Comparative In Vitro Antifungal Activity of Amphotericin B and Amphotericin B Methyl Ester

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Received for publication 26 August 1974

The in vitro antifungal activity of amphotericin B methyl ester (AME), a water-soluble derivative of amphotericin B, was compared to that of the parent compound against a variety of pathogenic and potentially pathogenic fungi. AME has a significant antifungal activity, but the activity of AME was slightly lower than that of amphotericin B. Among the yeast-like organisms, only the yeast cells of Sporothrix schenckii were more resistant than others to both antibiotics, with a minimal fungicidal concentration of 5 to 10 µg/ml. The yeast cells of other fungi were killed at concentrations of 1 µg or less of either antibiotic per ml. The filamentous forms of S. schenckii and Oidiodendron kairai were more resistant than the filamentous forms of other dimorphic fungi to both drugs. The minimal fungicidal concentration for S. schenckii was 10 µg/ml and for O. kairai, 50 µg/ml. The dermatophytes, phycomycetes, and dematocous and other potentially pathogenic fungi were inhibited fairly well by both drugs, but up to 50 µg/ml was required for fungicidal action. The water solubility and wide spectrum of antifungal activity of AME warrant evaluation of its chemotherapeutic activity against experimental fungal infections.

Amphotericin B, a polyene antibiotic produced by Streptomyces nodosus, is the most effective agent currently available for the treatment of many systemic mycoses. The drug is administered in a dispersed form (Fungizone), using sodium deoxycholate as the dispersing agent. The utility of amphotericin B, however, is seriously hampered by its nephrotoxicity and other side effects (2, 4, 10, 11), which in part may be associated with its insolubility in water and other body fluids. Previous attempts in the chemical alterations of the polyene antibiotic, with the hope of reducing the toxicity, have not been successful (2, 5). Chemical changes in the antibiotic, resulting in increased solubility or decreased toxicity, were usually associated with a decrease in antifungal activity.

Recently, Mechlinski and Schaffner (6) have prepared amphotericin B methyl ester (AME) hydrochloride, a water-soluble derivative of amphotericin B. AME when dissolved in water formed micelles, but the degree of dispersion was significantly higher than that of Fungizone (1). AME hydrochloride has been shown in experimental animals to be significantly less toxic than the parent compound (3).

The lower toxicity and higher solubility of AME and preliminary demonstration of its antifungal activity against Saccharomyces cerevisiae and Candida albicans (1) led us to a more comprehensive study of the antifungal activity of the new derivative. The study reported here describes the comparative in vitro antifungal activity of AME with that of amphotericin B against a number of pathogenic and potentially pathogenic fungi.

MATERIALS AND METHODS

Cultures. Sixty-one isolates of pathogenic and potentially pathogenic fungi used for susceptibility testing were obtained from our stock culture collection. These included: Aspergillus fumigatus, A. niger, Blastomyces dermatitidis, C. albicans, C. guillermondii, C. pseudotropicalis, Cladosporium sp., Coccioides immittis, Cryptococcus neoformans, Histoplasma capsulatum, H. duboisii, Microsporum canis, M. gypseum, Mucor pusillus, Oidiodendron kairai, Paracoccidioides brasiliensis, Phialophora compactum, P. dermatitidis, P. verrucosa, Rhizopus arrhizus, Sepedonium sp., Sporothrix schenckii, Syncephalastrum, Torulopsis glabrata, Trichophyton mentagrophytes, T. rubrum, and T. simii.

The yeast-phase cultures were maintained on modified brain heart infusion agar slants (Difco) supplemented with 1.0% glucose and 0.1% l-cysteine hydrochloride. Cultures were maintained at 4 C and transferred every 3 weeks. The mycelial-phase cultures were maintained on Sabouraud dextrose agar slants at 4 C and transferred every 3 months.

Media. The antibiotic susceptibility was per-
formed in a synthetic medium (pH 7.2) (8) with the following composition (grams per liter): K$_2$HPO$_4$, 2.5; NH$_4$Cl, 0.5; (NH$_4$)$_2$SO$_4$, 0.5; L-cysteine hydrochloride, 0.1; MgSO$_4$·7H$_2$O, 0.1; glucose, 5.0; glutamine, 0.1; and FeCl$_3$, 0.001. The solid synthetic medium contained 1.5% agar (Difco). The medium was dispensed in 100-ml quantities into 250-ml Erlenmeyer flasks and sterilized by autoclaving.

Preparation of inoculum. The yeast cells were grown on brain heart infusion agar slants at 37 C for 24 to 48 h depending on the organism. The growth was harvested with 10 ml of sterile saline, and 2 ml of this suspension was inoculated into flasks containing 50 ml of the liquid synthetic medium. The flasks were incubated at 37 C for 24 to 48 h on a gyratory shaker (New Brunswick Scientific Co.) with a shaking speed of 150 rpm. The cultures were harvested, washed twice with cold sterile 0.9% NaCl, and centrifuged in a refrigerated Sorvall centrifuge at 30 x g for 1 min to remove aggregated yeast cells.

The mycelial-phase cultures were grown on Sabouraud dextrose agar slants at 25 C for 1 to 4 weeks, depending on the organism. The cultures were harvested with sterile 0.9% NaCl and shaken in sterile vaccine bottles containing glass beads to obtain a uniform suspension. Both yeast and mycelial cell suspensions were standardized turbidimetrically to 0.5 optical density at 550 nm using a Spectronic 20 spectrophotometer (Bausch & Lomb).

Preparation of antibiotic dilutions. Amphotericin B and AME hydrochloride were dissolved in sterile dimethylsulfoxide and diluted with distilled water to obtain solutions containing 10,000 to 1.0 μg of the antibiotic per ml. Appropriate amounts of these solutions were dispensed into flasks containing 100 ml of the liquid and solid synthetic media to obtain final concentrations of the drugs ranging from 50.0 to 0.01 μg/ml. The liquid medium containing antibiotics was dispensed in 5-ml quantities into sterile test tubes, and the solid medium was poured into sterile petri dishes.

Susceptibility testing. Both tube and agar dilution techniques were used to determine the antibiotic susceptibility. The standardized cell suspension in 0.05-ml quantities was inoculated with a calibrated loop into liquid and solid media containing varying amounts of the antibiotics. Controls included: uninoculated sterility control, drug- and solvent-free growth controls, and growth controls containing 1% of the solvent. All tests were performed in triplicate.

Inoculated tubes and plates were incubated at 30 C in the dark until growth appeared in the controls. The minimal time of incubation was 48 h. After determining the minimal inhibitory concentrations (MIC), 0.05 ml of agar or broth was transferred from the test media showing no growth and from the first cultures in which growth was detectable to the synthetic agar plates. The plates were incubated at 30 C for 72 h or until growth appeared on the plates inoculated from test media containing visible growth.

The criteria used to determine the MIC and minimal fungicidal concentrations (MFC) were adapted from those described by Shadomy (8). The lowest concentration of antibiotic which completely inhibited growth in at least two of three test cultures was considered as the MIC. The lowest concentration of antibiotic for which subcultures from at least two of three initial test cultures were negative was regarded as the MFC.

RESULTS

The results showing the MIC and the MFC obtained by tube and agar dilution techniques have been arranged in two categories, the first showing the antifungal activity against the organisms in yeast form (Tables 1 and 2) and the second against organisms in mycelial form (Tables 3 and 4). The values obtained by agar and broth dilutions were similar.

AME was slightly less active against yeast cells of certain fungi, whereas with others the activity was comparable to that of amphotericin B (Table 1). Four isolates of H. capsulatum were inhibited by 0.05 μg of amphotericin B per ml and 0.1 μg of AME per ml. The MFC of AME was 0.5 μg/ml and that of amphotericin B was 0.1 μg/ml. B. dermatitidis and P. brasiliensis were less susceptible than Histoplasma to both antibiotics. Blastomyces and Paracoccidioides were inhibited at 0.1 μg of amphotericin B and 0.1 to 0.5 μg of AME per ml. Among the dimorphic fungi, the yeast form of S. schenckii was more resistant to both antibiotics. The MFC values of the two drugs ranged from 5 to 10 μg/ml. For O. kalrai, the two drugs were equally effective with an MIC of 0.5 μg/ml and an MFC of 1.0 μg/ml. Six isolates of C. albicans and two isolates of C. guilliermondii were equally susceptible to the antifungal activities of AME and amphotericin B. Both drugs were inhibitory at 0.5 μg/ml and fungicidal at 0.5 to 1.0 μg/ml. However, C. tropicalis and C. pseudotropicalis were slightly less susceptible to AME. Two isolates of C. tropicalis were inhibited by 0.1 μg of amphotericin B per ml as compared with 0.5 μg of AME per ml. The MICs of amphotericin B and AME for C. pseudotropicalis were 0.5 and 1.0 μg/ml, respectively. Four isolates of Cryptococcus neoformans were inhibited by 0.1 to 0.5 μg of each antibiotic per ml, whereas the MIC for Torulopsis glabrata was 0.5 μg/ml. Both antibiotics were fungicidal at 1.0 μg/ml for these organisms.

The filamentous fungi were more resistant than the yeast-like organisms to both antibiotics (Table 3). The MIC of amphotericin B ranged from 0.1 to 0.5 μg/ml for six isolates of H. capsulatum. The inhibitory concentration of AME was 0.1 to 1.0 μg/ml. H. duboisii, B. dermatitidis, P. brasiliensis, and Coccidioides immitis were equally susceptible to am-
TABLE 1. *In vitro* susceptibility of yeast-like organisms to amphotericin B and AME by the agar dilution technique

| Test organisms               | Strains tested | Susceptibility to Amphotericin B (MIC (μg/ml)* | MFC (μg/ml) | AME (MIC (μg/ml) | MFC (μg/ml) |
|------------------------------|----------------|----------------------------------------------|-------------|-----------------|-------------|
| *H. capsulatum*              | 4              | 0.05                                         | 0.1         | 0.1             | 0.5         |
| *B. dermatitidis*            | 2              | 0.1                                          | 0.5-1.0     | 0.1-0.5         | 1.0         |
| *P. brasiliensis*            | 1              | 0.5                                          | 0.5         | 0.5             | 1.0         |
| *S. schenckii*               | 2              | 0.5-1.0                                      | 5.0-10.0    | 1.0             | 10.0        |
| *O. krail*                   | 1              | 0.5                                          | 1.0         | 0.5             | 1.0         |
| *C. albicans*                | 6              | 0.5                                          | 0.5-1.0     | 0.5             | 0.5-1.0     |
| *C. tropicalis*              | 2              | 0.5                                          | 1.0         | 0.5             | 1.0         |
| *C. pseudotropicalis*        | 2              | 0.5                                          | 1.0         | 0.5             | 1.0         |
| *C. guilliermondii*          | 2              | 0.5                                          | 1.0         | 0.1-0.5         | 1.0         |
| *Cryptococcus neoformans*    | 4              | 0.1-0.5                                      | 0.5-1.0     | 0.1-0.5         | 1.0         |
| *T. glabrata*                | 1              | 0.5                                          | 1.0         | 0.5             | 1.0         |

* MIC was measured after 2 to 3 days of incubation at 30 C and MFC after an additional 2 to 3 days of incubation following subculture to antibiotic-free media.

TABLE 2. *In vitro* susceptibility of yeast-like organisms to amphotericin B and AME by tube dilution technique

| Test organisms               | Strains tested | Susceptibility to Amphotericin B (MIC (μg/ml)* | MFC (μg/ml) | AME (MIC (μg/ml) | MFC (μg/ml) |
|------------------------------|----------------|----------------------------------------------|-------------|-----------------|-------------|
| *H. capsulatum*              | 4              | 0.05                                         | 0.1         | 0.1             | 0.5         |
| *B. dermatitidis*            | 2              | 0.1                                          | 0.5-1.0     | 0.1-0.5         | 1.0         |
| *P. brasiliensis*            | 1              | 0.5                                          | 1.0         | 0.5             | 1.0         |
| *S. schenckii*               | 2              | 0.5-1.0                                      | 10.0        | 1.0             | 10.0        |
| *O. krail*                   | 1              | 0.5                                          | 1.0         | 0.5             | 1.0         |
| *C. albicans*                | 6              | 0.5                                          | 0.5-1.0     | 0.5             | 1.0         |
| *C. tropicalis*              | 2              | 0.1                                          | 0.5         | 0.5             | 0.5         |
| *C. pseudotropicalis*        | 1              | 0.5                                          | 1.0         | 0.5             | 1.0         |
| *C. guilliermondii*          | 2              | 0.5                                          | 1.0         | 0.5             | 1.0         |
| *Cryptococcus neoformans*    | 4              | 0.1-0.5                                      | 0.5-1.0     | 0.5             | 1.0         |
| *T. glabrata*                | 1              | 0.5                                          | 1.0         | 0.5             | 1.0         |

* MIC was measured after 2 to 3 days of incubation at 30 C and MFC after an additional 2 to 3 days of incubation following subculture to antibiotic-free media.

Amphotericin B and AME. Both drugs were inhibitory at a concentration of 0.5 μg/ml or less and fungicidal at 1.0 μg/ml. Among the dimorphic fungi, the filamentous forms of *S. schenckii* and *O. krail* were less susceptible to both antibiotics. One isolate of *S. schenckii* was inhibited by 0.5 μg of both antibiotics per ml, whereas the other was inhibited by 5 μg of amphotericin B and 10 μg of AME per ml. The MFC of the two antibiotics for the mycelial form of *Sporothrix* was 10 μg/ml. For *O. krail*, the MICs of amphotericin B and AME were 5 and 10 μg/ml, respectively. The fungicidal concentration of both antibiotics was 50 μg/ml.

The phycomycetes, *Aspergillus* and *Sepedonium*, were relatively more resistant to the antifungal activity of amphotericin B and AME, but amphotericin B was more effective against these fungi. For five isolates of *Rhizopus*, the MIC of amphotericin B was 0.5 μg/ml as compared with 5 μg/ml of AME. Amphotericin B inhibited both species of *Aspergillus* at 0.5 μg/ml, whereas 1 to 5 μg of AME per ml was required to inhibit growth. *Mucor pusillus* and *Syncephalastrum* sp., the other two phycomycetes, were more susceptible to amphotericin B than AME. The difference in the fungicidal activity of the two compounds was not as significant, and up to 50 μg of either antibiotic per ml was required for fungicidal action. The antibiotic susceptibilities of dermatophytes and dematacious fungi were comparable to those of phycomycetes. The MIC of amphotericin B ranged from 0.1 to 0.5 μg/ml.
and that of AME from 0.1 to 1.0 µg/ml. With the exception of *Phialophora compactum*, the MFCs of amphotericin B and AME ranged from 5 to 50 µg/ml. The MFC of amphotericin B for *P. compactum* was 0.5 µg/ml and that of AME was 1.0 µg/ml.

**DISCUSSION**

The results presented demonstrate that AME, a water-soluble derivative of amphotericin B, has significant antifungal activity against a variety of pathogenic and potentially pathogenic fungi. However, the antifungal activity of AME was slightly lower than that of the parent compound. Among the yeast-like organisms, only the yeast cells of *S. schenckii* were less susceptible than others to both antibiotics with MFCs of 5 to 10 µg/ml. The yeast cells of other fungi were killed at concentrations of 1 µg or less of either antibiotic per ml. The filamentous forms of *S. schenckii* and *O. kalaiae* were more resistant than the filamentous forms of other dimorphic fungi to both drugs. The MFC of the two antibiotics for *S. schenckii* was 10 µg/ml and for *O. kalaiae*, 50 µg/ml. The dermatophytes and dematacious and other potentially pathogenic fungi were inhibited fairly well by both drugs, but up to 50 µg/ml were required for fungicidal action. These findings extend the earlier report of the antifungal activity of AME against *S. cerevisiae* and *C. albicans* (1).

* S. schenckii and *Allescheria boydii* have been shown to be more resistant than other fungi to amphotericin B (8). The MFC of amphotericin B for *S. schenckii* was 25 µg/ml, and it was greater than 100 µg/ml for *A. boydii*. In the present study, amphotericin B and its water-soluble derivative (AME) also were less effective against *S. schenckii*, *O. kalaiae*, phycymycetes, and dematacious and certain other opportunistic fungi. The reasons for the differences in the susceptibility of different fungi for amphotericin B and AME are not known.

The lower antifungal activity of AME as compared with the parent compound could be due to lower stability or modification of chemical structure necessary for antifungal activity. Our observations on the inhibitory action of amphotericin B and AME on macromolecular synthesis by yeast cells of *H. capsulatum* support the contention that the lower antifungal activity of AME is due to decreased stability (unpublished data). Both antibiotics inhibited the incorporation of radioactive precursors into

| Test organisms       | Strains tested |Susceptibility to Amphotericin B | AME |
|----------------------|---------------|---------------------------------|-----|
|                      |               | MIC (µg/ml)*                     | MFC (µg/ml)* | MIC (µg/ml) | MFC (µg/ml) |
| *H. capsulatum*      | 6             | 0.1-0.5                         | 0.5-5.0    | 0.1-1.0    | 0.5-5.0    |
| *H. dubosii*         | 1             | 0.1                             | 0.5        | 0.1        | 0.5        |
| *B. dermatitidis*    | 2             | 0.1-0.5                         | 0.5-1.0    | 0.1-0.5    | 0.5-1.0    |
| *S. schenckii*       | 2             | 0.5-5.0                         | 10.0       | 0.5-10.0   | 10.0       |
| *O. kalaiae*         | 1             | 5.0                             | 50.0       | 10.0       | 50.0       |
| *P. brasiliensis*    | 1             | 0.1                             | 0.5        | 0.5        | 1.0        |
| *C. immitis*         | 2             | 0.5                             | 1.0        | 0.5        | 1.0        |
| *Rhizopus sp.*       | 3             | 0.5                             | 50.0       | 5.0        | 50.0       |
| *R. arrhizus*        | 2             | 0.5                             | 50.0       | 5.0        | 50.0       |
| *A. niger*           | 1             | 0.5                             | 10.0       | 5.0        | 10.0       |
| *A. fumigatus*       | 1             | 0.5                             | 5.0        | 1.0        | 5.0        |
| *M. pusillus*        | 1             | 0.1                             | 10.0       | 0.5        | 50.0       |
| *Sepedonium sp.*     | 1             | 0.5                             | 1.0        | 0.5        | 10.0       |
| *Syncephalastrum sp.*| 1             | 0.1                             | 10.0       | 0.5        | 50.0       |
| *P. compactum*       | 1             | 0.1                             | 0.5        | 0.1        | 1.0        |
| *P. dermatitidis*    | 1             | 0.5                             | 50.0       | 0.5        | 50.0       |
| *P. verrucosa*       | 1             | 0.5                             | 5.0        | 1.0        | 50.0       |
| *Cladosporum sp.*    | 1             | 0.5                             | 50.0       | 1.0        | 50.0       |
| *M. canis*           | 1             | 0.5                             | 5.0        | 1.0        | 10.0       |
| *M. gypseum*         | 1             | 0.5                             | 5.0        | 1.0        | 5.0        |
| *T. mentagrophytes*  | 1             | 0.5                             | 5.0        | 0.5        | 10.0       |
| *T. rubrum*          | 1             | 0.5                             | 10.0       | 0.5        | 10.0       |
| *T. simii*           | 1             | 0.5                             | 10.0       | 0.5        | 10.0       |

*a* MIC was measured after 2 to 7 days of incubation at 30°C and MFC after an additional 2 to 7 days of incubation following subculture to antibiotic-free media.
Table 4. In vitro susceptibility of fungi in mycelial form to amphotericin B and AME by tube dilution techniques

| Test organism      | Strains tested | Susceptibility to | AME |
|--------------------|----------------|-------------------|-----|
|                    |                | Amphotericin B    | MFC (µg/ml)* | MIC (µg/ml) | MFC (µg/ml) |
| H. capsulatum      | 6              | 0.1-0.5           | 0.5-1.0      | 0.1-0.5     | 0.5-1.0     |
| H. dubosii         | 1              | 0.1               | 0.5          | 0.1         | 1.0         |
| B. dermatitidis    | 2              | 0.1-0.5           | 0.1-1.0      | 0.1-0.5     | 0.5-1.0     |
| S. schenckii       | 2              | 0.5-1.0           | 5.0-10.0     | 1.0-5.0     | 10.0        |
| O. kairai          | 1              | 1.0               | 10.0         | 5.0         | 50.0        |
| P. brasiliensis    | 2              | 0.5               | 0.5          | 0.5         | 1.0         |
| C. immitis         | 1              | 0.5               | 1.0          | 0.5         | 1.0         |
| Rhizopus sp.       | 3              | 0.5               | 50.0         | 5.0         | 50.0        |
| R. arrhizus        | 2              | 0.5               | 10.0         | 5.0         | 50.0        |
| A. niger           | 1              | 0.5               | 10.0         | 1.0         | 10.0        |
| A. fumigatus       | 1              | 0.5               | 5.0          | 1.0         | 5.0         |
| M. pusillus        | 1              | 0.1               | 5.0          | 0.5         | 1.0         |
| Sepedonium sp.     | 1              | 0.1               | 1.0-5.0      | 0.5         | 10.0        |
| Syncephalastrum sp.| 1              | 0.1               | 10.0         | 0.5         | 50.0        |
| P. compactum       | 1              | 0.1               | 1.0          | 0.5         | 1.0         |
| P. dermatitidis    | 1              | 0.5               | 10.0         | 0.5         | 50.0        |
| P. verrucosum      | 1              | 0.5               | 10.0         | 1.0         | 10.0        |
| Cladosporum sp.    | 1              | 0.5               | 10.0         | 1.0         | 10.0        |
| M. canis           | 1              | 0.5               | 5.0          | 0.5         | 1.0         |
| M. gypseum         | 1              | 0.5               | 5.0          | 0.5         | 1.0         |
| T. mentagrophytes  | 1              | 0.5               | 5.0          | 1.0         | 10.0        |
| T. rubrum          | 1              | 0.5               | 5.0          | 1.0         | 10.0        |
| T. simii           | 1              | 0.5               | 10.0         | 1.0         | 10.0        |

* MIC was measured after 2 to 7 days of incubation at 30 C and MFC after an additional 2 to 7 days of incubation following subculture to antibiotic-free media.

Preliminary studies indicated that amphotericin B and AME have similar antifungal activities, which are studied in the present study. The agar dilution technique appears preferable because it permits reliable testing of higher concentrations of antibiotics as compared with the broth dilution technique.

The retention of the wide spectrum of antifungal activity of amphotericin B and the water solubility and the significant lower toxicity of AME in experimental animals (3) warrant evaluation of its chemotherapeutic activity against a variety of experimental fungal infections.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grants AI-11094 from the National Institute of Allergy and Infectious Diseases and FR-7068 from the Division of Research Facilities and Resources.

We are indebted to William Brown, the Squibb Institute for Medical Research, for the supply of amphotericin B and to Carl Schaffner, Rutgers University, for amphotericin B methyl ester.

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