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Antiviral Effects of Amphotericin B Methyl Ester

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The methyl ester of amphotericin B (AME) is water soluble, retains antifungal activity, and is significantly less toxic in mammals than amphotericin B. In contrast to amphotericin B, which is not water soluble, AME exhibits antiviral effects against vesicular stomatitis virus, herpes simplex virus types 1 and 2, Sindbis virus, and vaccinia virus in a plaque reduction assay. No antiviral effects could be demonstrated against the unenveloped adenovirus type 4 or echovirus type 11. The extent of virus inactivation was found to be dependent upon the AME concentration, contact time, and temperature. No consistent effect of the virus concentration on the probability of plaque-forming unit inactivation could be determined. The antiviral effects of AME were partially antagonized by the presence of serum. Binding of AME to vesicular stomatitis virus was demonstrated by the comigration of drug and virus in linear sucrose gradients. AME represents a new class of antiviral agents with activity at concentrations relevant to therapeutics. Sterol components of the host cell membrane that become incorporated into the viral envelope are postulated as the site of reaction with AME.

Amphotericin B is a polyene macrolide antimicrobical agent that is currently the most effective agent available for the treatment of many systemic fungal infections in man. The biological properties of the polyenes result primarily from interaction with sterol components of the cell membrane (6). The major disadvantages of amphotericin B are its poor solubility in water, necessitating stabilization of suspensions with deoxycholate, and its numerous toxic side effects. In contrast, the methyl ester of amphotericin B (AME) is water soluble and less toxic and retains antifungal activity (3, 8). Recently, activity of AME against herpesviruses has been reported (11). We describe the binding of AME to vesicular stomatitis virus (VSV), the kinetics of the antiviral action, and the spectrum of activity against several classes of enveloped and unenveloped viruses.

MATERIALS AND METHODS

Reagents. Cells were grown in Eagle basal medium with 10% fetal bovine serum (FBS). Maintenance medium consisted of Eagle basal medium with 2% FBS. Gentamicin (10 μg/ml) was added to all media. Hanks balanced salt solution with 0.5% gelatin, but no serum, was used for the dilution of viruses and AME.

The hydrochloride of AME was kindly supplied, as a yellow, dry powder, by Carl P. Schaffner, The Waksman Institute of Microbiology, Rutgers University, New Brunswick, N.J. Tritiated AME had a specific activity of 1.2×10^6 dpm/mg. One-half-milliliter portions of a stock solution of AME in sterile distilled water (2 mg/ml) were frozen at −70°C; no turbidity or precipitation was observed. Antifungal potency was assayed in the laboratory of Paul D. Hoeprich, Section of Infectious Diseases, University of California at Davis School of Medicine. No loss of antifungal or antiviral potency was observed during 6 months.

Cells. Fibroblasts derived from human foreskins were serially propagated in Eagle basal medium with 10% FBS in 250-ml plastic flasks. Primary chicken embryo cells were prepared by mincing and trypsinization of 10-day-old embryos. Human embryo kidney cells were purchased from Microbiological Associates, Inc., Bethesda, Md.

Viruses. Sindbis virus and the Indiana strain of VSV were kindly supplied by R. S. Chang, Department of Medical Microbiology, University of California School of Medicine, Davis, Calif. Herpes simplex viruses type 1 strain F (HS-1) and type 2 strain G (HS-2) were obtained from the American Type Culture Collection. Virus stocks were grown in monolayers of chicken embryo cells or human foreskin fibroblasts and were stored at −70°C. Adenovirus type 4 and echovirus type 11, obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md., were grown in human embryo kidney cells.

Reaction of AME with virus. Dilutions of AME and virus were combined at 4°C in a final volume of 0.8 ml. After reacting under various conditions, 0.2 ml of dilutions of the drug-virus mixture were inoculated in duplicate onto monolayers of cells in 35-mm plastic petri dishes. To determine the effect of serum on the antiviral action of AME, dilutions of drug and serum were combined before the addition of virus.

Plaquing technique. Adsorption was allowed for 1 h with frequent redistribution of the inoculum. Overlay medium consisted of Eagle basal medium with 2%

FBS, gentamicin, neutral red (final dilution, 1:30,000), and 0.8% Ionagar. Agarose (0.6%) was substituted for Inonagar for plaquing herpes simplex viruses. Liquid overlay medium was maintained at 45°C, and 4 ml was added to each petri dish. The cell monolayers were incubated for 40 h, and plaques were counted.

Density gradient centrifugation. Tritiated AME and VSV were layered over linear 10 to 50% sucrose gradients and were centrifuged at 100,000 × *g* in a Beckman SW-40 swinging-bucket rotor for 2 h. Fractions were collected dropwise by puncturing the bottom of the tube. Optical density at 280 nm of a 1:10 dilution of each fraction in distilled water was determined in a Beckman model 24 spectrophotometer. The presence of tritium was detected by adding 0.1 ml of each fraction to 10 ml of Beckman Ready-Solv and counting in a Beckman LS-330 liquid scintillation system. The densities of the fractions were determined by weighing 50-μl volumes on an analytical balance.

RESULTS

Antiviral effects of amphotericins. A comparison of amphotericin B and AME on the inactivation of VSV is shown in Table 1. Amphotericin B, as Fungizone, was toxic to human foreskin fibroblasts at concentrations above 12.5 μg/ml; no antiviral effects were observed. In contrast, AME exhibited antiviral effects, without toxicity, at concentrations up to 200 μg/ml.

Virus concentration. The inactivation of VSV by AME was studied over a range of virus concentrations from 5.7×10^6 to 340 plaque-forming units (PFU)/ml (Table 2). Virus and AME were allowed to interact at 35°C for 60 min. No systematic effect of inoculum size could be demonstrated for AME concentrations of 0.32 to 100 μg/ml, indicating that the proportion of PFU inactivated depended only upon the concentration of AME.

Kinetics of inactivation. The extent of inactivation of VSV and Sindbis virus was found to be a function of both the concentration of AME and the duration of drug-virus contact (Fig. 1). Dilutions of AME and virus were made at 4°C. Drug-virus combinations were thoroughly mixed and were incubated at the indicated temperature for various periods. Each sample was then placed on ice, and monolayers of cells were inoculated. Adsorption was carried out at 22°C for 1 h, during which time further inactivation may have occurred. However, as will be shown subsequently, inactivation by AME occurs much less rapidly at 22 than at 37°C (which was used for Sindbis virus). A greater degree of inactivation with increasing drug contact time was also found for the inaactivation of HS-1 virus (data not shown). The three enveloped viruses studied represent members of the rhabdovirus, togavirus, and herpesvirus groups. In all cases, prolonged contact with AME resulted in increased inactivation.

For VSV, the probit of the percentages of PFU recovered showed a linear relationship to the logarithm of the product of AME concentration and exposure time (Fig. 2). This finding is consistent with the hypothesis that AME concentration and exposure time, over the range studied, have equal and additive effects on the probability of PFU inactivation (5).

Effect of temperature. The extents of inactivation of Sindbis and HS-1 viruses, at given concentrations of AME, were temperature dependent (Fig. 3). For Sindbis virus, a small but significant difference was seen between the inactivation at 4°C and that at 22°C. However, inactivation of both Sindbis and HS-1 viruses was greatly enhanced at 35°C. Low temperature did not inhibit the reaction completely, as shown by the inhibition of Sindbis virus at 4°C in the presence of AME at a concentration of 100 μg/ml. Similar temperature effects were found for the inactivation of VSV (data not shown).

Effect of serum on antiviral activity. The addition of FBS antagonized the antiviral action of AME on both VSV and herpes simplex virus (data not shown). Bovine serum albumin, in comparison with FBS, was less antagonistic (Fig.

TABLE 1. *Effect of amphotericin on PFU of VSV*

Drug	PFU[a] appearing at drug concn of (μg/ml):			
	200	50	12.5	0
Amphotericin B	Tox[b]	Tox	61, 65	56, 51
AME	0, 1	2, 5	9, 14	

[a] Plaque counts shown in duplicate.

[b] Tox, Cell lysis (observed at 40 h) after exposure of human foreskin fibroblast monolayers for 1 h to 0.2 ml of inoculum containing the indicated drug concentration; the agar overlay was added without removal of inoculum.

TABLE 2. *Effect of VSV concentration on percentage of PFU inactivated by AME*

PFU/ml exposed	PFU inactivated (%)[a] at AME concn of (μg/ml):					
	0.32	1.0	3.2	10	32	100
5.7×10^6		44.7	51.8	84.2	95.6	99.6
6.2×10^5		22.3	66.9	95.3	98.4	99.7
5.9×10^4		20.3	67.8	96.9	98.3	99.6
6.4×10^3		48	77.2	97.3	98.6	99.7
6,050	19.0	29.7	64.5	89.6	95.4	97.6
3,300	13.6	41.7	54.5	87.9	93.3	96.9
1,200	0	31.5	59.4	88.1	98.3	98.3
605	6.6	32.1	63.4	88.5	93.4	91.8
340	16.2	22.8	81.6	90.7	97.8	99.3

[a] Results expressed as the percentage of PFU inactivated: $100 \times \{1 - [(\text{PFU/ml recovered})/(\text{PFU/ml exposed})]\}$. Duplicate plaque counts were averaged at each AME concentration.

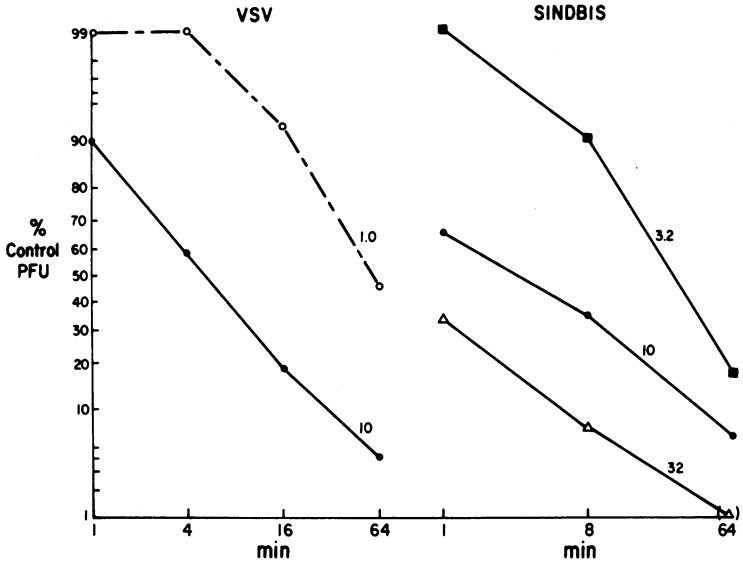

FIG. 1. *Effect of the contact time of AME with virus on the extent of inactivation. VSV was exposed to AME at 22°C; average plaque count on controls, 109. Sindbis virus was exposed to AME at 35°C; average plaque count on controls, 26; parentheses indicate "less than." AME concentrations (μg/ml): ○, 1.0; ■, 3.2; ●, 10; △, 32.*

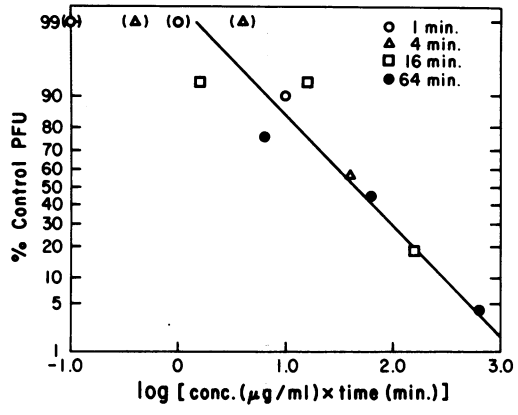

FIG. 2. *Combined effect of AME concentration and contact time on inactivation of VSV (data from Fig. 1).*

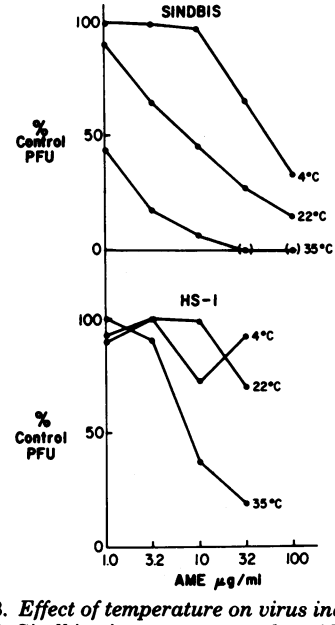

FIG. 3. *Effect of temperature on virus inactivation by AME. Sindbis virus was exposed to AME for 64 min; average plaque count on controls, 26; parentheses indicate "less than." HS-1 was exposed to AME for 30 min; average plaque count on controls, 21.*

4). The total protein concentration of the FBS was 4.0 g/liter, of which 2.2 g was albumin, which corresponds to 10% FBS. Bovine serum albumin was used at a concentration of 5.0 g/liter, that is, about the same amount of total protein and twice the amount of albumin as in FBS. As shown in Fig. 4, the albumin content alone can only partially account for the decreased activity of AME in serum.

Spectrum of activity. Having shown the necessity for standardizing the conditions of drug-virus contact, the susceptibilities of different viruses to inactivation by AME were compared. The concentrations expected to result in inactivation of 50% of the PFU of several different viruses are shown in Table 3. Virus stocks were grown in chicken embryo cells, and drug-

virus contact was carried out at 35°C for 60 min. The lack of activity of AME against echovirus type 11 and adenovirus type 4 is shown in Table 4.

Binding of AME to VSV. To demonstrate binding of virus and drug, tritiated AME at a concentration of 100 μg/ml was allowed to react with 10^9 PFU of VSV per ml for 1 h at 4°C. Under these conditions, 98.3% of the infectivity was lost. The mixture was then centrifuged on a linear 10 to 50% sucrose gradient to separate unbound drug and virus. An identical gradient was run with virus alone to establish the location of untreated virus and infectivity. As shown in Fig. 5, absorbance at 280 nm indicated the presence of VSV in the gradient in the presence or absence of AME (which also absorbs weakly at

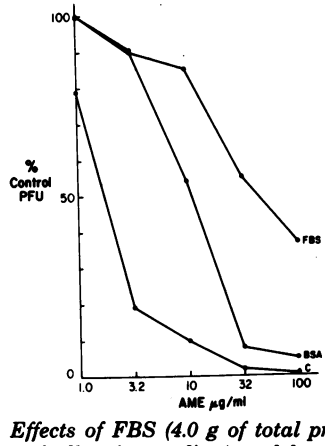

FIG. 4. *Effects of FBS (4.0 g of total protein per liter; 2.2 g of albumin per liter) and bovine serum albumin (BSA; 5.0 g/liter) on the inactivation of VSV by AME. c, Control curve in Hanks balanced salt solution with 0.5% gelatin. Average plaque count on controls, 99.5.*

TABLE 3. *Concentrations of AME inhibiting 50% of plaques*

Virus	AME concn (μg/ml)
VSV	1.3
Sindbis	3.8
HS-1	1.0
HS-2	1.0
Vaccinia	5.0
Newcastle disease	100

TABLE 4. *Effect of AME on echovirus type 11 and adenovirus type 4*

AME concn (μg/ml)	PFU	
	ECHO 11	Adeno 4
100	53	54.5
32	52.5	51.5
10	62.5	58.5
3.2	54.5	49.5
C_{35}[b]	50.5	59
C_4[b]	57	60.5

[a] Average of two plaque counts.
[b] Controls (no AME) maintained at 35 and 4°C.

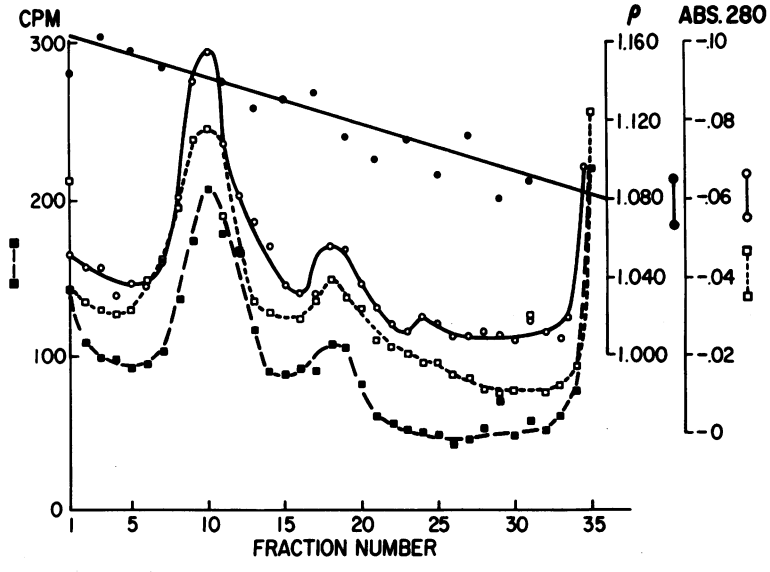

FIG. 5. *Location of AME-treated VSV in a 10 to 50% sucrose gradient centrifuged at 100,000 × g for 2 h. VSV exposed to AME: (○) absorbance (ABS) at 280 nm; (●) counts per minute. VSV control: (□) absorbance at 280 nm; (■) density (ρ).*

this wavelength). The virus migrated to a density of 1.14, as did the associated AME. There is also an association of AME with a second absorbance peak at a density of approximately 1.12, which probably represents binding of AME to defective particles of VSV. The association of AME-treated VSV with viral infectivity is shown in Fig. 6. AME-treated and untreated VSV preparations were centrifuged on identical gradients. These experiments show that AME is bound to VSV (29.6 and 26.6% [Fig. 5 and 6, respectively]) and that the resulting complex migrates to the same location as untreated virus without measurable changes in density under these conditions. An estimate of 1.7×10^7 molecules of AME per infectious unit can be calculated; this is a maximum estimate, because AME may also bind to noninfectious complete particles.

DISCUSSION

In comparison with the parent compound, AME is less toxic to animal cells in vitro and in vivo (8; Table 1). Amphotericin B (as Fungizone) is commonly used in cell culture media to suppress the growth of fungi, and under these conditions antiviral effects have not been observed. This may be due to the virtual lack of solubility of the drug in water at physiological pH. As a suspension stabilized with deoxycholate, amphotericin B is present in micelles, which may not be able to interact with particles in the size range of viruses. In contrast, AME, as a water-soluble compound, has both antifungal and antiviral properties at comparable concentrations.

Several lines of evidence point to a direct effect of AME on enveloped viruses. First, the comigration of AME and VSV in sucrose gradients indicates binding of drug to virus. Under the conditions of these experiments, changes in the density of drug-treated VSV were not observed. Second, inactivation of HS-1, VSV, and Sindbis virus was found to be a function of the temperature and duration of drug-virus contact before the inoculation of monolayers. Although amphotericin B has been shown to enhance interferon production (B. W. Booth and E. C. Borden, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S283, p. 251), the dependence of inactivation on the conditions of drug-virus contact is evidence against involvement of the interferon system in these results. Variation of the inoculum size over a wide range did not systematically affect the percentage of VSV inhibited by AME. These results differ in some respects from the findings of Stevens et al. (11), who studied the inactivation of several strains of herpes simplex by AME. They also observed a wide variation in the susceptibilities of different strains of herpesvirus, which may, in part, be responsible for the differences reported here.

The increased virus inactivation at 35°C is of interest in view of the demonstration that the polyene antibiotic-cholesterol interaction in *Acholeplasma laidlawii* occurred strongly at 0°C (4). However, subsequent biological effects, such as loss of potassium, occurred optimally at higher temperatures; i.e., there may be a temperature-dependent rearrangement of the initial polyene-sterol complexes, with ultimate disruption of membrane function. The antagonism of the antiviral effects of AME by serum could be only partially explained by the presence of albumin (Fig. 4). The effective AME concentra-

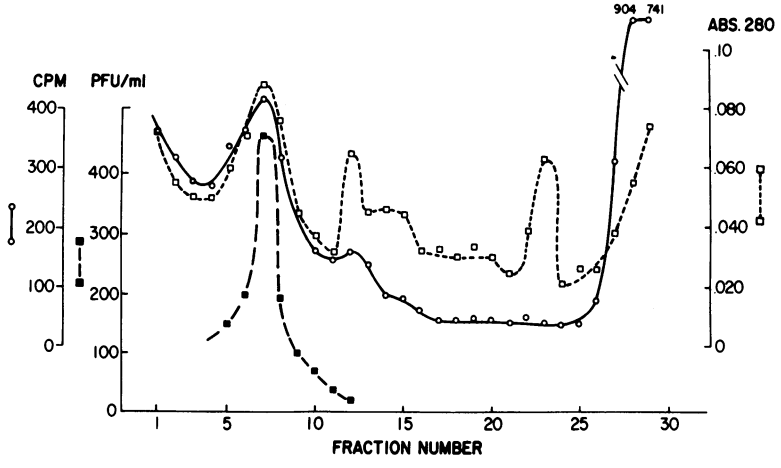

FIG. 6. *Migration of AME-treated VSV to the location of infectivity of untreated VSV in a 10 to 50% sucrose gradient centrifuged at 100,000 × g for 2 h. VSV exposed to AME:* (○) *counts per minute;* (□) *absorbance (ABS) at 280 nm. VSV control:* (■) *PFU/ml.*

tion may have been reduced by binding to sterols or other lipid components of serum (1). Alternatively, serum components may retard virus inactivation once interaction with AME has occurred.

Representative members of the rhabdovirus, togavirus, herpesvirus, and poxvirus groups were found to have an end point for 50% inactivation of PFU between 1 and 5 μg of AME per ml after 1 h of contact at 35°C. These AME concentrations are similar to the minimal inhibitory concentrations for susceptible fungi and are readily achievable, without limiting toxicity, in the sera of mice (9), monkeys (F. Jagdis, R. M. Lawrence, P. D. Hoeprich, and C. P. Schaffner, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 15th, Washington, D.C., Abstr. no. 215, 1975), and humans (7).

Although the viral envelope is derived from the host cell membrane, AME appears to be relatively more toxic to the virion than to the host cell. Certain differences between the virion and the host cell may be responsible for this finding. First, the host cell may be able to repair AME-induced membrane defects, whereas the virion cannot. Second, differences in physical properties, such as surface tension and internal pressure, may, in part, be responsible. Finally, the viral envelope is composed of a portion of the cell membrane that is altered from normal host cell composition and contains viral proteins. In this regard, experiments comparing the susceptibility of infected and uninfected cells to AME may be of interest. Differences in the composition of viral lipids depend not only on the virus and host cell but also upon the composition of the medium (2, 10). Viral lipid composition may be subject to manipulation by environmental factors, with the result that susceptibility to AME could be enhanced. The antiviral properties of AME represent a newly discovered activity of the polyene antibiotics. In addition to having antiviral effects at concentrations relevant to therapeutics, studies of the mechanism of action of AME on enveloped viruses may yield basic information on the properties and function of the viral envelope.

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