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In Vitro Activities of Nine Antifungal Drugs and Their Combinations against *Phialophora verrucosa*

Yali Li, Zhe Wan, Ruoyu Li

Department of Dermatology and Venereology, Peking University First Hospital, Research Center for Medical Mycology, Peking University, and Beijing Key Laboratory of Molecular Diagnosis of Dermatoses, Beijing, People's Republic of China

The *in vitro* activities of nine antifungal drugs and their combinations against 31 clinical and 15 environmental *Phialophora verrucosa* strains were tested. The MIC_{90}/90% minimum effective concentration (MIC/MEC_{90}) values (μg/ml) across all strains were as follows: for terbinafine, 0.25; for posaconazole, 0.5; for voriconazole, 1; for itraconazole, 2; for amphotericin B, 4; for caspofungin and micafungin, 16; and for fluconazole and flucytosine, 64. The highest synergy was shown by the combination of itraconazole plus caspofungin (with synergy against 100% of the 31 clinical strains), followed by amphotericin B plus flucytosine (45.2%) and itraconazole plus terbinafine or micafungin (25.8% or 12.9%, respectively).

Phialophora verrucosa is one of the major dematiaceous fungi causing intractable chromoblastomycosis, phaeohyphomycosis, and other infections such as mycetoma and endophthalmitis (1–3). With the chronic repetitious nature of the infections, traditional drugs and physical therapies cannot deal with the relapse, resistance, and incomplete cures of chromoblastomycosis and phaeohyphomycosis (1). Currently, clinical *P. verrucosa* infection cases and antifungal therapies, including drug combination therapies, have been reported (2, 4–10), but effective therapies are still limited. Previous studies have indicated that terbinafine combined with itraconazole, amphotericin B, or voriconazole showed synergy against dematiaceous molds such as *Fonsecaea pedrosoi* and *Exophiala jeanselmei* (11); amphotericin B combined with terbinafine also showed synergy for six *P. verrucosa* isolates (12). Our group had also explored the susceptibilities of three drugs against only 20 *P. verrucosa* isolates, including 1 clinical isolate, and no synergy or antagonism was observed when terbinafine was combined with itraconazole or amphotericin B (13). At present, there is limited information available on common antifungals and effective combinations against numerous clinical *P. verrucosa* isolates. This study aimed to investigate the *in vitro* susceptibilities of clinical and some environmental strains of *P. verrucosa* to nine antifungal drugs (fluconazole, flucytosine, amphotericin B, itraconazole, voriconazole, posaconazole, caspofungin, micafungin, and terbinafine) and the potential synergy and antagonism of these drugs when combined in pairs.

Forty-six *P. verrucosa* strains were obtained from the Research Center for Medical Mycology at Peking University, comprising 31 clinical isolates originating from patients with chromoblastomycosis ($n = 11$), phaeohyphomycosis ($n = 19$), and subcutaneous cysts ($n = 1$) and 15 isolates originating from environments in northern China ($n = 8$), southern China ($n = 3$), and the garden of a patient's house ($n = 4$) (see Table S1 in the supplemental material). Antifungals alone or in combination were tested against clinical strains. Environmental strains were used only in the single antifungal susceptibility test. All strains were identified by morphological methods and sequencing of the conserved ribosomal internal transcribed spacer (ITS) region (14–17).

In vitro susceptibilities were determined as described in the CLSI M38-A2 document (18). Synergy testing was evaluated using the checkerboard technique, as follows. Serial twofold dilutions with 50 μl each of drugs A and B were dispensed along the vertical and horizontal directions to yield 100 μl per well in a 96-well microtiter plate (19). Isolates were cultured on potato dextrose agar at 28°C for 7 days or longer, until the spores were rich. Inocula were prepared by gently scraping the surfaces of the fungal colonies by use of a sterile tip with 2.5 to 3 ml of sterile physiological saline containing 0.05% Tween 20. Large particles in the cell suspensions were allowed to settle for 3 to 5 min at room temperature, and the final concentration of spores dispensed into the wells was adjusted to approximately 2.5×10^4 CFU/ml, as determined by quantitative spore counts obtained using a hemocytometer.

Antifungal drugs were obtained as reagent-grade powders. When the drugs were used alone, the final concentrations of fluconazole (Sunve Pharm, Shanghai, China) and flucytosine (Sigma-Aldrich, Saint Louis, MO, USA) ranged from 0.5 to 256 μg/ml, those of amphotericin B (Sigma-Aldrich), itraconazole (Shouguang Pharm, Shangdong, China), voriconazole (Shouguang Pharm), and posaconazole (Merck, Rahway, NJ, USA) ranged from 0.031 to 16 μg/ml, those of caspofungin (Merck) and micafungin (Astellas Pharma, Tokyo, Japan) ranged from 0.063 to 32 μg/ml, and that of terbinafine (Novartis, Basel, Switzerland) ranged from 0.002 to 1 μg/ml. When the drugs were combined in pairs, the final concentrations of itraconazole, voriconazole, and posaconazole ranged from 0.031 to 2 μg/ml, that of amphotericin B ranged from 0.125 to 8 μg/ml, that of caspofungin ranged from 0.25 to 128 μg/ml, that of fluconazole ranged from 4 to 256 μg/ml, those of flucytosine and micafungin ranged from 0.5 to 256 μg/ml, and that of terbinafine ranged from 0.004 to 2 μg/ml. The quality control strains *Candida parapsilosis* ATCC 22019, *Candida*

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Address correspondence to Ruoyu Li, mycolab@126.com.
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krusei ATCC 6258, *Aspergillus flavus* ATCC 204304, and *Trichophyton mentagrophytes* ATCC-MYA 4439 were included in each assay run.

After 72 h of incubation at 35°C or 28°C (six isolates could not grow at 35°C), MICs were determined visually by comparison of the growth in the wells containing the drug to that of the drug-free control. When the drugs were tested alone, amphotericin B, itraconazole, voriconazole, and posaconazole were found to require the lowest drug concentrations for prevention of any discernible growth (100% inhibition), whereas fluconazole and flucytosine required the lowest concentrations for ≥50% inhibition and terbinafine required the lowest concentration for ≥80% inhibition (18). The minimum effective concentration (MEC) was read as the lowest concentration of drug causing the growth of small, rounded, compact hyphal forms in comparison to the hyphal growth seen in the growth control well for caspofungin and micafungin (see Fig. S1 in the supplemental material). For synergy testing, 100% inhibition was determined for all drugs, including echinocandins. The fractional inhibitory concentration index (FICI) was calculated using the equation FICI = $(A_c/A_a) + (B_c/B_a)$, where A_c and B_c are the MICs of drugs A and B in combination, respectively, and A_a and B_a are the MICs of the drugs alone, respectively. FICIs of ≤0.5 indicate synergy, FICIs of >4 indicate antagonism, and FICIs of >0.5 and ≤4 indicate no interaction (20). Each assay was performed twice for every isolate.

The geometric mean MIC/MECs, MIC/MEC_{50}s, MIC/MEC_{90}s, and ranges of MIC/MECs for *P. verrucosa* are presented in (Table 1). For all clinical strains, the MIC_{90}s of itraconazole, voriconazole, and posaconazole were low (≤0.5 μg/ml), but for environmental strains, itraconazole and voriconazole became less active (MIC_{90}s of ≥2 μg/ml). Additionally, among the nine drugs, only the MICs of itraconazole and voriconazole ($P < 0.001$) were significantly different between the clinical and environmental strains. In the treatment of dematiaceous fungal infections, MICs of ≤1 μg/ml are generally used as an indicator of potential susceptibility to most drugs (21). So, we can predict that the newer triazoles are active against *P. verrucosa* isolates from clinical samples, and this activity has already been observed in other dematiaceous fungi (22–24). Previous studies had showed that the MEC_{90}s of caspofungin and anidulafungin against *Fonsecaea* spp. were both 2 μg/ml (22) and that the MEC_{90}s of caspofungin and micafungin against *Cladophialophora carrionii* (23), *Cyphellophora* spp., and *Phialophora* spp. (24) (not including *P. verrucosa*) were 2 μg/ml and 4 μg/ml, respectively. The present study showed relatively high MECs for caspofungin and micafungin against *P. verrucosa*, and moreover, we did not observe the variability of caspofungin MICs in our *P. verrucosa* strains that had previously been reported for *Candida* spp. (25). Therefore, the *in vitro* activities of echinocandin drugs against clinically important dematiaceous fungi appeared to be weak.

Thirty-one clinical strains were chosen for the synergy studies. We tested 12 pairs of drug combinations: amphotericin B combined with fluconazole, terbinafine, flucytosine, itraconazole, voriconazole, posaconazole, caspofungin, or micafungin; itraconazole combined with terbinafine, caspofungin, or micafungin; and fluconazole combined with flucytosine. Synergy was observed for four pairs of combinations: itraconazole combined with terbinafine, caspofungin, or micafungin and amphotericin B combined with flucytosine (Table 2). Other combinations showed no interactions.

TABLE 1 MIC/MEC values of nine antifungal drugs against 46 *P. verrucosa* strains

Strain group (no. of strains) and drug	MIC/MEC (μg/ml)			
	Range	50%	90%	Geometric mean
Total ($n = 46$)				
Fluconazole	8–256	32	64	55.826
Flucytosine	2–256	16	64	32.609
Amphotericin B	2–4	4	4	3.261
Itraconazole	0.25–4	0.5	2	0.973
Voriconazole	0.063–4	0.25	1	0.58
Posaconazole	0.031–1	0.125	0.5	0.232
Caspofungin	2–16	8	16	9.174
Micafungin	0.5–32	4	16	8.63
Terbinafine	0.002–1	0.125	0.25	0.152
Clinical ($n = 31$)				
Fluconazole	8–256	32	64	43.097
Flucytosine	2–256	16	64	36.387
Amphotericin B	2–4	4	4	3.29
Itraconazole	0.25–1	0.5	0.5	0.476
Voriconazole	0.063–1	0.25	0.5	0.361
Posaconazole	0.031–1	0.125	0.5	0.196
Caspofungin	2–16	8	16	8.903
Micafungin	0.5–32	8	16	8.968
Terbinafine	0.002–1	0.125	0.25	0.143
Environmental ($n = 15$)				
Fluconazole	16–256	64	256	82.133
Flucytosine	2–64	16	64	24.8
Amphotericin B	2–4	4	4	3.2
Itraconazole	1–4	2	4	2
Voriconazole	0.25–4	1	2	1.033
Posaconazole	0.031–1	0.25	0.5	0.308
Caspofungin	2–16	8	16	9.733
Micafungin	1–32	4	16	7.933
Terbinafine	0.063–0.5	0.125	0.25	0.171

Combinations of itraconazole with caspofungin or micafungin were not only usually used to treat infections caused by *Aspergillus fumigatus* but also showed synergy against clinically important fungi *in vitro* (26–29). Synergy was observed in all 31 clinical isolates when itraconazole was combined with caspofungin, with FICIs ranging from 0.125 to 0.5. These results suggest that itraconazole with caspofungin seems to be the most potent combination against *P. verrucosa in vitro*.

When flucytosine was combined with amphotericin B, 45.2% of the clinical strains showed synergy. This combination had already been reported to observably ameliorate skin lesions and to achieve a mycological cure of chromoblastomycosis for a patient who had been infected by *P. verrucosa* and had not been responsive to itraconazole, fluconazole, or terbinafine (5). The present study showed that the combination of terbinafine and itraconazole had a synergistic response against a small portion of *P. verrucosa* isolates as well as other dematiaceous molds (11), but no interaction was observed when terbinafine was combined with amphotericin B. This result was unlike that observed in the previous study (12).

In conclusion, this study indicated that the newer triazoles had low MICs for *P. verrucosa* but that amphotericin B, fluconazole, flucytosine, caspofungin, micafungin, and terbinafine had rela-

TABLE 2 MIC ranges, geometric mean MICs, FICI ranges, and ratios for 31 clinical strains for which combinations of drugs showed synergy

Characteristic	Value for indicated antifungal(s)[a]											
	ITC + CAS	ITC	CAS	ITC + MCFG	ITC	MCFG	ITC + TRB	ITC	TRB	AMB + 5FC	AMB	5FC
MIC range (μg/ml)		0.031–0.25	0.5–16		0.031–1	0.5–64		0.031–0.5	0.063–1		0.125–4	0.5–256
GM[b] MIC (μg/ml)		0.088	5.435		0.347	3.161		0.152	0.371		1.21	47.951
FICI range	0.125–0.5			<0.141–1.25[c]			0.245–2			0.094–2		
Ratio (%)[d]	100			12.9			25.8			45.2		

[a] ITC, itraconazole; CAS, caspofungin; MCFG, micafungin TRB, terbinafine; AMB, amphotericin B; 5FC, flucytosine.
[b] GM, geometric mean.
[c] The MIC of MCFG used alone was >256 μg/ml for 100% inhibition.
[d] The higher the ratio, the more potent the combination.

tively high MICs/MECs *in vitro*. When itraconazole was combined with terbinafine, caspofungin, or micafungin and amphotericin B was combined with flucytosine, synergy but no antagonism was observed. However, the *in vitro* results presented here need to be confirmed by using the appropriate animal models of *P. verrucosa* infection or clinical validation *in vivo*.

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