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In Vitro Interaction of Terbinafine with Itraconazole against Clinical Isolates of *Scedosporium prolificans*

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In order to develop new approaches for the chemotherapy of invasive infections caused by *Scedosporium prolificans*, the in vitro interaction between itraconazole and terbinafine against 20 clinical isolates was studied using a checkerboard microdilution method. Itraconazole and terbinafine alone were inactive against most isolates, but the combination was synergistic against 95 and 85% of isolates after 48 and 72 h of incubation, respectively. Antagonism was not observed. The MICs obtained with the terbinafine-itraconazole combination were within levels that can be achieved in plasma.

Invasive infections caused by *Scedosporium* species are uncommon but are generally fatal in immunocompromised patients, especially when they are caused by *Scedosporium prolificans* (2, 13). Treatment includes surgical debridement, if possible, and antifungal chemotherapy, although the optimal choice and duration of therapy are unknown. Azoles, such as miconazole and itraconazole, have been used with some success for the treatment of invasive infections with *Scedosporium apiospermum* (6), but treatment failures have also been reported (15). *S. prolificans* is considered multiresistant since low in vitro activities have been reported for amphotericin B, flucytosine, and the azoles (3). Even novel antifungal agents such as the triazoles voriconazole, posaconazole (SCH56592), and Syn-2869 (5, 9) and the echinocandins LY303366 and caspofungin (MK-0991) show limited or no in vitro activity against this fungus (4, 5). We have previously reported a patient with pulmonary pseudallescheriosis who failed itraconazole therapy but responded after treatment was changed to terbinafine (15). The *S. apiospermum* isolate was resistant to either drug alone but was susceptible in vitro to the terbinafine-itraconazole combination (N. S. Ryder and I. Leitner, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. J-155, 1998). In the present study we investigated whether terbinafine and itraconazole act synergistically against 20 clinical *S. prolificans* isolates to determine if this combination is a potentially useful combination in the treatment of these infections.

All isolates were obtained from clinical specimens (3) and subcultured on Sabouraud glucose agar (SAB) plates with 0.5% chloramphenicol and incubated at room temperature for 7 days. They were then subcultured again on SAB plates and incubated for another 5 to 7 days at 37°C, and spores were collected. *Paecilomyces variotii* (ATCC 22319) was used as a quality control strain, and all isolates were tested in duplicate.

MICs were determined by a broth microdilution method according to National Committee for Clinical Laboratory Standards guidelines (proposed standard M38-P) (11). Briefly, a suspension of spores was adjusted with a spectrophotometer (Spectronic 20D; Milton Roy, Rochester, N.Y.) to 68 to 70% transmission at a wavelength of 530 nm and diluted 10-fold to yield a final inoculum of 1×10^4 to 5×10^4 CFU/ml. The spectrophotometer transmissions were verified by enumeration of colonies per milliliter of serial dilution on SAB plates that were incubated at 35°C for 48 h. These cultures showed that the final inoculum varied between 1.5×10^4 and 3.5×10^4 CFU/ml. Terbinafine (Novartis, Basel, Switzerland) and itraconazole (Janssen Research Foundation, Beerse, Belgium) were tested in RPMI 1640 medium with L-glutamine and without bicarbonate (GIBCO BRL, Life Technologies, Breda, The Netherlands), buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid). The final concentrations ranged from 0.5 to 32 μg/ml for itraconazole and 0.06 to 64 μg/ml for terbinafine. Growth was graded on a scale of 0 to 4 as follows: 4 indicated no reduction in growth, 3 indicated a 25% reduction of growth, 2 indicated a 50% reduction of growth, 1 indicated a 75% reduction of growth, and 0 indicated an optically clear well. The MIC was defined as the lowest concentration of antifungal compound that inhibited growth by 50% or more.

A two-dimensional, two-agent broth microdilution checkerboard technique was used to study the interactions between itraconazole and terbinafine. Serial twofold dilutions of itraconazole and terbinafine, alone and in combination, were tested against final inocula of 1×10^4 to 5×10^4 CFU/ml. In order to obtain an exact percentage of growth for each well, the dye 3-(4,5-dimethyl-2-thiazyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT; Sigma Chemical, St. Louis, MO.) was added together with the inoculum to each well at a final concentration of 0.1 mg/ml (8). After 48 or 72 h of incubation, the content of each well was removed and 200 μl of isopropanol containing 5% HCl (1 M) was added. After 30 min of incubation at room temperature and gentle agitation, the optical density (OD) was measured with a spectrophotometer (MS2 reader, Titertek-plus; ICN Biomedical Ltd., Basingstoke, United Kingdom) at 550 nm (8). The OD of the blank, to which a conidium-free inoculum had been added, was subtracted from the OD values. The percentage of growth for each well was calculated by comparing the OD of a well with that of the drug-free control. For each itraconazole-terbinafine combination the summation of the fractional inhibitory concentration (ΣFIC) was calculated as follows: (MIC of itraconazole plus terbinafine/MIC of itraconazole) + (MIC of itraconazole plus terbinafine/MIC of terbinafine). The interpretation of the ΣFIC was as follows: the

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TABLE 1. MICs and ΣFICs of itraconazole and terbinafine alone and in combination against *S. prolificans* after 48 and 72 h of incubation

Isolate	MIC (μg/ml)		Lowest ΣFIC at 48 h	MICs of the combination (itraconazole/terbinafine)	MIC (μg/ml)		Lowest ΣFIC at 72 h	MICs of the combination (itraconazole/terbinafine)
	Itraconazole	Terbinafine			Itraconazole	Terbinafine		
7898	16	0.5	0.25	2/0.06	>32	4	0.14	1/0.5
7906	16	0.5	0.25	2/0.06	>32	4	0.25	8/0.5
7902	16	0.5	0.25	2/0.06	>32	4	0.31	4/1
7921	>32	0.5	0.18	4/0.06	>32	4	0.51	0.5/2
7946	16	1	0.19	2/0.06	>32	4	0.26	0.5/1
7891	>32	1	0.12	4/0.06	>32	4	0.16	1/0.5
7940	>32	0.5	0.52	1/0.25	>32	32	0.09	2/2
7886	>32	1	0.27	1/0.25	>32	>64	0.02	0.5/0.5
7901	>32	1	0.09	2/0.06	>32	>64	0.05	2/1
7924	>32	1	0.15	1/0.125	>32	>64	0.05	1/2
7920	>32	0.5	0.27	1/0.125	>32	>64	0.04	0.5/2
7894	>32	1	0.12	4/0.06	>32	4	0.38	8/1
7905	16	1	0.19	2/0.06	>32	8	0.52	1/4
7888	16	2	0.13	1/0.125	>32	>64	0.07	0.5/4
7908	>32	1	0.14	1/0.125	>32	>64	0.05	2/1
7913	>32	0.5	0.28	2/0.125	>32	>64	0.05	1/2
7930	16	1	0.12	1/0.06	>32	>64	0.05	1/2
7903	>32	8	0.09	2/0.5	>32	>64	2	>32/>64
7900	>32	2	0.1	2/0.125	>32	>64	0.25	16/1
7912	>32	>64	0.04	0.5/2	>32	>64	0.04	0.5/2

synergistic effect was ≤0.5, the indifferent effect was >0.5 but ≤4, and the antagonistic effect was >4 (7). The results of experiments with the two agents alone and in combination were analyzed separately as well as together by calculating the mean ODs. Since all analyses yielded identical results, we present the results based on the mean OD from both experiments.

The MICs of terbinafine and itraconazole based on 50% reduction of growth for *P. variotii* were 0.125 and 0.25 μg/ml, respectively. Itraconazole was inactive in vitro against most isolates, with the MIC at which 90% of the isolates were inhibited being >32 μg/ml after both 48 and 72 h of incubation (Table 1). An attempt was made to establish the exact MIC of itraconazole by an agar dilution method. Serial dilutions ranging from 32 to 512 μg of itraconazole per ml were made in RPMI 1640 agar. The growth of none of the *S. prolificans* isolates was inhibited by any of these concentrations after 48 h of incubation. Therefore, a MIC of 64 μg/ml was chosen for calculations for those isolates that grew in the wells that contained the highest concentration of itraconazole. The MIC of terbinafine at which 90% of the isolates were inhibited was 2 μg/ml after 48 h but increased to 64 μg/ml after 72 h. Synergism was found for 19 of 20 (95%) of the *S. prolificans* isolates after 48 h and for 17 of 20 (85%) of the isolates after 72 h of incubation (Table 1). For three isolates the effect of the combination appeared to be indifferent after 72 h of incubation, and antagonism was not observed. Although drug interactions in vitro are difficult to assess, we believe that the observed synergism is significant for several reasons. (i) Despite the fact that we selected a stringent criterion for the definition of synergism, almost all isolates showed synergism after both 48 and 72 h of incubation. (ii) Because the MIC of itraconazole was set at 64 μg/ml for most isolates, the calculated ΣFIC underestimates the actual level of synergism. Calculations with higher MICs of single drugs would have resulted in even lower ΣFIC. (iii) Itraconazole and terbinafine block different steps of the same pathway of fungal ergosterol biosynthesis, which supports the possibility of synergistic action. The classic example of proven synergism in this respect is the combination of trimethoprim with sulfonamides, which also interacts with consecutive steps of a common pathway. Furthermore, the combination of terbinafine with azoles has been shown to be synergistic in vitro for other fungi, including *Candida albicans* (1), *Candida glabrata*, and *Cryptococcus neoformans* (A. W. Fothergill, I. Leitner, J. G. Meingassner, N. S. Ryder, and M. G. Rinaldi, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E53, 1996), and for *Pythium insidiosum* (14). Synergism has been reported for combinations of amphotericin B with azoles against *S. apiospermum* but the synergistic activity was less pronounced than for the terbinafine-itraconazole combination and occurred only for a limited number of isolates (16). In another study, the effect of amphotericin B combined with terbinafine was indifferent against *S. apiospermum*, but terbinafine with fluconazole was synergistic against the same isolate (Ryder and Leitner, 38th ICAAC, abstr. J-155). Terbinafine appears to interact synergistically with the class of azole antifungal drugs.

The MICs of the terbinafine-itraconazole combination are within the range that can be achieved in blood. The achievable maximum concentrations of terbinafine are approximately 1.7 μg/ml within 2 h of oral administration of a dose of 500 mg (10). Levels in serum above 3.0 μg/ml can be achieved with itraconazole (12), and even higher levels may be achieved with the oral solution or the intravenous formulation that is now undergoing clinical evaluation.

Invasive infections caused by *S. prolificans* are generally fatal, and at present there is no antifungal regimen that has been shown to be effective. The in vitro synergism of itraconazole and terbinafine that we demonstrated may prove effective for the treatment of these infections. Animal-model and clinical studies are warranted to further elucidate the potential of terbinafine-itraconazole combination therapy in difficult-to-treat infections by filamentous fungi.

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