IN VITRO ANTI-MYCOPLASMAL ACTIVITY OF AMPHOTERICIN B METHYL ESTER

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The polyene macrolide antibiotic amphotericin B (AB) and its chemically modified derivative amphotericin B methyl ester (AME) were tested for *in vitro* activity against *Acholeplasma laidlawii, Spiroplasma citri* and *Mycoplasma gallisepticum.* Both polyene macrolide preparations demonstrated anti-mycoplasmal activity. However, AME was mycoplasmacidal toward all three strains of mycoplasma at levels which previous studies have indicated would be permissible for most cell culture systems, whereas the levels of AB required for similar activity would be physiologically intolerable for tissue culture cells. In addition, AME was 100 fold more active than AB toward *A. laidlawii,* 10 fold more active than AB toward *S. citri* and demonstrated equivalent activity as AB toward *M. gallisepticum.* The *in vitro* anti-mycoplasmal activity of AME and AB was directly correlated with polyene macrolide antibiotic levels and the number of treated mycoplasma.

Mycoplasmal contamination is a major problem in tissue culture methodology and virology. Different strains of mycoplasma can: (a) alter cell metabolism, morphology and growth; (b) induce chromosomal abnormalities; (c) modify nucleic acid synthesis; (d) alter cell surface antigenicity; (e) produce virus-like changes in cell culture, including altered cell morphology and cytopathogenic effects; and (f) alter viral growth in infected cultures.[1~3] Attempts to eliminate mycoplasma have usually depended on treatment with antibiotics, such as tetracycline[4] or kanamycin.[5] However, presently used antibiotics have only resulted in marginal success and offer the further disadvantages of inducing drug resistance[4,6] and cell toxicity.[7]

Recently, MECHLINSKI and SCHAFFNER[8] synthesized the water-soluble derivative of amphotericin B (AB), amphotericin B methyl ester (AME). AME has been shown to: (1) be less toxic than the deoxycholate complex of AB, Fungizone^R (FZ) (E. R. Squibb & Sons), when administered intravenously to mice[9,10] and dogs;[10] (2) retain the antifungal properties of AB;[9,11,12] (3) be less toxic than AB or FZ toward a variety of eukaryotic tissue culture cells;[13~15] (4) be selectively toxic toward certain human and mouse tumor cells in comparison with cells derived from normal tissue;[13] (5) induce a growth stimulatory effect in L-M and Vero cells;[14] and (6) inactivate herpes zoster and five strains of herpes simplex virus.[16] In the present study, we report on the *in vitro* mycoplasmacidal activity of AME toward three mycoplasmas**: *Acholeplasma laidlawii, Spiroplasma citri* and *Mycoplasma gallisepticum.*

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** The general term mycoplasmas is used to describe all three microorganisms although it is recognized that they belong to three different genera.

Materials and Methods

Polyene Macrolide Antibiotics: Amphotericin B (AB) was kindly provided by E. R. Squibb and Sons, New Brunswick, N.J. Amphotericin B methyl ester (AME) was synthesized in our laboratory according to procedures previously described.[8] Both antibiotics were dissolved in dimethyl sulfoxide (DMSO).

Test Organisms: The three mycoplasmas used in this study were *A. laidlawii, S. citri* and *M. gallisepticum.* A common contaminant of bovine serums and cell culture systems[2] is *A. laidlawii.* A culture of this organism was originally obtained from the American Type Culture Collection, ATCC (#23206). The organism, *S. citri,* is the causative agent of citrus "Stubborn" disease[17,18] which affects orange trees. The microorganism, originally isolated from orange seeds, was obtained from the ATCC (#27556). The fowl mycoplasma, *M. gallisepticum,* has been isolated from primary chick embryo cell cultures and is a respiratory contaminant in chickens[2]. This mycoplasmal strain was also obtained from the ATCC (#19610).

Growth of Test Organisms: The mycoplasmas, *S. citri* and *A. laidlawii,* were grown at 28°C in ATCC plant mycoplasmal medium 675 (ATCC #27563) containing 20% fetal bovine serum (FBS) and 10% yeast extract. The mycoplasma, *M. gallisepticum,* was grown at 37°C in the same medium.

In Vitro Anti-mycoplasmal Assay: Mycoplasmas in the log phase of growth were pelleted by centrifugation at 12,800 × *g* for 30 minutes at 4°C. The cells were resuspended in HANKS' balanced salt solution (HBSS), pelleted as above, and 5 ml of HBSS were added to the pellet. Following dilution of each mycoplasmal strain (depending on the initial inoculum), a 1-ml aliquot of diluted cell suspension was pipetted into sterile test tubes and polyene macrolide antibiotics added in varying concentrations. In these studies 5, 10, and 20 μg of AB and 10, 50, and 100 μg of AME were added in 10 μl aliquots of DMSO to the cell suspensions; the final concentration of DMSO was 1%. The tubes were then incubated for 1 hour at 27°C, and a 0.1-ml aliquot removed and spread on agar prepared with ATCC plant mycoplasma medium #675. Both *A. laidlawii* and *S. citri* were incubated at 27°C and *M. gallisepticum* at 37°C. Colonies were counted under a dissecting microscope.

Results

Amphotericin B (AB) and its chemically modified derivative amphotericin B methyl ester (AME) were both found to be mycoplasmacidal toward *A. laidlawii, S. citri* and *M. gallisepticum* (Tables 1~3). The antimycoplasmal activity exhibited by AB and AME was directly related to the concentration of treated organisms. At the lowest concentration of mycoplasma both AB and AME were very effective in preventing mycoplasmal growth.

Differences in the anti-mycoplasmal activity of AB and AME toward the same test organisms were found. AME was more active than AB in killing *A. laidlawii* (Table 1) and *S. citri* (Table 2).

Table 1. Effect of amphotericin B (AB) and amphotericin B methyl ester (AME) on the *in vitro* growth of *A. laidlawii*

Number of test organisms CFU/ml	% Reduction in CFU induced by AB (μg/ml)*			% Reduction in CFU induced by AME (μg/ml)*		
	5	10	20	10	50	100
1.1×10^4	0	0	0	99	99.5	100
1.1×10^3	0	0	0	100	100	100
1.1×10^2	100	100	100	100	100	100

* Percent reduction in colony forming units (CFU) expressed in relation to control and DMSO (1%) treated cells which represent 0% reduction in CFU.

Table 2. Effect of AB and AME on the *in vitro* growth of *S. citri*

Number of test organisms CFU/ml	% Reduction in CFU induced by AB (μg/ml)*			% Reduction in CFU induced by AME (μg/ml)*		
	5	10	20	10	50	100
1.6×10^4	0	0	0	100	100	100
1.6×10^3	100	100	100	100	100	100

* Same as Table 1.

Table 3. Effect of AB and AME on the *in vitro* growth of *M. gallisepticum*

Number of test organisms CFU/ml	% Reduction in CFU induced by AB (μg/ml)*			% Reduction in CFU induced by AME (μg/ml)*		
	5	10	20	10	50	100
4.7×10^4	0	0	0	0	0	0
4.7×10^3	67	65	93	72	88	98
4.7×10^2	80	100	100	80	100	100

* Same as Table 1.

In both cases, AME was mycoplasmacidal against high concentrations of organisms, even at low drug levels, whereas AB was ineffective in preventing growth at the higher mycoplasma concentrations. In contrast, AB and AME were similarly mycoplasmacidal toward *M. gallisepticum* (Table 3). The drugs are considered mycoplasmacidal since no growth on agar or in broth tubes was observed following exposure to the drugs.

Variations in the effect of AB and AME toward the three different test organisms were also apparent. AB and AME were most effective against *S. citri*. However, AB was least effective against *A. laidlawii*, whereas AME was least effective against *M. gallisepticum*.

Discussion

In vitro anti-mycoplasmal activity has been demonstrated with certain polyene macrolide antifungal antibiotics.[19~21] In the present study, we evaluated the effect of amphotericin B (AB) and amphotericin B methyl ester (AME) on the *in vitro* growth of three mycoplasmas: *A. laidlawii*, *S. citri* and *M. gallisepticum*. Although both polyene macrolide preparations were mycoplasmacidal, AME would be a preferable antibiotic for possible use in tissue culture systems because of its reduced toxicity in comparison with AB (Table 4). In addition, the most widely used antifungal agent in tissue culture systems, Fungizone^R as the deoxycholate complex of AB, has been found to exhibit toxicity[13~15,22] and alter membrane permeability[15,22~24] in a number of cell systems. In contrast, AME which demonstrates similar antifungal activity as AB or Fungizone[9~11] is not as deleterious to cell culture systems and can be used at higher levels than either AB or Fungizone (Table 4).[12~15]

Mode of action studies implicate the cell membrane sterols as the site of polyene macrolide antibiotic action.[19,21,25,26] Polyene macrolide-membrane interactions can result in permeability alterations and a leakage of essential metabolites from cells resulting in death.[15,21] The differential effect of AB and AME toward the three strains of mycoplasma studied may result from differences in the amount, type and/or molecular orientation of specific sterol(s) in the membranes of these organisms.

The mycoplasmacidal activity of both AB and AME was directly related to the mycoplasmal inoculum size. This concentration-dependent phenomenon may result from competitive binding be-

Table 4. Comparative toxicity of amphotericin B (AB) and amphotericin B methyl ester (AME) toward different cell lines

Cell type	Origin*	TCD_{50}** AB (μg/ml)	TCD_{50}** AME (μg/ml)	Reference
L-M	Mouse areolar and adipose tissue (N)	1 1	250 180	(13) (14)
Vero	African green monkey kidney (N)	1.5	250	(14)
TH-1	Terrapin heart (N)	3	225	(15)
PTK2	Marsupial kidney (N)	4	200	(15)
PMK-6	Swiss mouse kidney passage #6 (N)	4	200	(14)
GMK-8	Green monkey kidney passage #8 (N)	5	180	(14)
HEL-8	Human embryon lung passage #8 (N)	5	120	(13)
WISH	Human amnion (N)	5	60	(13)
RK 13	Rabbit kidney (N)	2.5	10	(15)
BHK 21	Syrian hamster kidney (N)	1	10	(15)
MA 160	Human benign prostatic hypertrophy (T)	1	10	(15)
HeLa	Human cervical epidermoid carcinoma (T)	4	10	(13)
KB	Human oral epidermoid carcinoma (T)	1	7	(12, 13)
RAG	Mouse renal adenocarcinoma (T)	1	5	(13)

* (N) refers to origin from normal tissue;
(T) refers to origin from tumor tissue.

** TCD_{50} indicates the concentration of polyene macrolide antibiotic, in μg/ml, which results in approximately a 50% reduction in viable cell number in comparison with control cultures within 3 or 5 days.

tween the specific polyene macrolide and the mycoplasmal membrane. A specific number of polyene macrolide molecules per cell may be necessary to induce toxicity. When an excess of organisms are used insufficient levels of polyene macrolide may bind to each cell and consequently not kill the organism.

In comparing the mycoplasmacidal activity of AME and AB, differences in the activity of these antibiotics toward *A. laidlawii* (Table 1) and *S. citri* (Table 2) were noted. AME was more mycoplasmacidal than AB toward both organisms. This difference in activity may be related to the different physico-chemical properties of AME and AB.[8] AME is water soluble and approaches molecular dispersion in aqueous solutions, whereas AB is highly water insoluble and forms large micelles in aqueous solution.[8] As compared to AB higher levels of AME may therefore be available in solution to bind to the mycoplasmal membrane and kill these organisms. It is also possible that the membrane damage caused by AME is more extensive. The differences in the structure of the membranes of *A. laidlawii* and *S. citri* may also affect the binding and toxic properties of AME and AB. We are presently investigating the mechanism involved in the differential toxicity of AME and AB toward the

same and different mycoplasmas and the applicability of AME as an anti-mycoplasmal agent for use in various tissue culture systems.

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