

Toxicity of Amphotericin B and Its Methyl Ester toward Normal and Tumor Cell Lines¹

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SUMMARY

The toxicity of amphotericin B, amphotericin B methyl ester (AME), and Fungizone toward three "normal" and three tumor-derived human and mouse cell lines was evaluated in monolayer culture. AME was less toxic than amphotericin B and Fungizone to all cell lines, but the sensitivity of the normal and tumor lines was different. The human (HEL-8 and WISH) and mouse (L-M) cells derived from normal tissue were more resistant to AME than the tumor-derived human (KB and HeLa) and mouse (RAG) cells as indicated by: (a) increased 24-hr survival, (b) increased 72-hr viability, and (c) growth rates at higher AME concentrations. In contrast, no pattern of differential sensitivity was observed with amphotericin B and Fungizone.

INTRODUCTION

AME,² a synthetic derivative of AB, the polyene macro-lide antibiotic, exhibits full antifungal activity of the parent compound (16). It has also been shown in our laboratory and elsewhere that, when administered i.v., AME is significantly less toxic to mice (1) and dogs (11) than the parent compound, administered as FZ³ the commercial sodium deoxycholate formulation.

This study was designed to compare the cytotoxicities of AB, AME, and FZ against various cell lines in a tissue culture system. Six cell lines were used: human normal HEL-8 and WISH, tumor-derived KB and HeLa, and mouse normal L-M and mouse tumor RAG. The use of these cell lines in our work was motivated by indications of differential sensitivity to polyene antibiotics between normal and tumor cell lines reported by other researchers (18, 19). Because the polyene antibiotics used in this study are closely related chemically and the chemical and physical differences are well understood, it was possible to evaluate

the relationship between antibiotic structure and cytotoxicity.

MATERIALS AND METHODS

Polyene Antibiotics. AB and FZ were kindly supplied by E. R. Squibb and Sons, New Brunswick, N. J. AME was synthesized in our laboratory according to procedures previously described (16).

Cell Lines and Media. The L-M mouse cell line, originally derived from normal s.c. areolar and adipose tissue (14, 15) and the HeLa human cell line, derived from a carcinoma of the cervix (8), were gifts from Dr. Robert J. Kuchler, Microbiology Department, Rutgers University, New Brunswick, N. J. The RAG cell line, originated from a cloned population of mouse renal adenocarcinoma (4, 13), was kindly supplied by Dr. Frank H. Ruddle, Department of Biology, Yale University, New Haven, Conn. HEL-8 cells (human embryonic lung passage 8) derived from normal fetal lung tissue, were purchased from Flow Laboratories, Rockville, Md. The WISH cell line, originally derived from normal human-amnion tissue (9), and the KB cell line, originated from a human epidermoid carcinoma of the mouth (7), were purchased from North American Biologicals, Inc., Rockville, Md. All 6 cell lines were grown at 36° in Dulbecco's modified Eagle's medium, supplemented with nonessential amino acids and 10% uninactivated fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.).

Methods. Cell-survival studies on the 6 cell lines in either the absence or presence of various concentrations of the polyene antibiotics were performed over a 24-hr test period. Growth rates and population-doubling times of control and treated cultures were determined between 24 and 72 hr. The concentration of polyene antibiotic resulting in an approximate 50% reduction in viable cell number, as opposed to final control value, was determined after a 72-hr test period for each antibiotic for each cell line. This value was designated the TCD₅₀. Cell survival, growth rate, and TCD₅₀ studies were performed, using a minimum of 3 replicate experiments. Equal numbers of cells (1 to 4 × 10⁵) in 1 ml of medium were inoculated into replicate Leighton tubes or 30- × 15-mm tissue culture plates, followed by a single administration of a 10-μl volume of appropriate concentrations of the polyene antibiotics to yield: 1 to 5 μg of AB per ml, 1 to 20 μg of FZ per ml, or 1 to 400 μg of AME per ml. AB and AME were dissolved in DMSO while

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² The abbreviations used are: AME, amphotericin B methyl ester; AB, amphotericin B; FZ, Fungizone; TCD₅₀, the concentration of polyene antibiotic, in μg/ml, that results in approximately a 50% reduction in 72-hr viable cell number, in comparison with control cultures; DMSO, dimethyl sulfoxide.

³ "Fungizone" is the trademark of E. R. Squibb & Sons, Inc.

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FZ was dissolved in sterile distilled water. Control cultures received no drug treatment or 10 μ l of DMSO. Ten counts of viable cells were performed on Day 0 and 20 (10/replicate sample) counts were performed on Days 1, 2, and 3, with the use of hemocytometer and the trypan blue dye-exclusion technique. The growth rate (R) was determined by:

$$R = [\log_2 (y_2/y_1)]/(t_2 - t_1)$$

where y_1 = 24-hr (t_1) viable cell number and y_2 = 72-hr (t_2) viable cell number. Population-doubling time in hr was computed as $1/R$.

RESULTS

AME was found to be generally less cytotoxic than AB and FZ to all 6 cell lines (Table 1). This decreased cytotoxicity is especially manifest when one compares the response of the 3 normal lines with the 3 antibiotic preparations. The normal lines were anywhere from 6- to 250-fold more sensitive to AB and FZ than to AME. For the tumor-derived lines, the range in sensitivities to the 3 antibiotic preparations was not nearly as pronounced as that observed for the normal lines.

While there was variation, the TCD_{50} concentrations of AB and FZ are similar for both the normal and tumor-derived cells. AME, on the other hand, was substantially more cytotoxic to those lines of tumor origin than to those derived from normal tissue. For the 3 normal lines, the TCD_{50} values for AME were 250 (L-M), 120 (HEL-8), and 60 μ g/ml (WISH). For the tumor-derived cell lines, the corresponding values for AME were 7 (KB), 10 (HeLa), and 5 μ g/ml (RAG).

The dose-response curves of the 6 cell lines to increasing levels of AME after 24 hr of treatment (Chart 1) and after 72 hr of treatment (Chart 2) show the clear-cut concentration dependence and the differential sensitivity between the

Table 1

Comparative toxicity of AME, AB, and FZ toward normal and tumor-derived human and mouse cell lines

Equal numbers of cells (1 to 4×10^5) in 1 ml medium were grown for 72 hr in the presence of AB, FZ, or AME. Control cultures received antibiotic diluent. Cell viability was determined by the trypan blue dye-exclusion technique, as described in "Materials and Methods." A comparison of the level of polyene antibiotic, resulting in a 50% reduction in cell viability, with those of control cultures after 72 hr was determined from 3 replicate studies.

Cell type	Av. population-doubling time ^a (hr)	Origin	TCD_{50} (μ g/ml)		
			AB	FZ	AME
L-M	21.5 \pm 1.2	Normal mouse	1	3	250
HEL-8	33.4 \pm 3.1	Normal human	5	20	120
WISH	27.7 \pm 2.5	Normal human	5	8	60
RAG	22.9 \pm 0.9	Tumor-derived, mouse	1	3	5
HeLa	23.6 \pm 2.4	Tumor-derived, human	4	7	10
KB	22.9 \pm 1.6	Tumor-derived, human	1	3	7

^a Computed for control cells over 48 hr time period (Day 1 to Day 3). Expressed as average of replicate cultures from 3 separate studies.

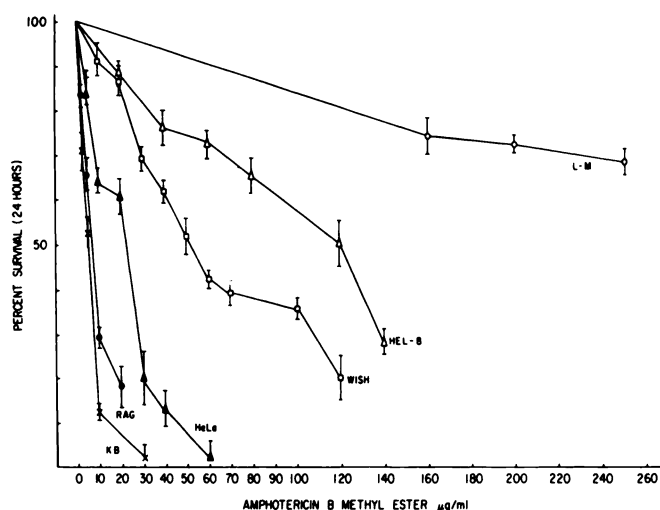


Chart 1. Survival (24 hr) of KB(\times), RAG(\bullet), HeLa(\blacktriangle), WISH(\square), HEL-8(\triangle), and L-M(\circ) cells as a function of AME concentration. Equal numbers of cells (1 to 4×10^5) in 1 ml of medium received 1 to 300 μ g of AME per ml. Control cultures received antibiotic diluent (DMSO). Cell viability was determined and survival expressed as percentage of control. Vertical bars, mean \pm S.D. from 3 replicate studies.

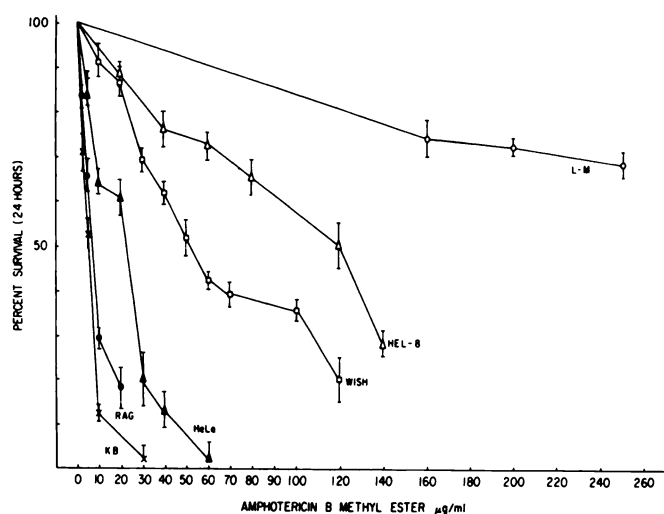


Chart 2. Effect of AME on 72-hr viability of KB(\times), RAG(\bullet), HeLa(\blacktriangle), WISH(\square), HEL-8(\triangle), and L-M(\circ) cells. Other conditions were as for Chart 1. Vertical bars, mean \pm S.D. from 3 replicate studies.

2 types of cells. Dose-response curves were also constructed for AB and FZ but, due to the similar response of the cell lines to these 2 drugs, no pattern of differential sensitivity was discernible.

Additional criteria used to substantiate a selective action of AME on tumor cells included a comparison of cell-growth rates and a comparison of the concentration of AME needed to ensure total cell death ($<1\%$ of control value) after 72 hr of treatment. In line with TCD_{50} values, low concentrations of AME (5 to 7 μ g/ml) reduced the growth rate of the tumor-derived cell lines, while the normal lines required substantially higher amounts (60 to 160 μ g/ml) to elicit the same response. The concentrations of AME required for total cell death after 72 hr of treatment reflected the same pattern of selective toxicity observed in

previous experiments. Populations of KB and RAG cells were rendered totally nonviable after treatment with 20 μg AME per ml, while 60 $\mu\text{g}/\text{ml}$ were required to completely kill the HeLa population. In contrast, for the same population, the WISH cells required 120 μg AME per ml, and the HEL-8 cells required 200 μg AME per ml. Moreover, the L-M line had surviving cells even after exposure to 500 μg AME per ml for 72 hr.

Daily spectrophotometric monitoring of the polyene antibiotics in the tissue culture systems demonstrated that more than 50% of the initial concentrations remained after the 3-day test period. Spectrophotometric changes in AB, FZ, and AME were found to parallel each other, although AME was slightly less stable. Greatest reductions in the levels of all 3 antibiotic preparations occurred between Days 1 and 3.

DISCUSSION

Mode of action studies demonstrate that the polyene macrolide antibiotics complex with cholesterol in mammalian cell membranes or with ergosterol in fungal cell membranes. While the possibility exists for interaction of the polyene antibiotics with sterol-containing components within the cell, the primary site of toxicity, for reasons of accessibility, is believed to lie at the cell surface. This interaction may result in pore formation and permeability changes within the membrane that can lead to cell death (2, 6, 10, 12, 17, 22, 24).

The extent of damage to the cell will depend, to a large degree, on the affinity of the polyene antibiotics to either the mammalian or fungal sterols and the concentration and distribution of the interacting compounds within the membrane. It has been shown (2, 6) that the interaction of polyene antibiotics with sterols is greatly dependent on the chemical and physicochemical nature of both substances. In a recently proposed molecular model for the polyene-sterol interaction within membranes (5), it has been suggested that not only must polyene be capable of interacting with membrane sterol but that polyene, sterol, and polyene-sterol complex must have sufficient mobility within the membrane to allow for a reaggregation into large complexes. Certainly, the composition and organization of the membrane with reference to sterols, other lipids, and even proteins will influence this mobility and therefore mediate what is observed as polyene toxicity.

In considering polyene-sterol complex formation, certain structural prerequisites must be kept in mind. On the sterol side, the 3 β -hydroxyl group must be free, and the molecule must be planar and in possession of a hydrophobic side chain at C-17 (5). Sterol esters, which are also present in cell membranes, complex poorly with polyene antibiotics. They may, however, affect complex formation by influencing the lateral translocation of interacting species. Differences in affinity to polyene antibiotics between cholesterol and ergosterol have been observed (25), and they may govern the important therapeutic factor in the treatment of fungal infections. In regard to structural characteristics of the polyene macrolides, it has been shown that the conjugated

system of double bonds in the closed macrolactone ring, as well as the presence of the free amino group, has an important bearing on the biological activity of these compounds (2, 16). In contrast, the free carboxyl group, at least in AB, is not critical for sterol binding and may be esterified. This derivative, AME, shows equivalent antifungal activity to the parent AB which would indicate a similar affinity for, and mobilization within, the fungal cell membrane. However, in model membrane studies involving cholesterol (2), while AB and AME showed equivalent ability in disrupting membrane permeability, the response of the membrane to the removal of these substances from the external reservoir after interaction was quite different. Membranes treated with AME returned much faster to a normal state than those treated with AB. This observation may indicate a difference in polyene antibiotic interaction kinetics with cholesterol within the membrane. Such an explanation considered in light of the differential ability of cells to repair membrane damage may account, in part, for the observed disparate toxicities of the drugs presented in this report.

A 2nd explanation, not necessarily completely disjointed from the 1st, may have to do with novel structural differences between parent compound and derivative. The zwitterionic structure, $\text{NH}_3^+ - \text{R} - \text{COO}^-$, present in the parent AB and FZ and not present in AME, restricts aqueous solubility. Unlike the parent, AME has a chemical character of a base and easily produces salts with acids that show good solubility in water. In the free-base form, the aqueous solubility of AME is also better than that of the parent compound.

We believe that this better solubility of AME in water in comparison with parent AB and also with FZ plays an important role in the lower toxicity. Although FZ dissolves in water to a greater extent than AB and may produce higher concentrations than AME as the free base (but not as the salts of AME), the solutions differ dramatically in the dispersion of molecules. Spectrophotometric studies reveal (21) that FZ forms large micelles in water, whereas AME approaches molecular dispersion. It is conceivable that this difference in micelle size for similar concentrations of FZ and AME may cause localized saturations of FZ, but not AME, in the vicinity of the cell membranes and, in the case of FZ, may result in lethal cell damage. The slightly lower toxicity of FZ *versus* AB demonstrated throughout these studies also seems to be indicative of their solubility properties. However, the participation of the free amino group of AME in specific interactions with the cell membrane cannot be excluded.

The basis for the apparent selective toxicity of AME toward neoplastic cells in culture must, at present, remain even more speculative. Changes in sterol composition and localization are possible in neoplastic membranes that interact with AME. Differences in surface membrane properties between normal and tumorous cells in regard to contact inhibition, surface antigens, and lectin agglutinability are well recognized and have been the subject of a recent review (20). There have also been reports as to altered sterol metabolism in some hepatomas (23) and leukemias (3), the

presence of which might influence sensitivity to the polyene antibiotics. The exact nature and extent of these changes is still under investigation.

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