JOURNAL OF CLINICAL MICROBIOLOGY, July 1999, p. 2343–2345 Vol. 37, No. 7
0095-1137/99/$04.00+0

In Vitro Amphotericin B Resistance in Clinical Isolates of *Aspergillus terreus*, with a Head-to-Head Comparison to Voriconazole

DEANNA A. SUTTON,[1]* STEPHEN E. SANCHE,[1] SANJAY G. REVANKAR,[1] ANNETTE W. FOTHERGILL,[1] AND MICHAEL G. RINALDI[1,2]

Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio,[1] and Audie L. Murphy Division, South Texas Veterans Health Care System,[2] San Antonio, Texas 78284

Received 22 December 1998/Returned for modification 26 January 1999/Accepted 26 March 1999

Amphotericin B therapy continues to be the "gold standard" in the treatment of invasive aspergillosis in the immunocompromised host. Although *Aspergillus fumigatus* and *Aspergillus flavus* constitute the major species, several reports have described invasive pulmonary or disseminated disease due to the less common *Aspergillus terreus* and dismal clinical outcomes with high-dose amphotericin B. We therefore evaluated 101 clinical isolates of *A. terreus* for their susceptibility to amphotericin B and the investigational triazole voriconazole by using the National Committee for Clinical Laboratory Standards M27-A method modified for mould testing. Forty-eight-hour MICs indicated 98 and 0% resistance to amphotericin B and voriconazole, respectively. We conclude that *A. terreus* should be added to the list of etiologic agents refractory to conventional amphotericin B therapy and suggest the potential clinical utility of voriconazole in aspergillosis due to this species.

There are increasing reports of invasive or disseminated disease (6, 10, 11, 14, 15, 18, 21, 26, 28, 29, 34, 36, 37), primary or secondary cutaneous manifestations (17, 31, 38), endophthalmitis or keratitis (4, 13, 30), and otitis media (35) caused by *Aspergillus terreus*. The apparent refractoriness of this organism to amphotericin B therapy (11, 14) and a review of our own in vitro antifungal susceptibility data (32, 33) prompted a large-scale evaluation of the in vitro susceptibility data for this species of *Aspergillus*. In addition to obtaining data for amphotericin B, we also sought to determine in vitro susceptibility to voriconazole, a new investigational triazole with promising activity against the other, more common *Aspergillus* species, *A. fumigatus* and *A. flavus* (2, 5, 7, 9, 16, 19, 23).

(This work was presented in part at the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, Calif., 24 to 27 September 1998.)

The isolates evaluated were clinical isolates submitted to the Fungus Testing Laboratory, for either susceptibility testing or identification or both, through the years 1992 to 1997 and the early months of 1998. The majority of the isolates (68%) were from respiratory sites, including lung biopsies. Other areas of recovery included bone, blood, kidney, cutaneous lesions, bile, cornea, maxillary sinus, and cerebrospinal fluid. Macroscopically, *A. terreus* is easily differentiated from the more common *A. fumigatus*, *A. flavus*, *A. niger*, and *Emericella nidulans* by its buff to cinnamon granular front and its yellow reverse on potato flakes agar (PFA) prepared in-house (25). Microscopically, *A. terreus* produces a delicate, columnar fruiting structure. Conidiophores are smooth and 70 to 300 μm long; vesicles are variably shaped and 7 to 20 μm wide. Metulae and phialides (biseriate) cover the upper portion of the vesicles. Conidia are small (2 to 2.5 μm in diameter), globose, and smooth. Globose, sessile, hyaline aleurioconidia (2 to 6 μm in diameter) are frequently produced on submerged hyphae (32).

Isolates were evaluated by use of the National Committee for Clinical Laboratory Standards broth macrodilution method M27-A (20). Briefly, isolates were grown on PFA for 7 to 10 days at 25°C to induce conidial formation. Mature PFA *A. terreus* slant cultures and the control strain *Paecilomyces* UTHSC 90-450 were overlaid with sterile distilled water, and suspensions were made by gently scraping the colonies with the tip of a Pasteur pipette. Heavy hyphal fragments were allowed to settle, and the upper, homogeneous conidial suspensions were removed. Conidia were counted with a hemacytometer, and the inoculum was standardized to 1.0×10^5 CFU/ml. Conidial suspensions were further diluted 1:10 in medium for a final inoculum concentration of 1.0×10^4 CFU/ml.

Final drug concentrations were 0.03 to 16 μg/ml for amphotericin B (E. R. Squibb & Sons, Princeton, N.J.) and 0.03 or 0.125 to 16 μg/ml for voriconazole (Pfizer Inc., New York, N.Y.). Amphotericin B was tested in antibiotic medium 3 (Difco Laboratories, Detroit, Mich.), while voriconazole was tested in RPMI 1640 with L-glutamine and morpholinepropanesulfonic acid (MOPS) buffer at 165 mM and without sodium bicarbonate (American Biorganics, Inc., Niagara Falls, N.Y.). Previously prepared frozen-drug tubes containing 0.1 ml of drug were allowed to thaw and were inoculated with 0.9 ml of the conidial-medium suspension. A drug-free growth control tube was included with each isolate and the control organism. Tubes were incubated at 35°C, and MICs were read at the first 24-h interval when growth was observed in the drug-free control tube. MICs were defined as the first tube with a score of 0 (optically clear) for amphotericin B and a score of 2 (≥80% reduction in turbidity compared to that in the drug-free control tube) for voriconazole. The minimum lethal concentrations (MLCs) were determined by plating 100 μl from the drug-free control tube and each negative tube onto a drug-free Sabouraud dextrose agar plate. The MLC was defined as the lowest concentration of antifungal compound resulting in five or fewer colonies, which corresponded to 0.1% of the control inoculum or a 99.9% reduction (1, 27). Both MICs and MLCs were evaluated after 48 h of incubation, the time currently being used for *Aspergillus* species in ongoing studies of

* Corresponding author. Mailing address: Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284-7750. Phone: (210) 567-4131. Fax: (210) 567-4076. E-mail: suttond@uthscsa.edu.

TABLE 1. Geometric mean MICs and MLCs for *A. terreus* clinical isolates

Drug	h	MIC (μg/ml)	MLC (μg/ml)	No. of isolates tested
Amphotericin B[a]	24	1.17[c]	7.03[c]	101
	48	3.37[c]	13.4[c]	101
Voriconazole[b]	24	0.12	5.39	101
	48	0.22	17.4	51

[a] Tested in antibiotic medium 3 at 35°C.
[b] Tested in RPMI 1640 at 35°C.
[c] Resistant in vitro.

filamentous fungi by the National Committee for Clinical Laboratory Standards Subcommittee on Antifungal Susceptibility Testing.

Antifungal susceptibility data were obtained for 101 clinical isolates of *A. terreus*. As we sought to determine the lethal as well as the static activity of amphotericin B, both MICs and MLCs were determined for this agent. Moreover, prior reports indicating fungicidal activity of voriconazole against *Aspergillus* species (2) prompted us to test a random subset of isolates for MLCs. The completed study consisted of amphotericin B MIC and MLC data for 101 isolates and voriconazole MIC data for 101 isolates and MLC data for 51 isolates. The geometric means are displayed in Table 1. For amphotericin B, only 1.98% of the 48-h MICs appeared susceptible (MIC, ≤1 μg/ml), with a mean 48-h MIC of 3.37 μg/ml; MLCs were elevated beyond achievable levels, with mean 24- and 48-h concentrations of 7.03 and 13.4 μg/ml, respectively. Conversely, 48-h voriconazole MICs were all well within achievable levels, based upon a therapeutic range of 2 to 10 μg/ml (23a), with a mean 48-h MIC of 0.22 μg/ml; mean MLCs at 24 and 48 h were 5.39 and 17.4 μg/ml, respectively. For the control strain, the mean 48-h MIC and MLC were 0.38 and 2 μg/ml for amphotericin B and 0.02 and 8 μg/ml for voriconazole, respectively. The distribution of MICs and MLCs of both drugs is displayed in Table 2. The 48-h amphotericin B and voriconazole MICs at which 90% of the isolates were inhibited (MIC_{90}s) were 4 and 0.25 μg/ml, respectively. The MLC at which 90% of the isolates were killed (MLC_{90}) was 16 μg/ml for both drugs.

TABLE 2. Distribution of 48-h amphotericin B and voriconazole MICs and MLCs for *A. terreus*

Drug and concn (μg/ml)	Distribution of isolates[a] at the listed	
	MIC	MLC
Amphotericin B[b]		
1	3	
2	30	8
4	58	16
8	9	14
16	1	63
Voriconazole[c]		
0.06	4	
0.125	18	
0.25	74	
0.5	5	
1		
2		
4		2
8		12
16		37

[a] Values are numbers of isolates.
[b] Tested in antibiotic medium 3 at 35°C.
[c] Tested in RPMI 1640 at 35°C.

Despite the empiric use of amphotericin B in systemic, life-threatening mycoses, several reports have described in vitro tolerance or resistance of *Aspergillus* species (3, 22, 32, 33), particularly *A. terreus* (11, 14), and in vivo clinical failures for other genera, for which MLCs are elevated (22, 39). Although numerous factors outlined by Rex et al. (24) contribute to overall clinical efficacy with antifungal drug therapy (pharmacokinetics of the drug, general host factors, site of infection, and virulence of the pathogen), in vitro antifungal susceptibility data suggesting resistance appear useful in predicting clinical response. The survival rate for immunocompromised hosts with invasive pulmonary or disseminated aspergillosis caused by *A. terreus* is dismal (such infection is frequently fatal), and a review of the literature documents that therapy with amphotericin B frequently fails to eradicate the organism.

The in vitro data generated by this study appear to substantiate the resistance of this species of *Aspergillus* to amphotericin B. In addition to elevated MICs, MLCs were also elevated beyond levels achievable for the standard formulation of amphotericin B, suggesting a lack of fungicidal activity as well. This finding is significant, as amphotericin B is usually considered fungicidal, but it frequently fails to "kill" organisms when host immune defenses are lacking. The data for voriconazole appear more encouraging with regard to fungistatic properties. Mean 24- and 48-h MICs of 0.12 and 0.22 μg/ml, respectively, indicate activity against *A. terreus* at levels easily achievable with standard dosing regimens of voriconazole (48-h MIC_{90}, 0.25 μg/ml). Despite previous reports of the in vitro fungicidal activity of voriconazole against *Aspergillus* species (2, 8, 12), mean 24- and 48-h MLCs of 5.39 and 17.4 μg/ml, respectively, against 51 clinical isolates of *A. terreus* failed to support these data (MLC_{90}, 16 μg/ml), as values were beyond achievable levels. This result may be due in part to a lack of a consensus for the definition of MLC for antifungal testing. Our method requires a 99.9% kill rate, while other investigators defined their MLC methods as having kill rates as low as 95%. This less stringent definition would produce MLCs lower than those that we reported. In conclusion, our data appear to confirm that *A. terreus* demonstrates in vitro resistance to amphotericin B. Voriconazole has consistently low MICs, while MLCs remain high. Further animal studies and prospective in vitro-in vivo correlations are warranted.

REFERENCES

1. **Amsterdam, D.** 1996. Susceptibility testing of antimicrobials in liquid media, p. 103. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams & Wilkins, Baltimore, Md.
2. **Clancy, C. J., and M. H. Nguyen.** 1998. In vitro efficacy and fungicidal activity of voriconazole against *Aspergillus* and *Fusarium* species. Eur. J. Clin. Microbiol. Infect. Dis. **17:**573–575.
3. **Colombo, A. L., D. A. McGough, and M. G. Rinaldi.** 1993. Amphotericin B (AMB) tolerance with *Aspergillus* species, abstr. 748, p. 256. *In* Programs and abstracts of the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
4. **Das, T., P. Vyas, and S. Sharma.** 1993. *Aspergillus terreus* postoperative endophthalmitis. Br. J. Ophthalmol. **77:**386–387.
5. **Denning, D., A. De Favero, E. Gluckman, D. Norfolk, M. Ruhnke, S. Yonren, P. Troke, and N. Sarantis.** 1995. UK-109-496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: clinical efficacy in acute invasive aspergillosis, abstr. F80, p. 126. *In* Program and abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
6. **Drexler, L., M. Rytel, M. Keelan, L. I. Bonchek, and G. N. Olinger.** 1980. *Aspergillus terreus* infective endocarditis on a porcine heterograft valve. J. Thorac. Cardiovasc. Surg. **79:**269–274.
7. **Espinel-Ingroff, A.** 1998. In vitro activity of the new triazole voriconazole (UK-109-496) against opportunistic filamentous and dimorphic fungi and

common and emerging yeasts. J. Clin. Microbiol. **36:**198–199.
8. **Espinel-Ingroff, A.** 1998. Evaluation of antifungal susceptibility testing parameters for amphotericin B, itraconazole, voriconazole, SCH56592, and BMS-207147 against *Aspergillus* spp., abstr. J-7, p. 452. *In* Program and abstracts of the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
9. **George, D., P. Miniter, and V. T. Andriole.** 1996. Efficacy of UK-109496, a new azole antifungal agent, in an experimental model of invasive aspergillosis. Antimicrob. Agents Chemother. **40:**86–91.
10. **Hara, K. S., and J. H. Ryu.** 1989. Disseminated *Aspergillus terreus* infection in immunocompromised hosts. Mayo Clin. Proc. **64:**774–775.
11. **Iwen, P. C., M. E. Rupp, A. N. Langnas, E. C. Reed, and S. H. Hinrichs.** 1998. Invasive pulmonary aspergillosis due to *Aspergillus terreus*: 12-year experience and review of the literature. Clin. Infect. Dis. **26:**1092–1097.
12. **Johnson, E. M., A. Szekely, and D. W. Warnock.** 1998. In vitro fungicidal activity of voriconazole, itraconazole, and amphotericin B against filamentous fungi, abstr. J-3, p. 451. *In* Program and abstracts of the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
13. **Kalina, P. H., and R. J. Campbell.** 1991. *Aspergillus terreus* endophthalmitis in a patient with chronic lymphocytic leukemia. Arch. Ophthalmol. **109:**102–103.
14. **Lass, C., D. Niederwieser, G. Kofler, and M. Dierich.** 1996. Isolation of *Aspergillus terreus* in neutropenic patients associated with resistance to amphotericin B, abstr. 36. *In* Abstracts of Focus on Fungal Infections 6.
15. **Latham, M. N., and J. L. Carpenter.** 1982. *Aspergillus terreus*, a pathogen capable of causing infective endocarditis, pulmonary mycetoma, and allergic bronchopulmonary aspergillosis. Am. Rev. Respir. Dis. **125:**769–772.
16. **Martin, M. V., J. Yates, and C. A. Hitchcock.** 1997. Comparison of voriconazole (UK-109-496) and itraconazole in prevention and treatment of *Aspergillus fumigatus* endocarditis in guinea pigs. Antimicrob. Agents Chemother. **41:**13–16.
17. **McCarty, J. M., M. S. Flam, G. Pullen, R. Jones, and S. H. Kassel.** 1986. Outbreak of primary cutaneous aspergillosis related to intravenous arm boards. J. Pediatr. **108:**721–724.
18. **Moore, C. K., M. A. Hellreich, C. L. Coblentz, and V. L. Roggli.** 1988. *Aspergillus terreus* as a cause of invasive pulmonary aspergillosis. Chest **94:**889–891.
19. **Murphy, M., E. M. Bernard, T. Ishimaru, and D. Armstrong.** 1997. Activity of voriconazole (UK-109,496) against clinical isolates of *Aspergillus* species and its effectiveness in an experimental model of invasive pulmonary aspergillosis. Antimicrob. Agents Chemother. **41:**696–698.
20. **National Committee for Clinical Laboratory Standards.** 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard M27-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
21. **Neumeister, B., W. Hartmann, M. Oethinger, B. Heymer, and R. Marre.** 1994. A fatal infection with *Alternaria alternata* and *Aspergillus terreus* in a child with agranulocytosis of unknown origin. Mycoses **37:**181–185.
22. **Nguyen, M. H., C. J. Clancy, V. L. Yu, Y. C. Yu, A. J. Morris, D. R. Snydman, D. A. Sutton, and M. G. Rinaldi.** 1998. Do in vitro susceptibility data predict the microbiologic response to amphotericin B? Results of a prospective study of patients with *Candida* fungemia. J. Infect. Dis. **177:**425–430.
23. **Patterson, T. F., W. R. Kirkpatrick, and R. K. McAtee.** 1998. The efficacy of voriconazole in a guinea pig model of disseminated invasive aspergillosis, abstr. B-14, p. 29. *In* Program and abstracts of the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
23a. **Pfizer Inc.** Unpublished data.
24. **Rex, J. H., M. A. Pfaller, J. N. Galgiani, M. S. Bartlett, A. Espinel-Ingroff, M. A. Ghannoum, M. Lancaster, F. C. Odds, M. G. Rinaldi, T. J. Walsh, A. L. Barry, and Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards.** 1997. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. Clin. Infect. Dis. **24:**235–247.
25. **Rinaldi, M. G.** 1982. Use of potato flakes agar in clinical mycology. J. Clin. Microbiol. **15:**1159–1160.
26. **Rinaldi, M. G.** 1983. Invasive aspergillosis. Rev. Infect. Dis. **5:**1061–1077.
27. **Rinaldi, M. G., and A. W. Howell.** 1988. Antifungal antimicrobics: laboratory evaluation, p. 325–356. *In* B. Wentworth (ed.), Diagnostic procedures for mycotic and parasitic infections, 7th ed. American Public Health Association, Washington, D.C.
28. **Russack, V.** 1990. *Aspergillus terreus* myocarditis: report of a case and review of the literature. Am. J. Cardiovasc. Pathol. **3:**275–279.
29. **Schett, G., B. Casati, B. Willinger, G. Weinländer, T. Binder, F. Grabenwöger, W. Sperr, K. Geissler, and U. Jäger.** 1998. Endocarditis and aortal embolization caused by *Aspergillus terreus* in a patient with acute lymphoblastic leukemia in remission: diagnosis by peripheral-blood culture. J. Clin. Microbiol. **36:**3347–3351.
30. **Singh, S. M., S. Sharma, and P. K. Chatterjee.** 1990. Clinical and experimental mycotic keratitis caused by *Aspergillus terreus* and the effect of subconjunctival oxiconazole treatment in an animal model. Mycopathologia **112:**127–137.
31. **Suseelan, A. V., H. C. Gugnani, and J. O. Ojukwu.** 1976. Primary cutaneous aspergillosis due to *Aspergillus terreus*. Arch. Dermatol. **112:**1468.
32. **Sutton, D. A., A. W. Fothergill, and M. G. Rinaldi.** 1998. Guide to clinically significant fungi. Williams & Wilkins, Baltimore, Md.
33. **Sutton, D. A., J. H. Shin, and M. G. Rinaldi.** 1998. *In vitro* resistance of *Aspergillus terreus* to amphotericin B: data from 64 U.S./Korean clinical isolates, abstr. 15. *In* Abstracts of Focus on Fungal Infections 8. Imedex USA, Inc., Alpharetta, Ga.
34. **Thamlikitkul, V., K. Prachuabmoh, S. Sukroongreung, and S. Danchaivijitr.** 1983. *Aspergillus terreus* endocarditis—a case report. J. Med. Assoc. Thailand **66:**723–726.
35. **Tiwari, S., S. M. Singh, and S. Jain.** 1995. Chronic bilateral suppurative otitis media caused by *Aspergillus terreus*. Mycoses **38:**297–300.
36. **Tracy, S. L., M. R. McGinnis, J. E. Peacock, Jr., M. S. Cohen, and D. H. Walker.** 1983. Disseminated infection with *Aspergillus terreus*. Am. J. Clin. Pathol. **80:**728–733.
37. **Tritz, D. M., and G. L. Woods.** 1993. Fatal disseminated infection with *Aspergillus terreus* in immunocompromised hosts. Clin. Infect. Dis. **16:**118–122.
38. **van Burik, J. H., R. Colvin, and D. H. Spach.** 1998. Cutaneous aspergillosis. J. Clin. Microbiol. **36:**3115–3121.
39. **Walsh, T. J., G. P. Melcher, M. G. Rinaldi, J. Lecciones, D. McGough, J. Lee, D. Callender, M. Rubin, and P. A. Pizzo.** 1990. Disseminated trichosporonosis resistant to amphotericin B. J. Clin. Microbiol. **28:**1616–1622.