>64)	>64 (>64)	0.84 (0.13–2)	2.38 (2–4)
ICZ and TB				
7898	>32 (>32)	>64 (>64)	1 (0.5–2)	16 (16)
7901	>32 (>32)	>64 (64–>64)	10.01 (4–16)	12.7 (8–16)
7902	>32 (>32)	>64 (64–>64)	4 (0.5–16)	8 (8)
7906	>32 (>32)	>64 (>64)	3.17 (1–16)	8 (2–16)
7918	>32 (>32)	>64 (>64)	4 (1–16)	10.08 (2–16)

[a] Based on the MIC-1 and SP72 data. Abbreviations: VCZ, voriconazole; MCZ, miconazole; ICZ, itraconazole; TB, terbinafine.

data, 75% growth inhibition was not observed for all strains at high concentrations of itraconazole and terbinafine up to 32 and 64 mg/liter, respectively, based on the results of SP72. When the two drugs were combined, the GM of the MIC-1 decreased up to 64-fold for itraconazole and 16-fold for terbinafine based on FIC-1 indices. The MIC-1 of itraconazole and terbinafine were then shifted down to 0.5 and 2 mg/liter, respectively (Table 1).

(i) LA-based models. (a) Nonparametric approach. The median FIC indices for all strains regarding the growth- and metabolism-inhibitory effects ranged from 0.04 to 0.75, indicating synergy. This was significant in all cases with mCOL method, but not with SP and COL methods since FIC indices up to 1.02 were found for some strains (Table 4). The highest degree of synergy was found after 72 h reading the MIC-1. The values of the FIC indices represented the minimal values since the off-scale MICs of both itraconazole and terbinafine alone did not allow the calculation of exact values.

(b) Parametric approach. The α interaction parameters of the fully parametric Greco model were positive, and the 95% CI did not overlap 0, indicating SS synergy for all strain-method-incubation period combinations (Table 4). The interaction parameter α ranged from 3.3 to 7.6 $\times$ 10^3, indicating very strong synergy. High α values were obtained with the mCOL72 data. In some cases none of the tested concentrations of itraconazole resulted in 50% of growth. In these cases the Greco model resulted in IC_{50}s outside the range of concentrations tested, and this affected the fit. Therefore, the IC_{50} of itraconazole was fixed to the next-highest doubling concentration.

(ii) BI-based models. SS interactions ($P < 0.05$) were also found with the two approaches based on BI. High levels of synergy were found with mCOL method (ΣSYN up to 1,354%) followed by the SP data (ΣSYN up to 536%). After 72 h of incubation the levels of synergy were higher than after 48 h. Strong SS antagonistic interactions (ΣANT up to -404%) were found with the COL method, particularly with the nonparametric approach. The semiparametric approach resulted in a higher percentage of synergy than the nonparametric approach. SS antagonistic interactions, which were strong in some cases (>200%), were found, particularly with the parametric approach after 72 h of incubation.

DISCUSSION

Strong synergistic interactions were found in vitro between terbinafine and three azoles—namely, voriconazole, miconazole, and itraconazole—against five clinical *S. prolificans* isolates based on antifungal growth- and metabolism-inhibitory effects after 48 and 72 h of incubation. These results were confirmed by parametric and nonparametric approaches of the LA and the BI no-interaction theories, respectively.

This confirms previous studies in which terbinafine was found to interact synergistically with itraconazole for 90% of *S. prolificans* isolates (32) and 43% of *Candida albicans* isolates (3), with voriconazole against *Aspergillus* species (41), and with fluconazole for 40% of *C. albicans* isolates (3) based on the FIC index model. However, the results obtained with the FIC index model are dependent on the MIC endpoints (Meletiadis et al., submitted) used particularly for filamentous fungi, and these are not clearly established. In this study the results based on both MIC endpoints showed significant synergy, with MIC-1 resulting in levels of synergy that were higher and more consistent between the two incubation periods compared to those of MIC-2. The off-scale MIC-1, however, precluded the precise estimation of synergy at this level of growth inhibition. Despite the fact that experiments were repeated using higher drugs concentrations and agar dilution tests, a precise determination of the MIC-1 was not possible due to solubility and

TABLE 2. In vitro interaction between voriconazole and terbinafine against *S. prolificans*

Method and strain	Result according to theory						
	LA			BI[a]			
	Nonparametric [median (range)]		Parametric[b] ($\alpha \pm 95\%$ CI)	Nonparametric		Semiparametric[c]	
	FICi-2	FICi-1		ΣSYN (*n*)	ΣANT (*n*)	ΣSYN (*n*)	ΣANT (*n*)
SP48							
7898	0.13 (0.07–0.19)	0.10 (0.06–0.28)	13.9 ± 1.6	700 (29)	0 (0)	1,118 (51)	0 (0)
7901	0.25 (0.08–0.75)	0.10 (0.05–0.13)	10.8 ± 1.8	428 (29)	0 (0)	742 (48)	0 (0)
7906	0.26 (0.13–0.38)	0.13 (0.06–0.25)	16.3 ± 4.5	457 (26)	0 (0)	566 (36)	0 (0)
7918	0.09 (0.04–0.13)	0.09 (0.04–0.09)	2,479.8 ± 289.1	409 (21)	0 (0)	702 (39)	0 (0)
7902	0.14 (0.05–0.27)	0.09 (0.06–0.13)	42.2 ± 7.3	709 (44)	0 (0)	987 (53)	0 (0)
Median	0.14	0.10	16.30	457	0	742	0
SP72							
7898	0.07 (0.05–0.31)	0.14 (0.13–0.75)	13.0 ± 3.4	1,582 (49)	0 (0)	942 (42)	0 (0)
7901	0.32 (0.04–0.75)	0.13 (0.07–0.25)	32.4 ± 6.0	195 (14)	−3 (1)	702 (43)	0 (0)
7906	0.38 (0.13–0.50)	0.28 (0.19–0.38)	8.4 ± 3.3	57 (8)	0 (0)	67 (4)	0 (0)
7918	0.08 (0.02–0.28)	0.27 (0.16–0.50)	1,517.5 ± 334.8	770 (29)	−9 (2)	520 (21)	−12 (1)
7902	0.29 (0.13–0.31)	0.19 (0.08–0.25)	6.6 ± 2.1	185 (15)	0 (0)	579 (41)	−10 (2)
Median	0.29	0.19	14.04	195	0	579	0
COL72							
7898	0.25 (0.02–0.63)	0.13 (0.04–0.16)	8.9 ± 4.0	165 (10)	0 (0)	442 (20)	0 (0)
7901	0.75 (0.50–1.00)	0.16 (0.13–0.16)	29.9 ± 16.8	0 (0)	0 (0)	23 (4)	0 (0)
7906	0.53 (0.07–0.75)	0.17 (0.07–0.19)	8.0 ± 3.0	34 (4)	0 (0)	72 (7)	0 (0)
7918	0.13 (0.08–0.25)	0.20 (0.04–0.31)	40.7 ± 16.5	254 (8)	0 (0)	49 (3)	0 (0)
7902	0.16 (0.08–0.50)	0.11 (0.04–0.19)	42.0 ± 12.2	619 (22)	0 (0)	903 (32)	0 (0)
Median	0.25	0.16	29.90	165	0	72	0
mCOL48							
7898	0.25 (0.25–0.26)	0.25 (0.25–0.50)	1,754.9 ± 947.1	299 (15)	−4 (1)	373 (19)	−5 (2)
7901	0.25 (0.25)	0.25 (0.19–0.25)	33.7 ± 11.5	413 (25)	−5 (2)	564 (34)	0 (0)
7906	0.25 (0.25)	0.27 (0.25–0.38)	2.7 ± 1.5	532 (35)	0 (0)	753 (40)	0 (0)
7918	0.25 (0.25–0.26)	0.13 (0.13–0.25)	9.9 ± 4.1	214 (10)	0 (0)	75 (5)	0 (0)
7902	0.26 (0.25–0.50)	0.27 (0.13–0.38)	8.2 ± 2.5	72 (5)	0 (0)	236 (11)	0 (0)
Median	0.25	0.25	9.9	299	0	373	0 (0)
mCOL72							
7898	0.13 (0.08–0.13)	0.07 (0.06–0.07)	10.3 ± 7.6	279 (6)	0 (0)	56 (3)	0 (0)
7901	0.25 (0.09–0.38)	0.13 (0.07–0.13)	5.0 ± 2.6	795 (16)	0 (0)	379 (8)	0 (0)
7906	0.31 (0.13–0.31)	0.13 (0.06–0.13)	3.9 ± 2.4	566 (180)	0 (0)	1,072 (26)	0 (0)
7918	0.13 (0.07–0.13)	0.14 (0.07–0.14)	4.7 ± 2.9	579 (11)	0 (0)	513 (11)	0 (0)
7902	0.13 (0.07–0.25)	0.09 (0.06–0.13)	14.8 ± 5.0	464 (9)	0 (0)	468 (10)	0 (0)
Median	0.13	0.13	5.0	566	0	468	0

[a] The median absolute coefficient of variation of the differences between observed and predicted percent growth at each drug combination among the replicates of all strains ranged from 41 to 232% (median, 57%) for the SP method, 68 to 166% (median, 123%) for the COL method, and 44 to 83% (median, 53%) for the mCOL method with the semiparametric approach and from 49 to 165% (median, 88%) for the SP method, 86 to 180% (median, 105%) for the COL method, and 45 to 64% (median 51%) for the mCOL method with the nonparametric approach.

[b] The R^2 from the nonlinear regression analysis for the fully parametric Greco model ranged from 0.83 to 0.96 (median, 0.90). The IC_{50}s ranged from 0.96 to 2342 mg/liter (geometric mean, 5.9 mg/liter) of terbinafine and from 1.2 to 25 mg/liter (geometric mean, 4.2 mg/liter) of voriconazole. The slope (*m*) ranged from −0.16 to −1.46 (median, −0.61) for terbinafine and from −0.45 to −1.79 (median, −0.94) for voriconazole.

[c] The R^2 from the nonlinear regression analysis for the E_{max} model of the semiparametric approach ranged from 0.74 to 0.99 (median, 0.90). The IC_{50}s ranged from 1.6 to 67.4 mg/liter (geometric mean, 10.6 mg/liter) of terbinafine and from 0.24 to 16.7 mg/liter (geometric mean, 2.9 mg/liter) of voriconazole. The slopes m ranged from −0.44 to −4.38 (median, −0.92) for terbinafine and from −0.13 to −5.2 (median, −1.2) for voriconazole.

quantification problems, respectively. Nevertheless, by using the next-highest doubling concentration for the MIC-1 of the single drugs, the only effect on the results was the underestimation of the degree of synergy, resulting in relatively higher FIC indices than the actual indices, and consequently the synergy based on MIC-1 would have been even stronger than that based on MIC-2.

The interpretation of the FIC index in concluding synergy

TABLE 3. In vitro interaction between miconazole and terbinafine against *S. prolificans*

Method and strain	Result according to theory						
	LA			BI[a]			
	Nonparametric [median (range)]		Parametric[b] (α ± 95% CI)	Nonparametric		Semiparametric[c]	
	FICi-2	FICi-1		ΣSYN (*n*)	ΣANT (*n*)	ΣSYN (*n*)	ΣANT (n)
SP48							
7898	0.13 (0.02–0.28)	0.04 (0.01–0.05)	302.1 ± 32.6	173 (15)	0 (0)	242 (17)	0 (0)
7901	0.11 (0.02–0.14)	0.05 (0.02–0.06)	3,072.0 ± 441.7	243 (15)	0 (0)	396 (22)	0 (0)
7902	0.17 (0.14–0.28)	0.08 (0.01–0.28)	111.1 ± 17.5	163 (20)	0 (0)	206 (22)	−4 (1)
7906	0.17 (0.09–0.28)	0.04 (0.01–0.13)	565.0 ± 110.7	160 (11)	0 (0)	284 (18)	0 (0)
7918	0.06 (0.03–0.15)	0.03 (0.02–0.08)	9,462.1 ± 1,194.5	460 (33)	0 (0)	615 (40)	0 (0)
Median	0.13	0.04	565.0	178	0	284	0
SP72							
7898	0.06 (0.02–0.09)	0.05 (0.03–0.08)	552.1 ± 77.6	57 (5)	0 (0)	135 (13)	0 (0)
7901	0.05 (0.02–0.08)	0.05 (0.03–0.06)	5,516.6 ± 966.9	146 (10)	0 (0)	365 (21)	0 (0)
7902	0.06 (0.03–0.28)	0.06 (0.03–0.14)	1,374.9 ± 242.9	10 (2)	0 (0)	78 (8)	−6 (1)
7906	0.09 (0.03–0.13)	0.05 (0.02–0.07)	434.5 ± 124.9	67 (4)	0 (0)	138 (11)	0 (0)
7918	0.06 (0.03–0.16)	0.05 (0.03–0.27)	5,464.7 ± 1,082.9	392 (27)	0 (0)	505 (33)	0 (0)
Median	0.06	0.05	1,374.9	67	0	138	0
COL72							
7898	0.06 (0.03–0.09)	0.04 (0.02–0.06)	817.5 ± 297.5	7 (1)	0 (0)	14 (1)	0 (0)
7901	0.19 (0.06–0.28)	0.03 (0.02–0.04)	180.1 ± 50.3	17 (3)	0 (0)	92 (11)	−25 (2)
7902	0.05 (0.02–0.08)	0.05 (0.02–0.08)	128.2 ± 28.4	8 (1)	0 (0)	0 (0)	0 (0)
7906	0.03	0.05 (0.03–0.16)	55.5 ± 23.8	0 (0)	0 (0)	0 (0)	0 (0)
7918	0.08 (0.03–0.19)	0.04 (0.02–0.09)	633.2 ± 162.2	57 (3)	0 (0)	0 (0)	0 (0)
Median	0.06	0.04	180.1	8	0	0	0
MCOL48							
7898	0.27 (0.27–0.28)	0.05 (0.04–0.13)	9.8 ± 2.2	556 (49)	0 (0)	567 (43)	−6 (1)
7901	0.16 (0.09–0.31)	0.05 (0.04–0.05)	44.3 ± 11.4	955 (55)	0 (0)	941 (49)	0 (0)
7902	0.27 (0.27–0.50)	0.04 (0.03–0.05)	34.1 ± 8.2	354 (41)	0 (0)	392 (35)	0 (0)
7906	0.38 (0.31–0.51)	0.04 (0.04–0.05)	18.5 ± 5.0	539 (35)	0 (0)	526 (37)	0 (0)
7918	0.27 (0.13–0.28)	0.31 (0.05–0.31)	3,807.5 ± 1,479.4	259 (32)	0 (0)	372 (33)	0 (0)
Median	0.27	0.05	34.1	539	0	526	0
MCOL72							
7898	0.03 (0.03–0.04)	0.06 (0.06–0.13)	9,505.2 ± 2,824.2	487 (30)	0 (0)	518 (28)	−9 (1)
7901	0.03 (0.02–0.04)	0.09 (0.06–0.09)	6,768.1 ± 2,078.9	1,051 (37)	0 (0)	1,006 (36)	0 (1)
7902	0.03 (0.02–0.03)	0.06 (0.06–0.13)	7,740.3 ± 1,887.3	1,751 (61)	0 (0)	1,295 (46)	0 (0)
7906	0.03 (0.02–0.04)	0.13 (0.06–0.13)	7,872.5 ± 3,421.3	606 (39)	0 (0)	517 (38)	0 (0)
7918	0.06 (0.03–0.09)	0.08 (0.06–0.08)	534.6 ± 141.8	595 (45)	0 (0)	538 (34)	0 (0)
Median	0.03	0.08	7,740.3	606	0	538	0

[a] The median absolute coefficient of variation of the differences between observed and predicted percent growth at each drug combination among the replicates of all strains ranged from 60 to 124% (median, 95%) for the SP method, 142 to 227% (median, 173%) for the COL method, and 24 to 49% (median, 40%) for the mCOL method with the semiparametric approach and from 69 to 191% (median, 123%) for the SP method, 118 to 343% (median, 236%) for the COL method, and 23 to 56% (median, 36%) for the mCOL method with the nonparametric approach.

[b] The R^2 from the nonlinear regression analysis for the fully parametric Greco model ranged from 0.76 to 0.93 (median, 0.88). The IC_{50}s ranged from 0.23×10^3 to -7.2×10^3 mg/liter (geometric mean, 22.1 mg/liter) of terbinafine and from 0.53 to 32.9 mg/liter (geometric mean, 4.2 mg/liter) of miconazole. Relatively high IC_{50}s of terbinafine were obtained after 72 h of incubation. The slope (m) ranged from −0.1 to −0.53 (median, −0.16) for terbinafine and from −0.27 to −4.4 (median, −0.44) for miconazole.

[c] The R^2 from the nonlinear regression analysis for the E_{max} model of the semiparametric approach ranged from 0.73 to 0.99 (median, 0.88). The IC_{50}s ranged from 0.57 to 1,028 mg/liter (geometric mean, 11.4 mg/liter) of terbinafine and from 0.19 to 43.74 mg/liter (geometric mean, 4.43 mg/liter) of miconazole. The slope (m) ranged from −0.10 to −0.86 (median, −0.35) for terbinafine and from −0.24 to −1.63 (median, −0.45) for miconazole.

or antagonism is a problem in itself. Since the determination of the MIC is sensitive to dilution errors, and twofold dilutions are used in determining MICs, the MIC is usually taken to be accurate to within 1 dilution. Since the FIC indices are determined from two antifungals, the value of the FIC index may also differ between experiments. In the interpretation of a single FIC index, therefore, a value of >4 is usually considered to indicate antagonism, and a value of ≤0.5 is usually consid-

TABLE 4. In vitro interaction between itraconazole and terbinafine against *S. prolificans*

Method and strain	Result according to theory						
	LA			BI[a]			
	Nonparametric [median (range)]		Parametric[b] (α ± 95% CI)	Nonparametric		Semiparametric[c]	
	FICi-2	FICi-1		ΣSYN (*n*)	ΣANT (*n*)	ΣSYN (*n*)	ΣANT (*n*)
SP48							
7898	0.13 (0.07–0.16)	0.19 (0.13–0.19)	4.3 ± 1.3	19 (2)	−20 (1)	244 (18)	−45 (3)
7901	0.52 (0.51–0.52)	0.13 (0.13–0.19)	9.3 ± 1.8	36 (3)	−25 (2)	90 (15)	−27 (2)
7902	0.07 (0.02–0.56)	0.13 (0.13–0.25)	203.8 ± 49.1	49 (4)	0 (0)	144 (8)	0 (0)
7906	0.16 (0.04–0.51)	0.14 (0.13–0.19)	3.3 ± 0.8	230 (14)	0 (0)	291 (16)	0 (0)
7918	0.51 (0.07–0.51)	0.13 (0.09–0.38)	25.1 ± 9.6	52 (5)	0 (0)	167 (11)	0 (0)
Median	0.16	0.13	9.3	49	0	167	0
SP72							
7898	0.13 (0.07–0.56)	0.14 (0.13–0.16)	25.9 ± 8.2	76 (6)	−31 (2)	202 (16)	−178 (11)
7901	0.75 (0.52–1.00)	0.31 (0.31–0.38)	57.2 ± 14.7	10 (1)	−85 (4)	7 (1)	−98 (4)
7902	0.02 (0.02–0.28)	0.19 (0.07–0.38)	182.9 ± 48.1	41 (4)	0 (0)	90 (7)	0 (0)
7906	0.13 (0.06–0.38)	0.16 (0.14–0.27)	8.1 ± 2.5	536 (26)	0 (0)	428 (23)	0 (0)
7918	0.51 (0.06–0.52)	0.14 (0.09–0.38)	457.6 ± 148.5	81 (8)	0 (0)	180 (17)	0 (0)
Median	0.13	0.16	57.2	76	0	180	0
COL72							
7898	0.07 (0.02–0.26)	0.16 (0.09–0.16)	202.9 ± 130.7	0 (0)	−46 (40)	0 (0)	−109 (8)
7901	0.13 (0.01–0.16)	0.26 (0.25–0.27)	5.2 ± 3.3	7 (1)	−83 (8)	0 (0)	0 (0)
7902	0.13 (0.01–1.02)	0.19 (0.14–0.25)	29.1 ± 20.5	10 (1)	−53 (4)	179 (11)	−3 (1)
7906	0.04 (0.02–0.13)	0.13 (0.09–0.25)	23.7 ± 13.7	6 (1)	−209 (26)	16 (3)	−404 (13)
7918	0.16 (0.08–0.26)	0.16 (0.13–0.16)	69.9 ± 59.7	380 (12)	0 (0)	384 (15)	−4 (1)
Median	0.13	0.16	29.1	7	−53	16	−4
MCOL48							
7898	0.34 (0.19–0.50)	0.27 (0.27–0.28)	105.4 ± 39.1	25 (5)	−1 (1)	69 (8)	0 (0)
7901	0.14 (0.13–0.15)	0.20 (0.14–0.26)	802.1 ± 310.8	276 (22)	0 (0)	417 (20)	0 (0)
7902	0.27 (0.25–0.28)	0.20 (0.16–0.25)	23.9 ± 9.4	105 (12)	0 (0)	209 (20)	−0 (0)
7906	0.20 (0.16–0.25)	0.23 (0.19–0.27)	81.6 ± 28.0	31 (4)	0 (0)	88 (6)	0 (0)
7918	0.11 (0.06–0.16)	0.08 (0.02–0.14)	795.3 ± 435.9	79 (5)	0 (0)	49 (2)	0 (0)
Median	0.20	0.20	105.4	79	0	88	0
MCOL72							
7898	0.21 (0.14–0.28)	0.18 (0.04–0.31)	959.7 ± 542.4	146 (5)	0 (0)	529 (18)	0 (0)
7901	0.04 (0.02–0.07)	0.15 (0.05–0.25)	5,091.5 ± 3,287.1	942 (31)	0 (0)	372 (15)	0 (0)
7902	0.30 (0.28–0.31)	0.05 (0.04–0.06)	404.7 ± 242.3	533 (22)	0 (0)	616 (23)	−5 (10)
7906	0.28 (0.25–0.31)	0.06 (0.05–0.07)	2,351.3 ± 1,445.1	83 (5)	0 (0)	300 (19)	0 (0)
7918	0.04 (0.03–0.05)	0.07 (0.05–0.09)	7,673.7 ± 389.4	263 (10)	0 (0)	1,354 (44)	0 (0)
Median	0.21	0.07	959.7	263	0	529	−16

[a] The median absolute coefficient of variation of the differences between observed and predicted percent growth at each drug combination among replicates of all strains ranged from 58 to 157% (median, 106%) for the SP method, 59 to 222% (median, 106%) for the COL method, and 51 to 69% (median, 61%) for mCOL method with the semiparametric approach and from 57 to 287% (median, 154%) for the SP method, 51 to 195% (median, 115%) for the COL method, and 58 to 89% (median, 71%) for the mCOL method with the non-parametric approach.

[b] The R^2 from the nonlinear regression analysis for the fully parametric Greco model ranged from 0.69 to 0.95 (median, 0.88). The IC_{50}s ranged from 0.15 to 93.6 mg/liter (geometric mean, 2.98 mg/liter) of terbinafine and 17.5×10^3 to 7×10^3 mg/liter (geometric mean, 404.4 mg/liter) of itraconazole. The slope (m) ranged from −0.15 to −1.7 (median, −0.68) for terbinafine and −0.16 to −26.7 (median, −0.28) for itraconazole.

[c] The R^2 from the nonlinear regression analysis for the E_{max} model of the semiparametric approach ranged from 0.77 to 0.99 (median, 0.95). The IC_{50}s ranged from 0.23 to 155 mg/liter of terbinafine (geometric mean, 2.85 mg/liter) and from 5.2 to 5011 mg/liter of itraconazole (geometric mean, 298.64 mg/liter). The slope (m) ranged from −0.11 to −1.22 (median, −0.52) for terbinafine and −0.06 to −0.99 (median, −0.43) for itraconazole.

ered to indicate synergy (22). This is one of the approaches we also took in interpreting the results. However, because we performed replicate experiments (four times) for each strain combination studied, we were also able to interpret the results of the FIC index model for all replicates as one outcome (synergy, antagonism, or additivity), thereby constraining the interexperimental error. Thus, when the results of all four replicates were concordant, synergy or antagonism was claimed

if FIC indices were below 1 or above 1, respectively. In all other cases additivity was concluded. Another alternative approach would have been a more statistical approach, using the mean of the four replicates and the 95% CI; if the 95% CI does not include 1 synergism or antagonism could be concluded. However, because the distribution of the FICs is not normal, we preferred the approach described above.

Assessing the nature of drug interactions using the FIC index model presents several other problems besides the choice of the FIC indices and MIC endpoints, such as the lack of a good summary and statistical interpretation of the results and the imprecise approximation of the real FIC index when off-scale MICs are present. Therefore, the data were analyzed by a fully parametric approach described by Greco et al. (20). This model is based on the assumption that the concentration-effect curves follow the E_{max} model (sigmoid curve with variable slopes); includes statistical significance levels of the interactions; and summarizes them with a nonunit, concentration-independent interaction parameter. The model fitted to the data well since narrow 95% CIs for all parameters and a low sum of the squares were obtained and no SS deviation from the data was found when the residuals were analyzed (data not shown). SS synergy was found in all cases. The model resulted in high interaction parameters, particularly when the IC_{50}s of the individual drugs were off-scale. This indicates very strong synergy, since a drug with poor activity (very high IC_{50}) acquires activity when it is combined. However, to obtain a reasonable fit, the IC_{50}s had to be fixed to certain values in some cases. The value of the interaction parameter for these cases might be uncertain, because the extrapolation of the E_{max} model outside the range of effects caused by a drug alone (e.g., itraconazole, for which <50% inhibition was not observed at any concentration of the drug alone) is very dangerous. The values of interaction parameters found should then be regarded with extreme caution, since the E_{max} model may not describe the concentration effect curve of itraconazole appropriately in the whole range of effects. However, results of previous studies have shown that the E_{max} model fitted well to concentration-effect curves of itraconazole against other filamentous fungi (i.e., *Aspergillus* species) (29). In addition, the Greco model fitted to the entire data set (21) where effects of 0% up to 100% of inhibition were observed. Regardless, problems of fitting may arise when none of the tested combinations reduces the growth to 50%. Finally, results obtained with the Greco model, as for most of the regression analysis models, are dependent on various factors associated with the fitting procedure, such as the initial parameters, the algorithms used for calculation the sum of the squares, variance models, and weighting methods. In a preliminary analysis, we used different weighting and optimization methods, and we obtained the lowest sum of the squares with nonweighted analysis; therefore, the latter analysis was used. However, when data were analyzed with both weighted and nonweighted methods, similar α interaction parameters were obtained.

The fully parametric Greco model does not require replicates to obtain statistical measures (although the mean with the standard error of replicates can be included as a weight factor) and is less sensitive to intraexperimental errors than the FIC index model. The fully parametric Greco model can be applied in frugal experiments using the D-optimal design theory and then can be easily used in order to convert concentration-time triplets to time-effect relationships (13, 25).

From the mechanistic point of view, the synergistic interaction between allylamines and azoles can be explained in a similar manner as the synergistic interaction between the antibacterials trimethoprim and the sulfonamides, which are acting at different steps of the same pathway. Allylamines inhibit the squalene epoxidase, a non-cytochrome P-450 enzyme in the pathway of ergosterol biosynthesis, whereas azoles act at a more distal point in the same pathway by inhibiting the 14a-demethylase of lanosterol (16, 40, 48). Among the three azoles, miconazole showed the strongest synergy, with FIC indices as low as 0.03 using the MIC-1. The strong synergy of miconazole could be associated with its multiple mechanisms of action, which probably involve alteration of fungal cell wall, alteration of the RNA and DNA metabolism, and intracellular accumulation of peroxides (4, 12), thereby increasing the sites of potential interactions. Although sequential blocking of a biochemical pathway might often result in synergy (17, 44), the appropriateness of using the LA theory as the no-interaction theory for analyzing interactions of drugs with dissimilar actions was questioned first by Bliss (8). Furthermore, it was found that BI of the effects of the two drugs acting in such ways can be achieved (46, 47) even when the concentration-effect curves of the two drugs followed the E_{max} model (23) despite Berenbaum's critical arguments (6).

Even when BI was used in the no-interaction theory to analyze the interaction between the three azoles and terbinafine, strong SS synergy was found in most cases. A single integrated numerical measure that clearly summarizes the behavior of the entire interaction surface is not available, and replicates are required in order to obtain SS results. Combinations with different interactions can be obtained, and a full checkerboard design is required. Between the semiparametric and the nonparametric approach, higher levels of synergy were found with the former, which was also less variable between the two incubation periods. Although both models are sensitive to interexperimental variations, the semiparametric approach might reduce the intraexperimental errors, particularly in single drug-containing wells, but not in the entire response surface like the fully parametric model did.

Among the three azoles, the strongest synergy was produced with miconazole, followed by itraconazole and voriconazole, according to LA-based models and mainly the Greco model, since the calculation of the exact FIC indices for the interaction of itraconazole and terbinafine was not possible due to off-scale MICs of the former drug. According to BI-based models, voriconazole produced the strongest synergy, followed by miconazole and itraconazole. This discrepancy, which might reflect different theoretical mechanisms, is caused by the different isobols obtained from the two models. As it was presented in Fig. 12 of the review by Greco et al. (20), the isobols based on BI are dependent on the magnitude of the slopes of the individual concentration-effect curves. The smaller the slopes are, the weaker the Bliss synergistic interactions are. Therefore, voriconazole, with the largest absolute median slope, resulted in the strongest synergy, and itraconazole, with the smallest absolute median slope, resulted in the weakest synergy.

Using spectrophotometric and colorimetric methods, the

three azoles interacted synergistically with terbinafine not only by restricting the fungal biomass but also by reducing the metabolic activity of the fungi. However, lower levels of synergy were found with the standard colorimetric method compared to the spectrophotometric method and to the modified colorimetric method. This difference was more pronounced when the combinations were analyzed with BI-based models. Fewer and weaker SS synergistic interactions were found with the standard colorimetric method (fungi are exposed for 3 h to the dye MTT after 72 h incubation with the drug) despite the fact that the spectrophotometric method after 72 h resulted in strong SS synergistic interactions for all the strains. This was caused mainly by the large variation between the replicates obtained with the standard colorimetric method, resulting in fewer SS interactions. Interestingly, the modified colorimetric method showed the lowest levels of interexperimental variability, thus increasing its potential for application in the in vitro combination studies of antifungal drugs.

The dynamic aspect of synergy between azoles and terbinafine was assessed by using measurements after 48 and 72 h of incubation. The BI-based models resulted in higher levels of synergy after 48 h than after 72 h based on the spectrophotometric methods, while the opposite was observed with the modified colorimetric methods, particularly for the miconazole-terbinafine and voriconazole-terbinafine interactions. This could be associated with the relatively higher interexperimetal variation observed after 72 h for the spectrophotometric methods and after 48 h for the modified colorimetric methods between the two incubation periods. The LA-based models resulted in higher levels of synergy after 72 h than after 48 h, particularly for the miconazole-terbinafine and itraconazole-terbinafine interactions. This could be explained with the growth curves of *S. prolificans* recently published (28), in which after 72 h the growth control inserts the second transition period while the drug-containing wells might be in the log phase, which exhibits the highest growth rate. Therefore, at that time the synergistic effects are more pronounced and larger differences in growth between the single-drug- and the drug-combination-containing wells could be observed.

The synergistic interaction between these three azoles and terbinafine might have a great clinical impact on the control of infections caused by *S. prolificans*. Peak levels in human serum have been reported for terbinafine at concentrations up to 3.6 mg/liter (2), for voriconazole up to 2.5 mg/liter (http://www.aspergillus.man.ac.uk), for miconazole up to 21.9 mg/liter (12), and for itraconazole up to 1 mg/liter (1). These levels are within the range of concentrations of these drugs that produced synergistic effects when they were combined in vitro. Furthermore, successful outcome was reported in humans with invasive infections treated with itraconazole and terbinafine, which might be due to in vivo synergy between these two drugs (42, 45). Therefore, the combination therapy of an azole with terbinafine could be beneficial for the management of *S. prolificans* infections.

REFERENCES

1. **Bailey, E. M., D. J. Krakovsky, and M. J. Rybak.** 1990. The triazole antifungal agents: a review of itraconazole and fluconazole. Pharmacotherapy **10:**146–153.
2. **Balfour, J. A., and D. Faulds.** 1992. Terbinafine. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in superficial mycoses. Drugs **43:**259–284.
3. **Barchiesi, F., L. F. Di Francesco, P. Compagnucci, D. Arzeni, A. Giacometti, and G. Scalise.** 1998. In-vitro interaction of terbinafine with amphotericin B, fluconazole and itraconazole against clinical isolates of *Candida albicans*. J. Antimicrob. Chemother. **41:**59–65.
4. **Barriere, S. L.** 1990. Pharmacology and pharmacokinetics of traditional systemic antifungal agents. Pharmacotherapy **10:**134S-140S.
5. **Berenbaum, M. C.** 1985. The expected effect of a combination of agents: the general solution. J. Theor. Biol. **114:**413–431.
6. **Berenbaum, M. C.** 1989. What is synergy? Pharmacol. Rev. **41:**93–141.
7. **Berenguer, J., J. L. Rodriguez-Tudela, C. Richard, M. Alvarez, M. A. Sanz, L. Gaztelurrutia, J. Ayats, J. V. Martinez-Suarez, et al.** 1997. Deep infections caused by *Scedosporium prolificans*. A report on 16 cases in Spain and a review of the literature. Medicine (Baltimore) **76:**256–265.
8. **Bliss, C. I.** 1939. The toxicity of poisons applied jointly. Ann. Appl. Biol. **26:**585–615.
9. **Carrillo, A. J., and J. Guarro.** 2001. In vitro activities of four novel triazoles against *Scedosporium* spp. Antimicrob. Agents Chemother. **45:**2151–2153.
10. **Cormican, M. G., and M. A. Pfaller.** 1996. Standardization of antifungal susceptibility testing. J. Antimicrob. Chemother. **38:**561–578.
11. **Cuenca-Estrella, M., B. Ruiz-Diez, J. V. Martinez-Suarez, A. Monzon, and J. L. Rodriguez-Tudela.** 1999. Comparative in-vitro activity of voriconazole (UK-109,496) and six other antifungal agents against clinical isolates of Scedosporium prolificans and *Scedosporium apiospermum*. J. Antimicrob. Chemother. **43:**149–151.
12. **Daneshmend, T. K., and D. W. Warnock.** 1983. Clinical pharmacokinetics of systemic antifungal drugs. Clin. Pharmacokinet. **8:**17–42.
13. **D'Argenio, D. Z.** 1990. Incorporating prior parameter uncertainty in the design of sampling schedules for pharmacokinetic parameter estimation experiments. Math Biosci. **99:**105–118.
14. **Drusano, G. L., D. Z. D'Argenio, W. Symonds, P. A. Bilello, J. McDowell, B. Sadler, A. Bye, and J. A. Bilello.** 1998. Nucleoside analog 1592U89 and human immunodeficiency virus protease inhibitor 141W94 are synergistic in vitro. Antimicrob. Agents Chemother. **42:**2153–2159.
15. **Espinel-Ingroff, A., F. Barchiesi, K. C. Hazen, J. V. Martinez-Suarez, and G. Scalise.** 1998. Standardization of antifungal susceptibility testing and clinical relevance. Med. Mycol. **36:**68–78.
16. **Georgopapadakou, N. H., and T. J. Walsh.** 1996. Antifungal agents: chemotherapeutic targets and immunologic strategies. Antimicrob. Agents Chemother. **40:**279–291.
17. **Goldin, A., and N. Mantel.** 1957. The employment of combination drugs in the chemotherapy of neoplasia: a review. Cancer Res. **17:**635–654.
18. **Gosbell, I. B., M. L. Morris, J. H. Gallo, K. A. Weeks, S. A. Neville, A. H. Rogers, R. H. Andrews, and D. H. Ellis.** 1999. Clinical, pathologic and epidemiologic features of infection with *Scedosporium prolificans*: four cases and review. Clin. Microbiol. Infect. **5:**672–686.
19. **Graybill, J. R.** 1996. The future of antifungal therapy. Clin. Infect. Dis. **22**(Suppl. 2)**:**S166–S178.
20. **Greco, W. R., G. Bravo, and J. C. Parsons.** 1995. The search for synergy: a critical review from a response surface perspective. Pharmacol. Rev. **47:**331–385.
21. **Greco, W. R., H. S. Park, and Y. M. Rustum.** 1990. Application of a new approach for the quantitation of drug synergism to the combination of cis-diamminedichloroplatinum and 1-beta-D-arabinofuranosylcytosine. Cancer Res. **50:**5318–5327.
22. **Hindler, J.** 1995. Antimicrobial susceptibility testing, p. 5.18.11–15.18.20. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook, American Society for Microbiology Press, Washington, D.C.
23. **Jackson, R. C.** 1991. Synergistic and antagonistic drug interactions resulting from multiple inhibition of metabolic pathway, p. 363–408. *In* T. C. Chou, and D. C. Rideout (ed.), Synergism and antagonism in chemotherapy. Academic Press, New York, N.Y.
24. **Kauffman, C. A., and P. L. Carver.** 1997. Antifungal agents in the 1990s. Current status and future developments. Drugs **53:**539–549.
25. **Levasseur, L. M., W. R. Greco, Y. M. Rustum, and H. K. Slocum.** 1997. Combined action of paclitaxel and cisplatin against wildtype and resistant human ovarian carcinoma cells. Cancer Chemother. Pharmacol. **40:**495–505.
26. **Meletiadis, J., J. F. Meis, J. W. Mouton, J. P. Donnelly, and P. E. Verweij.** 2000. Comparison of NCCLS and 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) methods of in vitro susceptibility testing of filamentous fungi and development of a new simplified method. J. Clin. Microbiol. **38:**2949–2954.
27. **Meletiadis, J., J. F. Meis, J. W. Mouton, J. L. Rodriquez-Tudela, J. P. Donnelly, and P. E. Verweij.** 2002. In vitro activities of new and conventional antifungal agents against clinical *Scedosporium* isolates. Antimicrob. Agents Chemother. **46:**62–68.
28. **Meletiadis, J., J. F. Meis, J. W. Mouton, and P. E. Verweij.** 2001. Analysis of growth characteristics of filamentous fungi in different nutrient media. J. Clin. Microbiol. **39:**478–484.
29. **Meletiadis, J., J. W. Mouton, J. F. Meis, B. A. Bouman, J. P. Donnelly, and P. E. Verweij.** 2001. Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. J. Clin. Microbiol. **39:**3402–3408.
30. **Meletiadis, J., J. W. Mouton, J. F. Meis, B. A. Bouman, J. P. Donnelly, and P. E. Verweij.** 2000. Comparison of spectrophotometric and visual reading of

NCCLS method and evaluation of a colorimetric method based on the reduction of a soluble tetrazolium/formazan, XTT, for antifungal susceptibility testing of *Aspergillus* species. J. Clin. Microbiol. **39:**4256–4263.
31. **Meletiadis, J., J. W. Mouton, J. F. Meis, and P. E. Verweij.** 2000. Combination chemotherapy for the treatment of invasive infections by *Scedosporium prolificans*. Clin. Microbiol. Infect. **6:**336–337.
32. **Meletiadis, J., J. W. Mouton, J. L. Rodriguez-Tudela, J. F. Meis, and P. E. Verweij.** 2000. In vitro interaction of terbinafine with itraconazole against clinical isolates of *Scedosporium prolificans*. Antimicrob. Agents Chemother. **44:**470–472.
33. **National Committee for Clinical Laboratory Standards.** 1998. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi; proposed standard. Document M-38P. National Committee for Clinical Laboratory Standards, Wayne, Pa.
34. **Pfaller, M. A., J. H. Rex, and M. G. Rinaldi.** 1997. Antifungal susceptibility testing: technical advances and potential clinical applications. Clin. Infect. Dis. **24:**776–784.
35. **Polak, A.** 1999. The past, present and future of antimycotic combination therapy. Mycoses **42:**355–370.
36. **Prichard, M. N., L. E. Prichard, W. A. Baguley, M. R. Nassiri, and C. Shipman, Jr.** 1991. Three-dimensional analysis of the synergistic cytotoxicity of ganciclovir and zidovudine. Antimicrob. Agents Chemother. **35:**1060–1065.
37. **Prichard, M. N., L. E. Prichard, and C. Shipman, Jr.** 1993. Strategic design and three-dimensional analysis of antiviral drug combinations. Antimicrob. Agents Chemother. **37:**540–545.
38. **Prichard, M. N., and C. Shipman, Jr.** 1990. A three-dimensional model to analyze drug-drug interactions. Antivir. Res. **14:**181–205.
39. **Rex, J. H., M. A. Pfaller, T. J. Walsh, V. Chaturvedi, A. Espinel-Ingroff, M. A. Ghannoum, L. L. Gosey, F. C. Odds, M. G. Rinaldi, D. J. Sheehan, and D. W. Warnock.** 2001. Antifungal susceptibility testing: practical aspects and current challenges. Clin. Microbiol. Rev. **14:**643–658.
40. **Ryder, N. S.** 1992. Terbinafine: mode of action and properties of the squalene epoxidase inhibition. Br. J. Dermatol. **126**(Suppl. 39)**:**2–7.
41. **Ryder, N. S., and I. Leitner.** 2001. Synergistic interaction of terbinafine with triazoles or amphotericin B against *Aspergillus* species. Med. Mycol. **39:**91–95.
42. **Shenep, J. L., B. K. English, L. Kaufman, T. A. Pearson, J. W. Thompson, R. A. Kaufman, G. Frisch, and M. G. Rinaldi.** 1998. Successful medical therapy for deeply invasive facial infection due to *Pythium insidiosum* in a child. Clin. Infect. Dis. **27:**1388–1393.
43. **Suhnel, J.** 1990. Evaluation of synergism or antagonism for the combined action of antiviral agents. Antivir. Res. **13:**23–39.
44. **Veldstra, H.** 1956. Synergism and potentiation with special reference to the combination of structural analogues. Pharmacol. Rev. **8:**339–387.
45. **Verweij, P. E., N. J. Cox, and J. F. Meis.** 1997. Oral terbinafine for treatment of pulmonary *Pseudallescheria boydii* infection refractory to itraconazole therapy. Eur. J. Clin. Microbiol. Infect. Dis. **16:**26–28.
46. **Webb, J. L.** 1963. Effect of more than one inhibitor, p. 66–79. Enzymes and metabolic inhibitors, vol. 1. Academic Press, New York, N.Y.
47. **Webb, J. L.** 1963. Effect of more than one inhibitor, p. 487–512. Enzymes and metabolic inhibitors, vol. 1. Academic Press, New York, N.Y.
48. **White, T. C., K. A. Marr, and R. A. Bowden.** 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. **11:**382–402.