ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2003, p. 3161–3164 Vol. 47, No. 10
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.10.3161–3164.2003

In Vitro Activities of New Antifungal Agents against *Chaetomium* spp. and Inoculum Standardization

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Received 11 July 2003/Returned for modification 14 July 2003/Accepted 20 July 2003

***Chaetomium* is an unusual etiological agent of human infections, but the mortality rate among immunocompromised patients is considerably greater than that among nonimmunocompromised individuals. We investigated the in vitro antifungal susceptibilities to novel antifungal agents of 19 strains belonging to three species of *Chaetomium* which have been involved in human infections, i.e., *Chaetomium globosum*, *C. atrobrunneum*, and *C. nigricolor*, and one strain of the closely related species *Achaetomium strumarium*. A modification of the NCCLS reference microdilution method (M38-A) was used to evaluate the in vitro activities of ravuconazole, voriconazole, albaconazole, and micafungin. Micafungin was not active at all, while the geometric mean MICs and minimum effective concentrations of the three triazoles were less than 0.5 and 0.4 μg/ml, respectively.**

Several species of the ascomycete genus *Chaetomium* are able to cause human infections, and these mainly affect the nails or skin (5). However, disseminated infections have also been reported, especially in neutropenic patients (11). Despite antifungal treatments with amphotericin B (AMB) alone or in combination with itraconazole, the mortality rate among patients with severe infections caused by *Chaetomium* is high (M. A. Barron, D. A. Sutton, R. Veve, J. Guarro, M. Rinaldi, E. Thompson, P. J. Cagnoni, and N. E. Madinger, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 870, 2002). The most prevalent clinical species is *Chaetomium globosum*, although other species have also been reported (11). Clinical and in vitro data on the activities of antifungal drugs against the different species of *Chaetomium* are scarce. In previous studies, using broth macro- and microdilution methods, we demonstrated the inhibitory activities of miconazole, ketoconazole, and itraconazole in vitro (11, 17). However, the susceptibility of *Chaetomium* to the novel antifungal drugs is practically unknown.

In this work we have evaluated the in vitro activities of the new triazoles albaconazole (ABC), voriconazole (VRC), and ravuconazole (RVC) and the echinocandin micafungin (MFG) against a representative number of strains of *Chaetomium* species of clinical interest and one strain of *Achaetomium strumarium*, which has also been involved in human infections. We followed the guidelines of NCCLS reference method M38-A for antifungal susceptibility testing of molds (14). However, since this reference method does not apply to fungi that reproduce by ascospores instead of conidia, we have made a few modifications to the type and the size of the inoculum and the incubation conditions.

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MATERIALS AND METHODS

Isolates. Nineteen isolates of *Chaetomium* spp. (11 isolates of *C. globosum*, 7 isolates of *C. atrobrunneum*, 1 isolate of *C. nigricolor*) and 1 isolate of *A. strumarium* were tested; all of them were from clinical sources. The isolates were stored on oatmeal agar (OA; oatmeal, 30 g; agar, 20 g; tap water, 1 liter) slants covered with paraffin oil, subcultured on OA plates, and incubated at 30°C for 15 to 20 days. *Paecilomyces variotti* ATCC 36257 was included in each batch of tests as a reference strain.

Antifungal agents. Antifungal agents were obtained as pure powders. AMB (USP, Rockville, Md.), ABC (J. Uriach & Cía, Barcelona, Spain), VRC (Pfizer Inc., Madrid, Spain), and RVC (Bristol-Myers Squibb Company, New Brunswick, N.J.) were diluted in dimethyl sulfoxide (Panreac Química S.A., Barcelona, Spain). MFG (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) was diluted in sterile distilled water. Microplates were prepared as described in document M38-A (14). Final drug concentrations ranged from 32 to 0.06 μg/ml for MFG and from 16 to 0.03 μg/ml for the other drugs.

Inoculum preparations. Inocula were prepared by scraping the fungal colonies growing on the agar plates with a culture loop and suspending them in sterile saline. The fungal suspensions were vigorously vortexed for 15 s and filtered through sterile gauze to remove the hyphae and obtain suspensions composed almost exclusively of ascospores. These suspensions were adjusted with a hemacytometer to 10^7 ascospores/ml. The viability of the ascospores was verified by plating 100 μl of serial dilutions of each inoculum onto Sabouraud dextrose agar (Pronadisa, Madrid, Spain) plates. The adjusted suspensions were centrifuged, the supernatant was discarded, and the ascospore pellets were resuspended in the same volume of saline. The optical densities (ODs) of the ascospore suspensions were determined spectrophotometrically at 530 nm. All the isolates were tested three times.

MIC determination. The MIC was the lowest drug concentration that produced a complete absence of growth. MIC readings were taken visually with the aid of an inverted mirror.

MEC determination. The minimum effective concentration (MEC) was the lowest drug concentration that produced morphological changes in fungal hyphae. Macroscopically it was recognized as the observation of very compact and round colonies in the microtiter wells. MEC readings were made with the aid of a stereoscopic microscope.

MLC determination. The minimum lethal concentration (MLC) was the lowest drug concentration that resulted in 99.9% killing of spores. Briefly, 10-μl

TABLE 1. Correlation between ODs of ascospore suspensions[a] and the number of CFU per milliliter

Species	No. of isolates[b]	OD		CFU/ml	
		Range	Geometric mean	Range	Geometric mean
C. globosum	11	1.05–1.40	1.20	5.9×10^5–4.1×10^6	2.3×10^6
C. atrobrunneum	7	1.10–1.50	1.42	5.0×10^5–4.4×10^6	1.4×10^6
C. nigricolor	1	1.10–1.25	1.18	5.0×10^5–1.3×10^6	8.0×10^5
A. strumarium	1	1.05–1.40	1.25	8.0×10^5–1.2×10^6	9.8×10^5

[a] The ascospore suspensions contained 10^7 ascospores/ml.
[b] All the isolates were tested three times.

aliquots were subcultured from each well that showed complete inhibition of growth (an optically clear well) onto Sabouraud dextrose agar plates. The plates were incubated at 30°C for 96 h. The MLC was the lowest antifungal concentration that resulted in either no growth or fewer than three colonies per plate (6).

MICs, MECs, and MLCs were determined after 72 h of incubation at 30°C. The MIC and MEC ranges, the corresponding geometric means, and the MICs and MECs at which 90% of the isolates tested were inhibited (MIC_{90}s and MEC_{90}s, respectively) were determined for each combination of fungal species and drug and for all the isolates and drugs. For MLCs, only geometric means were determined.

RESULTS

Inocula standardization. The ODs of the ascospore suspensions adjusted to 10^7 ascospores/ml with a hemacytometer ranged from 1.05 to 1.50 (Table 1). The ascospore germination rate was very low for all species, with *C. globosum* being the species that showed the highest mean germination rate (15.3%). The range of growth for all isolates tested was 5×10^5 to 4.4×10^6 CFU/ml.

Antifungal data. All isolates tested produced detectable growth at 72 h of incubation. Therefore, MICs were read after this time. Table 2 shows the MICs, MECs, and MLCs of the five antifungal drugs for the 20 isolates tested. AMB showed the broadest MIC range (0.5 to 8 μg/ml). In general, the MICs, MECs, and MLCs of this drug were high. The geometric mean MICs were identical to the geometric mean MECs, and the MIC_{90}s and MEC_{90}s were also identical. MFG showed a very high mean MIC (64 μg/ml), although the mean MEC was considerably lower (9.2 μg/ml). The MLCs of MFG were not evaluated because the MICs were always greater than the highest concentration tested. The three azoles were generally active. The mean MICs of these drugs, with a few exceptions, were similar to each other and were less than 0.5 μg/ml. The mean MECs of the three azoles were also similar to each other and were only slightly lower than the mean MICs. The MLCs of all antifungals tested were high and generally exceeded the concentrations normally achievable in the host.

In general, for the two species for which more than one isolate was assayed, all the drugs tested, with the exception of MFG, displayed slightly lower (1 or 2 dilutions) MICs and MECs for *C. atrobrunneum* than for *C. globosum.*

DISCUSSION

Inoculum standardization by counting with a hemacytometer for antifungal susceptibility testing of filamentous fungi has

TABLE 2. MICs, MECs, and MLCs of five antifungal agents for 20 strains of *Chaetomium* and *Achaetomium* isolates

Species	No. of isolates	Antifungal agent	MIC (μg/ml)			MEC (μg/ml)			Geometric mean MLC (μg/ml)
			Range	Geometric mean	90%	Range	Geometric mean	90%	
C. globosum	11	AMB	2–8	4	8	2–8	4	8	29.7
		MFG	64	64	64	0.5–64	9	64	
		VRC	0.5	0.5	0.5	0.25–0.5	0.41	0.5	32
		ABC	0.12–1	0.34	1	0.12–0.5	0.25	0.5	32
		RVC	0.12–1	0.26	0.5	0.06–0.25	0.12	0.25	32
C. atrobrunneum	7	AMB	0.5–4	1.48	2	0.5–4	1.48	2	28
		MFG	64	64	64	8–16	12	16	
		VRC	0.12–0.5	0.23	0.5	0.12–0.25	0.18	0.25	32
		ABC	0.12–1	0.18	0.25	0.12–0.25	0.13	0.12	29.3
		RVC	0.06–1	0.16	0.25	0.06–0.25	0.10	0.25	32
C. nigricolor	1	AMB	4			4			8
		MFG	64			8			
		VRC	0.5			0.5			1
		ABC	0.5			0.12			32
		RVC	1			0.5			32
A. strumarium	1	AMB	1			1			32
		MFG	64			8			
		VRC	0.12			0.06			32
		ABC	0.06			0.06			32
		RVC	0.06			0.03			32

been considered an accurate and reproducible method. After the suspensions were cultured on agar plates, the spore counts and the corresponding CFU obtained by this method differed, in general, by less than 10% (1, 10, 16), but this was true only for inocula made up of conidia. When the molds tested were reproduced as ascospores, such as *Chaetomium*, the situation was different. Conidia are propagules that spread and that are produced in massive numbers, and they germinate quickly when the environmental conditions are favorable. By contrast, ascospores are resistant cells which can remain dormant for a long time, and a germination activator may be required. In coprophilous fungi, for example, the activation of the ascospores comes after passage through the gut of an animal. In the laboratory, some alcoholic, acid, or phenolic solutions or simply heating can induce ascospore germination (8). With adequate activation, most of the spores will germinate; if not, they will never abandon their dormant state. As no pretreatment was applied to inocula in our study, their size had to be increased to 10^7 spores/ml in order for the number of germinated ascospores to fit into the range recommended by the NCCLS (14). A preliminary test, performed with some of the strains used in the present study, allowed us to determine the rates of germination of the different species, which proved to be wide and variable, but in general very low (approximately 10%), with the exception of that of *C. nigricolor*, whose germination rate was considerably lower.

We also developed a spectrophotometric procedure in order to facilitate inoculum preparation for the testing of these types of fungi in future studies. Most strains of *Chaetomium* and *Achaetomium* produce soluble dark pigments, however, which can influence the OD readings. To rectify this interference, inoculum suspensions were centrifuged and the supernatant was changed and replaced by the same volume of sterile saline. By this procedure, the OD required to obtain the final inoculum concentration suggested by the NCCLS was 1.3, which was considerably higher than that required for fungi that produce conidia (0.09 to 0.17). These important differences can be explained by the fact that the inocula of ascospores are 10 times more concentrated than those made up of conidia and by the fact that the ascospores of *Chaetomium* are bigger than most conidia of any of the genera included in the M38-A document. By this procedure, the inoculum used to test isolates of *Chaetomium* and related genera can be easily standardized.

The antifungal activity of AMB obtained here agrees with those found by other investigators (4, 11, 13). In a previous study we reported AMB MICs similar to those obtained here (MIC_{90}, 9.23 μg/ml at 72 h of incubation), even though the results were generated by the macrodilution method. The activities of the azoles ABC and VRC against *C. globosum* were previously tested by Capilla et al. (4) and McGinnis and Pasarell (13), respectively, and their results were also comparable to ours.

In this study, in addition to the more traditional MICs and MLCs, we have also used MECs to evaluate the antifungal activities of the drugs tested. The MEC was defined by Kurtz et al. (12) for the testing of lipopeptides. Morphological changes can be scored macroscopically in the wells, by recording the presence of very compact colonies forming clumps (2, 7, 12), or by detecting more subtle changes such as variations in the breadth of the hyphae when they are observed microscopically (9). MECs can be 1 to 2 dilutions lower when the last criterion is used (7). The method that is the more predictive of the clinical outcome, however, has not been determined. We used the macroscopic reading because it is easier to perform in a clinical laboratory. It is remarkable that, in our study, while the MICs of MFG were always out of range, the MECs of this drug were as low as 0.5 μg/ml for some of the isolates. Although MEC endpoints were initially limited to the testing of lipopepticidal antifungal agents, they have recently been used for azoles and AMB (3, 18). In our study, the MECs of AMB were identical to the MICs and the MECs of the azoles were similar to the MICs, which generally confirmed the results obtained by other investigators (3, 15, 18).

Neither AMB deoxycholate, alone or in combination with itraconazole, nor its liposomal formulation has been effective treatment for any of the six patients with *Chaetomium* infections treated with antifungal drugs (Barron et al., 42th ICAAC). The good in vitro activities of the three triazoles obtained in our study encourage us to perform further studies with animal models to confirm their potential for the treatment of *Chaetomium* infections, for which there is no effective therapy.

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