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Enhancement of the In Vitro Activity of Amphotericin B Against *Aspergillus* spp. by Tetracycline Analogs

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Strains of *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* were tested for in vitro susceptibility to amphotericin B alone and in combination with fixed concentrations of tetracycline, doxycycline, or minocycline, using buffered minimal essential medium in microtiter plates. Enhanced inhibitory activity was seen, especially with combinations of amphotericin B and minocycline. Synergistic activity between amphotericin B and minocycline was observed in each of five isolates of each species when tested in a checkerboard dilution scheme. Time-kill curves demonstrated killing an *A. fumigatus* isolated at concentrations of amphotericin B that were four- or eightfold lower in the presence of 5 or 15 μg of minocycline per ml than with amphotericin B alone. Of the tetracycline analogs tested, minocycline has the greatest activity against *A. fumigatus*, *A. flavus*, and *A. niger* conidia when potentiated by amphotericin B.

The addition of flucytosine or rifampin to amphotericin B has previously been shown (10, 13) to enhance the in vitro activity of amphotericin B against *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*, despite the apparent lack of useful activity of either flucytosine or rifampin alone against these organisms. The efficacy of these combinations was also shown in an experimental animal model of aspergillus infection (2). Amphotericin B binds to sterols in the fungal cell membrane and increases permeability (12), allowing entry and subsequent interference with RNA synthesis by flucytosine or rifampin, as shown by Medoff and co-workers against yeast cells (17).

Tetracycline inhibits yeast protein synthesis in cell extracts but not in whole cells due to its inability to penetrate the cell (4). In the presence of a subinhibitory concentration of amphotericin B, tetracycline reduces colony counts of *Saccharomyces cerevisiae* by specifically inhibiting protein synthesis (14). Amphotericin B has also previously been shown to potentiate the in vitro activity of tetracycline analogs doxycycline and minocycline against *Candida albicans* (1, 15).

In this study, we evaluated strains of *A. fumigatus*, *A. flavus*, and *A. niger* for in vitro susceptibility to combinations of amphotericin B with tetracycline, doxycycline, or minocycline. In addition, the synergistic and fungicidal potentials of these drug combinations were determined.

MATERIALS AND METHODS

Organisms. Strains of *A. fumigatus*, *A. flavus*, and *A. niger* were obtained from clinical isolates collected by the mycology laboratories at the Minneapolis Veterans Administration Medical Center and the University of Minnesota Hospital.

Drugs. Stock aqueous solutions of amphotericin B (Fungizone; E. R. Squibb & Sons, Inc., New Brunswick, N.J.), doxycycline hyclate (Pfizer Inc., New York, N.Y.), tetracycline hydrochloride (Bristol Laboratories, Syracuse, N.Y.), and minocycline hydrochloride (Lederle Laboratories, Pearl River, N.Y.) were diluted in water to the appropriate concentrations and diluted 100-fold in media for dispensation into microtiter plates.

Preparation of microtiter plates. Plates (Dynatech Laboratories, Inc., Alexandria, Va.) were dispensed with an MIC-2000 98-channel dispenser, which dispensed 0.1 ml of drug-containing media into each well. Each row contained amphotericin B in doubling dilutions from 50 to 0.05 μg/ml. In combination rows, a constant amount of tetracycline, minocycline, or doxycycline at 5 or 15 μg/ml was added to each well. Tetracycline analogs were tested alone at concentrations of 100, 50, 25, 15, and 5 μg/ml. Growth control wells contained media with and without desoxycholate, an additive in the amphotericin B preparation.

Media. Microtiter plates were prepared with Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) with a final pH of 6.5. Our previous studies (10) have shown this medium to produce comparable, although slightly higher, MICs against *Aspergillus* spp. than buffered yeast nitrogen base in testing with a variety of antifungal agents and combinations.

Inoculation. Strains of *A. fumigatus* (12), *A. flavus* (5), and *A. niger* (5) were grown on potato glucose agar at 37°C for 3 to 7 days. Conidia were harvested by lightly swabbing the surface of the plate and suspended in saline; the resulting suspensions contained virtually all conidia by microscopic examination. The suspensions were then shaken in saline with glass beads, washed twice, and suspended in saline; final suspensions were adjusted to 95% transmittance at 530 nm (~5×10^5 to 1×10^6 spores per ml by plate count). Microtiter plates were inoculated in duplicate with each suspension by using a disposable inoculator that delivered 0.01 ml into each well. Plates were incubated at 37°C for 24 h.

Susceptibility determination. Microtiter plates were examined for fungal growth at 24 h. The MIC was defined as the first well showing no visible growth.

Killing curves. Suspensions of a typical *A. fumigatus* isolate were inoculated in duplicate into microtiter plates as described above. At 0 and 4 h, entire wells (100 μl) were emptied into 10 ml of saline and vigorously shaken, and 100-μl samples were plated onto blood agar plates for counting. At 24 h, entire wells showing no visible growth

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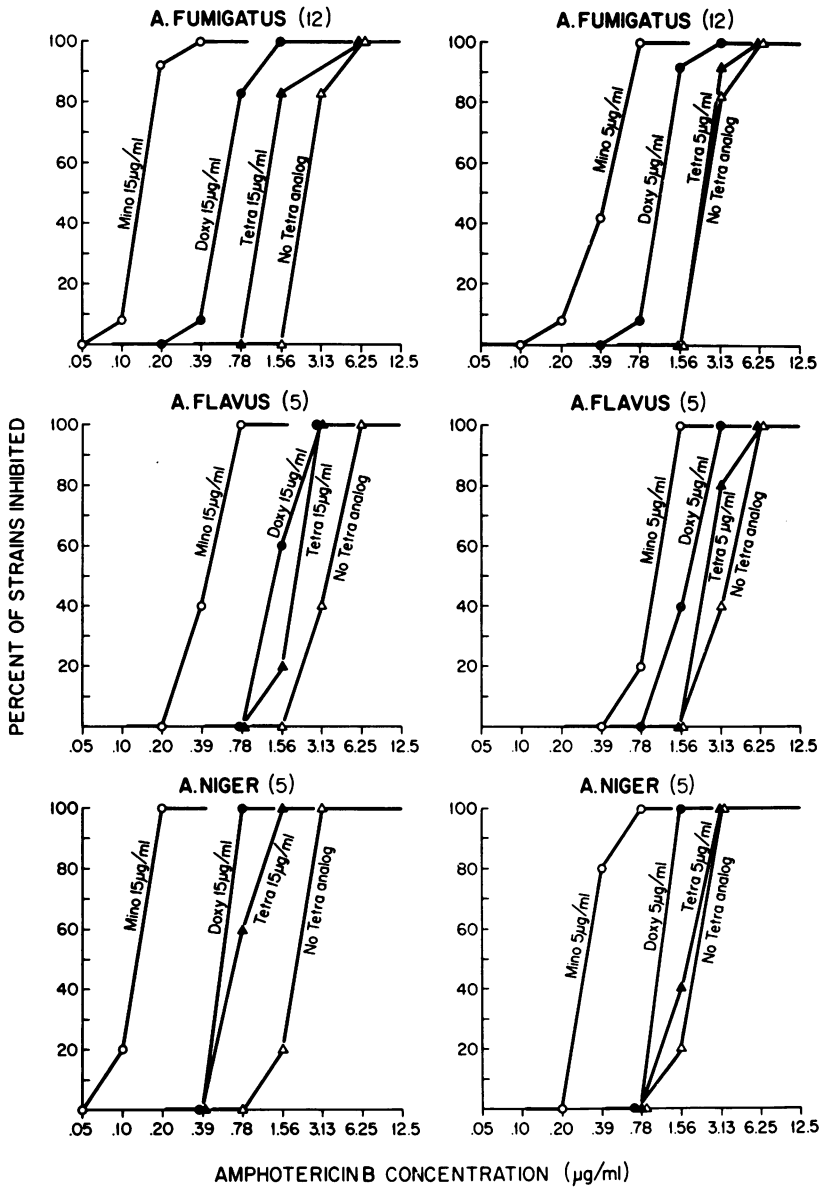

FIG. 1. Percentage of *Aspergillus* strains inhibited by increasing concentrations of amphotericin B alone and in the presence of minocycline (Mino), doxycycline (Doxy), or tetracycline (Tetra) at a concentration of 15 or 5 μg/ml. The number of strains tested is in parentheses.

were plated directly onto blood agar plates; wells showing visible growth were diluted 1:10 and 1:100 with saline and vigorously shaken, and 100-μl samples were plated onto blood agar plates. Plates were counted when colonies were large enough to read, after ca. 36 h of incubation at 37°C.

Synergism studies. Five isolates each of *A. fumigatus*, *A. flavus*, and *A. niger* were inoculated in duplicate into microtiter plates containing a checkerboard dilution scheme with doubling dilutions of amphotericin B (0.05 to 50 μg/ml) and minocycline (3.9 to 15.6 and 250 to 2,000 μg/ml). MICs were read after incubation at 37°C for 24 h. The fractional inhibitory concentration (FIC) was determined for each row of the checkerboard and was defined as the MIC of a drug in combination divided by the MIC of the drug alone (9). Synergism was defined as the sum of FICs (ΣFIC) being ≤0.5; antagonism was defined as ΣFIC ≥ 2.0.

RESULTS

Figure 1 shows the cumulative percentage of strains inhibited by doubling concentrations of amphotericin B alone and with each of the tetracycline analogs at concentrations of 15 or 5 μg/ml for *A. fumigatus*, *A. flavus*, and *A. niger*. There was no inhibition of growth by any concentration of tetracycline analog alone or by desoxycholate. The endpoints were easily read.

Killing curves for amphotericin B alone and with 5 μg of each tetracycline analog per ml against an *A. fumigatus* isolate are shown in Fig. 2. Cidal activity was achieved at fourfold-lower levels of amphotericin B in the presence of minocycline but not the other tetracyclines tested. Killing was observed at even lower levels of amphotericin B in the presence of 15 μg of minocycline per ml (Fig. 3). With

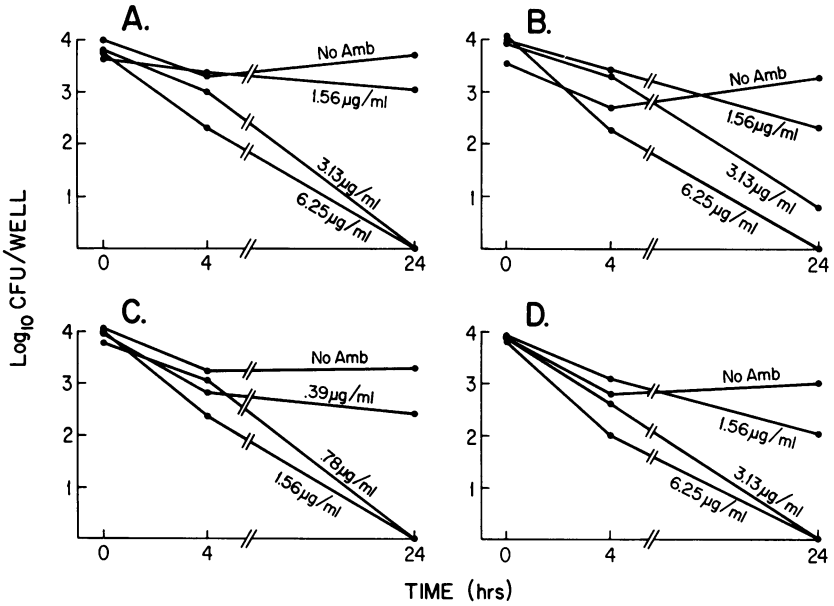

FIG. 2. Killing of an *A. fumigatus* strain by stated concentration of amphotericin B (Amb) alone (A) or in the presence of a 5-μg/ml concentration of tetracycline (B), minocycline (C), or doxycycline (D).

doxycycline at a concentration of 15 μg/ml (data not shown), a twofold reduction in amphotericin B concentration required for cidal activity was seen. There was generally good agreement between replicate plate counts and 10-fold dilutions.

The checkerboard studies demonstrated synergism between amphotericin B and minocycline against all five isolates of *A. fumigatus*, *A. flavus*, and *A. niger* (Table 1). The lowest ΣFICs were observed in the rows containing 7.8 or 15.6 μg of minocycline per ml.

DISCUSSION

In a study similar to ours with *C. albicans*, Lew et al. (15) found the greatest synergistic inhibitory activity between amphotericin B and minocycline. Doxycycline was intermediate, and tetracycline was potentiated least by amphotericin B. We found the same order of activity against *Aspergillus* spp. Interestingly, the lipophilic nature of the drugs, as measured by their apparent partition coefficients, is greatest for minocycline; doxycycline is intermediate, and tetracycline is least lipophilic (3). In addition, minocycline is unique in that it is most lipophilic at a near-neutral pH, which may account for its relatively greater penetration into brain, thyroid, and fat tissues. Amphotericin B is thought to interact with sterols in fungal cell membranes, forming complexes that allow enhanced cell membrane permeability (16). The lipophilic nature of minocycline may allow entry into cells that are relatively less permeable, perhaps by interacting with the free fatty acids that are found in fungal cell membranes (8).

There has been concern that tetracycline and analogs may have a detrimental effect on the phagocytic and chemotactic activities of leukocytes (5). Recent work indicates that these in vitro effects are mediated by the divalent cation-chelating activity of the drugs (6) and are highly dependent on cation concentrations. Hence, the in vivo significance of these observations is not known. The small amount of in vivo data addressing amphotericin B-tetracycline combinations against experimental infections with organisms other than *Aspergillus* spp. suggests additive activity (11, 18) or no difference (7) when compared with amphotericin B alone.

Aspergillus infections present formidable problems in therapy due to the toxicity and marginal efficacy of amphotericin B administration. The addition of flucytosine to amphotericin B adds the risk of bone marrow suppression in patients who are often already neutropenic. Thus, there is a need for less toxic agents which have activity against *Aspergillus* spp. when potentiated by amphotericin B. Based on our study and previous work with other fungi, evaluation of in vivo

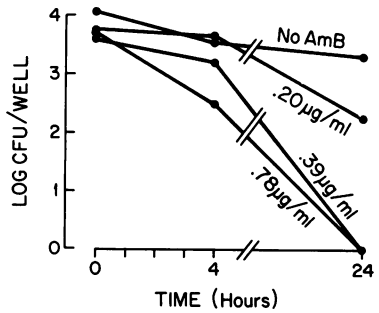

FIG. 3. Killing of same *A. fumigatus* strain as that shown in Fig. 2 by stated concentration of amphotericin B (AmB) in the presence of 15 μg of minocycline per ml.

TABLE 1. ΣFICs[a] for amphotericin B and minocycline against *Aspergillus* spp.[b]

Minocycline concn (μg/ml)	Mean ΣFIC[c] (range) against:		
	A. fumigatus	*A. flavus*	*A. niger*
3.9	0.45 (0.25–0.50)	0.45 (0.25–0.50)	0.70 (0.50–1.00)
7.8	0.09 (0.07–0.13)	0.30 (0.25–0.50)	0.12 (0.07–0.13)
15.6	0.08 (0.04–0.13)	0.21 (0.13–0.26)	0.09 (0.07–0.13)

[a] Sum of FICs.
[b] $n = 5$ for each species.
[c] Measured in micrograms per milliliter.

efficacy of minocycline and amphotericin B combinations in an experimental model of *Aspergillus* infection seems warranted.

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LITERATURE CITED

1. **Ansehn, S., S. Granstrom, H. Hojer, L. Nilsson, E. Akesson, A. Lundin, and A. Thore.** 1976. *In vitro* effects of *Candida albicans* of amphotericin B combined with other antibiotics. Scand. J. Infect. Dis. Suppl. **9**:S62–66.
2. **Arroyo, J., G. Medoff, and G. S. Kobayashi.** 1977. Therapy of murine aspergillosis with amphotericin B in combination with rifampin or 5-fluorocytosine. Antimicrob. Agents Chemother. **11**:21–25.
3. **Barringer, W. C., W. Schultz, G. Sieger, and R. Nash.** 1974. Minocycline hydrochloride and its relationship to other tetracycline antibiotics. Am. J. Pharm. **146**:179–191.
4. **Battaner, E., and D. Vasquez.** 1971. Inhibitors of protein synthesis by ribosomes of the 8-S type. Biochim. Biophys. Acta **254**:316–330.
5. **Forsgren, A., D. Schmeling, and P. G. Quie.** 1974. Effect of tetracycline on the phagocytic function of human leukocytes. J. Infect. Dis. **130**:412–415.
6. **Glette, J., S. Sandberg, G. Hopen, and C. O. Solberg.** 1984. Influence of tetracyclines on human polymorphonuclear leukocyte function. Antimicrob. Agents Chemother. **25**:354–357.
7. **Graybill, J. R., and L. Mitchell.** 1980. Treatment of murine cryptococcosis with minocycline and amphotericin B. Sabouraudia **18**:137–144.
8. **Griffin, D. H.** 1981. Fungal physiology, p. 47. John Wiley & Sons, Inc., New York.
9. **Hallander, H. O., K. Dornbusch, L. Gezelius, K. Jacobson, and I. Karlsson.** 1982. Synergism between aminoglycosides and cephalosporins with antipseudomonal activity: interaction index and killing curve method. Antimicrob. Agents Chemother. **22**:743–752.
10. **Hughes, C. E., C. Harris, J. A. Moody. L. R. Peterson, and D. N. Gerding.** 1984. In vitro activities of amphotericin B in combination with four antifungal agents and rifampin against *Aspergillus* spp. Antimicrob. Agents Chemother. **25**:560–562.
11. **Huppert, M., S. H. Sun, and K. R. Vukovich.** 1974. Combined amphotericin B-tetracycline therapy for experimental coccidioidomycosis. Antimicrob. Agents Chemother. **5**:473–478.
12. **Kinsky, S. C.** 1970. Antibiotic interactions with model membranes. Annu. Rev. Pharmacol. **10**:119–142.
13. **Kitahara, M., V. K. Seth, G. Medoff, and G. S. Kobayashi.** 1976. Activity of amphotericin B, 5-fluorocytosine, and rifampin against six clinical isolates of *Aspergillus*. Antimicrob. Agents Chemother. **9**:915–919.
14. **Kwan, C. N., G. Medoff, G. S. Kobayashi, D. Schlessinger, and H. J. Raskas.** 1972. Potentiation of the antifungal effects of antibiotics by amphotericin B. Antimicrob. Agents Chemother. **2**:61–65.
15. **Lew, M. A., K. M. Beckett, and M. S. Levin.** 1977. Antifungal activity of four tetracycline analogues *in vitro*: potentiation by amphotericin B. J. Infect. Dis. **136**:263–270.
16. **Medoff, G., J. Brayburg, and G. S. Kobayashi.** 1983. Antifungal agents in therapy of systemic fungal infections. Annu. Rev. Pharmacol. Toxicol. **23**:303–330.
17. **Medoff, G., G. S. Kobayashi, C. N. Kwan, D. Schlessinger, and P. Venkov.** 1972. Potentiation of rifampicin and 5-fluorocytosine as antifungal antibiotics by amphotericin B. Proc. Natl. Acad. Sci. U.S.A. **69**:196–199.
18. **Thong, Y. H., B. Rowan-Kelly, A. Ferrante, and C. Shephard.** 1978. Synergism between tetracycline and amphotericin B in experimental amoebic encephalitis. Med. J. Aust. **1**:663–664.