Biochemical Pharmacology, Vol 26, pp 177–180 Pergamon Press, 1977 Printed in Great Britain.

ERGOSTEROL-CONFERRED SENSITIVITY TO AMPHOTERICIN B IN CULTURED L1210 MOUSE LEUKEMIA CELLS

FRED J. SCHIFFMAN, JOYCE M. FISHER and MARCO RABINOVITZ

Laboratory of Medicinal Chemistry and Biology, Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A.

(*Received* 15 *May* 1976; *accepted* 19 *July* 1976)

Abstract—The sensitivity of cellular membranes to disruption by amphotericin B (AMP-B) depends on the sterol content of such membranes. L1210 murine leukemia cells, grown in medium containing ergosterol and then briefly treated with AMP-B, were more sensitive to the antibiotic than control populations. Cell lysis and damage were assessed by estimation of cell number, trypan blue uptake and cell viability by cloning in soft nutrient-agar. Cells grown in ergosterol required less than half the AMP-B needed for equivalent damage to controls, and were reduced in number and viability by over 99.9 per cent at an AMP-B concentration which barely affected the reproductive capacity of control cell populations.

Amphotericin B (AMP-B) is a polyene antibiotic which creates aqueous pores in thin lipid membranes [1, 2] and whose activity depends upon the sterol content of membranes [3]. It has been used with equivocal success in the treatment of astrocytoma [4, 5]. Thin lipid membranes containing ergosterol are more sensitive to polyene antibiotics than those containing cholesterol [6]. Depending upon the liposomal system employed, ergosterol confers more or less AMP-B sensitivity than cholesterol [7, 8]. Ergosterol content is critical for membrane disruption by AMP-B in fungal [9], protozoal [10] and mycoplasmal [11] membranes.

The antibiotic, its methyl ester, or a water-soluble complex with desoxycholate, Fungizone (Squibb), as well as other polyenes have shown variable cytotoxic activity in cultures of normal or neoplastic cells [12–17]. Since the structure of mammalian cell membranes can be altered by incubation with sterols which are dissolved in growth medium [10], or contained in phospholipid vesicles [18], we have exposed L1210 murine leukemia cells in culture to ergosterol and examined the subsequent effects of AMP-B treatment.

EXPERIMENTAL

AMP-B was purchased from CalBiochem; ergosterol and cholesterol from the Sigma Chemical Co. The L1210 mouse leukemia cells were kindly provided by Dr. V. Bono of the National Cancer Institute and RPMI 1630 medium was supplied by the National Institutes of Health Media Unit. All media were supplemented with 20% fetal calf serum (Flow Laboratories), and gentamicin (Schering), 40 μg/ml. Cell number was estimated with a Coulter counter.

Cholesterol and ergosterol were freshly prepared in absolute ethanol to give an 8 mg/ml stock solution. Ethanol concentration in incubation mixtures did not exceed 0.5% (v/v). AMP-B was freshly prepared in dimethylsulfoxide (DMSO) to give a 20 mg/ml stock solution. The DMSO concentration in incubation mixtures did not exceed 0.1% (v/v).

Two sequential incubations were carried out at 37°. During the first incubation period, cells which had been in stationary phase for 5 hr were seeded at 2.5×10^4 to 1×10^5 cells/ml in 20 ml medium containing ethanol, cholesterol or ergosterol in Falcon flasks, 75 cm^2. Sterols were present as fine dispersions at levels of 5–40 μg/ml. Cells were harvested after 4–44 hr, centrifuged at 300 g for 5 min, and washed twice with fresh medium. During the second incubation period, the cells were seeded at 1×10^5 cells/ml in 10 ml medium containing 1 to 12.5 μg/ml of AMP-B in Falcon flasks, 25 cm^2. They were harvested 0–3 hr after treatment, centrifuged at 300 g for 5 min and washed twice with fresh medium.

After treatment with AMP-B, one or more of the following techniques was used to assess cell survival: (1) cell number determined immediately after incubation in medium containing AMP-B; (2) trypan blue exclusion (washed cells in 0.5 ml medium were mixed with 0.1 ml of 0.4% trypan blue in normal saline. After 5 min, 200 cells were counted and the number of cells excluding the stain was determined); (3) cell number determined after resuspension in fresh medium and 45 hr of additional incubation at 37°; and (4) colony number as demonstrated by clonal growth for 2 weeks at 37° in RPMI 1630 medium containing 0.1% Noble agar (DIFCO).

The procedure for clonal growth in soft agar was based upon a previously described technique [19]. Noble agar (DIFCO) was suspended in distilled water at a concentration of 40 mg/ml and autoclaved at 120° and 15–20 psi for 15 min. This concentrated agar solution was added to RPMI 1630 complete medium to give an agar concentration of 0.133%. Three ml of the nutrient-agar was dispensed into 12 × 75 mm Falcon tubes and allowed to equilibrate at 37°. Ten-fold dilutions (10^5–10^2 cells/ml) were prepared from control and drug-treated cell populations and 1 ml of the appropriate cell dilution was added to the tubes

containing 3 ml nutrient-agar. Three tubes were prepared for each dilution. The tubes were mixed by inversion, placed in an ice bath for 15 min and kept at room temperature for 45 min. Tubes containing 25–100 colonies were scored after 14 days of incubation at 37°. Cloning efficiency was 95–100 per cent.

RESULTS

Cells which had been grown in ergosterol were more sensitive to lysis during a 2-hr treatment with AMP-B than those which had been grown in cholesterol or the solvent alone, ethanol (Fig. 1A). The slight growth at the lower levels of AMP-B which occurred during this 2-hr period accounts for the values of greater than 100 per cent of cells resisting lysis shown in Fig. 1A. Further support that growth in ergosterol potentiated membrane damage by AMP-B is seen in Fig. 1B, where an estimate is given of the cells surviving lysis which could be stained with trypan blue. No cells were found adherent to vessel surfaces. Phase contrast microscopy of treated cells revealed many that were deformed and in various stages of disruption with much cellular debris.

Cells first grown in ergosterol, then exposed to AMP-B, washed and permitted to grow in the absence of AMP-B either in liquid medium (Fig. 2) or as colonies in soft nutrient-agar (Fig. 3) for extended time periods were markedly sensitive to the earlier action of the antibiotic. Ergosterol-grown cells exhibited greater than a three log kill (99.9 per cent

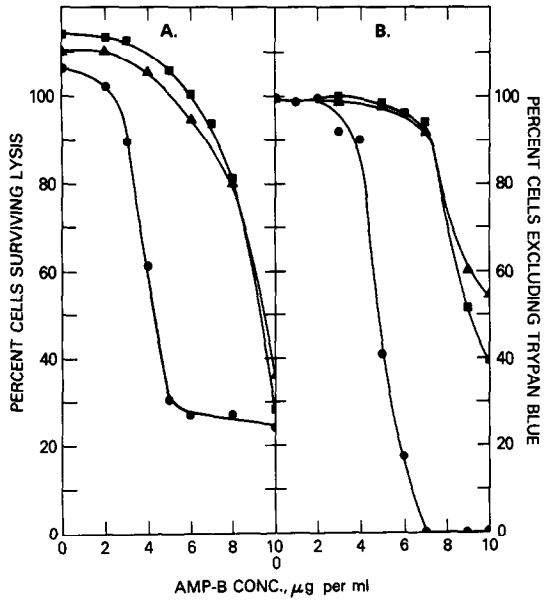

Fig. 1. Effect of growth in sterol on cell lysis and exclusion of trypan blue after a 2-hr incubation with AMP-B. L1210 cells were grown for 22 hr in medium containing the solvent ethanol, 0.5% v/v, or sterol, 40 μg/ml, and harvested in log phase. After washing twice with fresh medium, they were incubated for 2 hr in medium containing AMP-B. They were washed twice again, one aliquot was counted immediately (A) and another aliquot was stained with trypan blue (B). One hundred per cent equals 1×10^5 cells/ml. Key: (▲——▲) ethanol; (■——■) cholesterol; and (●——●) ergosterol.

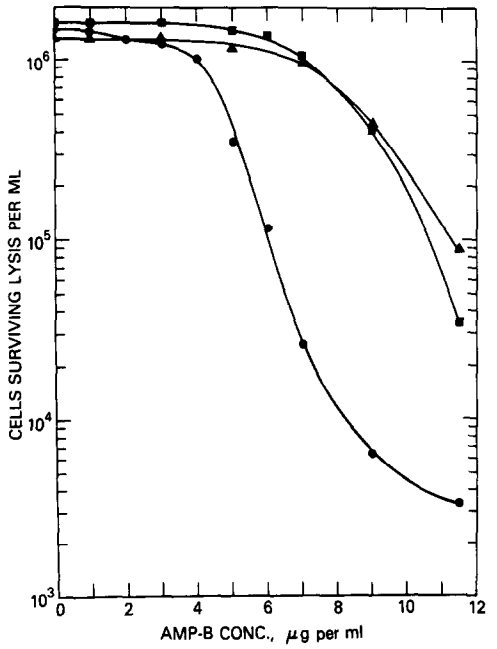

Fig. 2. Effect of growth in sterol and AMP-B treatment on lysis of L1210 cells during 45 hr of incubation in fresh medium. Cells were grown for 22 hr in medium containing the solvent ethanol, 0.5% v/v, or sterol, 40 μg/ml, and harvested in log phase. After washing twice with fresh medium, cells were incubated for 2 hr in medium containing AMP-B. After washing twice more in fresh medium, they were incubated for 45 hr in fresh medium and counted. Key: (▲——▲) ethanol; (■——■) cholesterol; and (●——●) ergosterol.

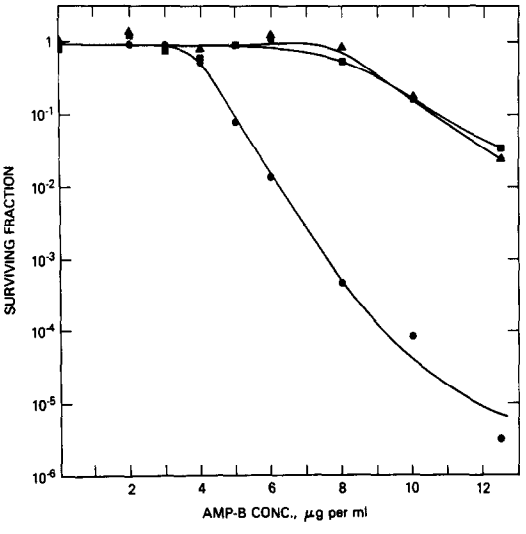

Fig. 3. Effect of growth in sterol and AMP-B treatment on clonal growth of L1210 cells. Cells were grown for 22 hr in medium containing the solvent ethanol, 0.5% v/v, or sterol, 40 μg/ml, and harvested in log phase. After washing twice with fresh medium they were incubated for 2 hr in medium containing AMP-B. After washing twice more with fresh medium, they were incubated for 2 weeks in nutrient-agar and individual colonies were counted. One represents 10^5 cells/ml present before incubation with AMP-B. Key: (▲—▲) ethanol; (■——■) cholesterol; and (●——●) ergosterol.

Table 1. Effect of growth in various levels of ergosterol on amphotericin-B cytotoxicity*

AMP-B concn (μg/ml)	Ergosterol level (μg/ml)				
	0	5	10	20	40
	Cells excluding trypan blue (%)				
0	98.6	100	97.2	97.7	100
2.5	99.3	99.5	96.2	99.4	98.8
5.0	94.6	97.2	96	50	16.1
10.0	54	51.8	45.1	0	0
	Colony-forming cells/ml				
0	98,600	91,500	95,700	90,300	104,700
2.5	95,000	97,300	90,000	96,000	112,300
5.0	112,000	98,700	82,600	56,300	21,300
10	6,800	686	337	217	11

* L1210 cells were grown for 22 hr in medium containing the solvent, ethanol (0.5%, v/v) or ergosterol and harvested in log phase. After washing twice with fresh medium, they were incubated for 2 hr in medium containing DMSO (0.1%, v/v) or AMP-B and then washed twice more. One aliquot of cells was stained with trypan blue and another was incubated for 2 weeks in soft nutrient-agar and individual colonies were counted.

mortality) at an AMP-B concentration of 8 μg/ml, which barely affected the reproductive capacity of control cell populations.

The cloning technique gives a more accurate assessment of cell viability than the other methods used, since its endpoint of visible colony formation estimates the reproductive ability of the cells. Cell number, determined immediately after AMP-B exposure or after 45 hr of additional growth, gives no indication whether some of the cells counted have sustained membrane damage, thus rendering them unable to reproduce. Trypan blue dye exclusion may, under certain conditions, underestimate the degree of cell kill, when compared with cloning (Table 1, 10 μg AMP-B level).

Increasing either the level of ergosterol in which the cells were grown or the time of growth in ergosterol conferred greater sensitivity to AMP-B (Table

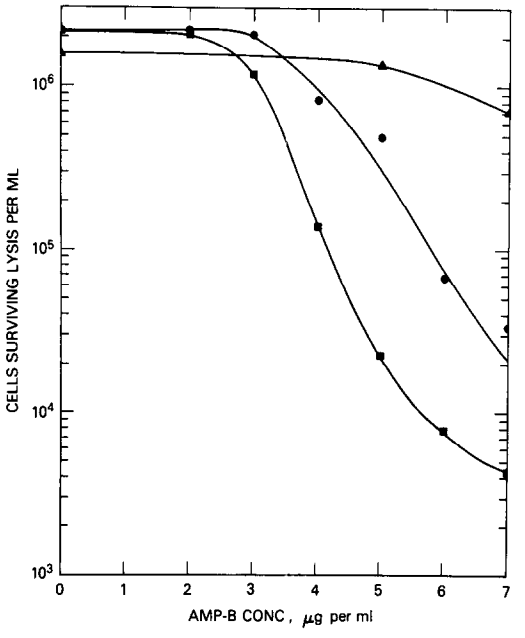

Fig. 4. Effect of time of growth in ergosterol on AMP-B-induced cell lysis during 45 hr of incubation in fresh medium. L1210 cells were grown for various time periods in medium containing ergosterol, 40 μg/ml, and harvested in log phase. After washing twice with fresh medium, cells were incubated for 2 hr in medium containing AMP-B. After washing twice more in fresh medium, they were incubated for 45 hr in fresh medium and counted. Key: (▲——▲) 4 hr; (●——●) 22 hr; and (■——■) 44 hr.

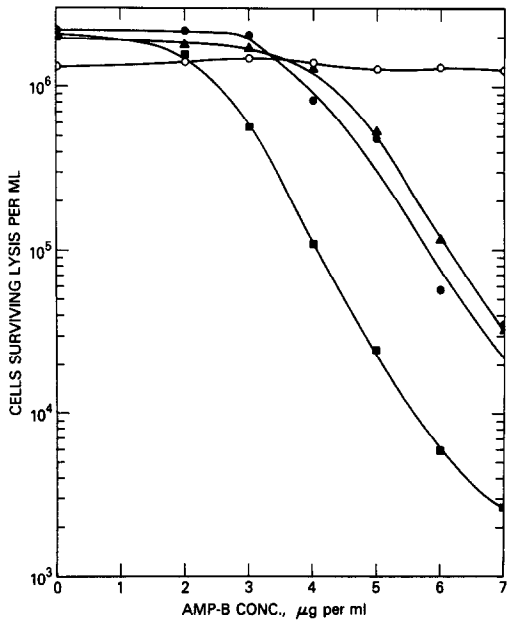

Fig. 5. Effect of duration of AMP-B treatment on cell lysis during 45 hr of incubation in fresh medium. L1210 cells were grown for 22 hr in medium containing ergosterol, 40 μg/ml, and harvested in log phase. After washing twice with fresh medium, cells were incubated for various time periods in medium containing AMP-B. After washing twice more in fresh medium, they were incubated for 45 hr in fresh medium and counted. Key: (○——○) 15 min; (▲——▲) 1 hr; (●——●) 2 hr; and (■——■) 3 hr.

1, Fig. 4). Also, increasing either the AMP-B concentration or the time cells were incubated with the antibiotic caused a corresponding increase in cell lysis (Table 1, Fig. 5).

DISCUSSION

The precise nature of the membrane–sterol association, and the characteristics of the membrane–sterol–polyene complex, have not yet been fully delineated. It has been suggested that a rigid or more ordered membrane configuration is a prerequisite for the activity of AMP-B and that the role of the sterol may be to confer increased membrane rigidity [8, 20]. Such rigidity is also dependent on the number and distribution of unsaturated sites in the acyl chains of the membrane phospholipids [21] as well as in the sterol nucleus and side chain [22, 23].

We have demonstrated that AMP-B sensitivity can be conferred by first treating cells with the phytosterol, ergosterol. The mechanism of action of ergosterol might involve the production of a stiff membrane substrate upon which AMP-B may subsequently act. It has been shown previously that ergosterol is capable of increasing membrane order [22]. A unique interaction between AMP-B and ergosterol (or the ergosterol–membrane complex) may also account for the effect of ergosterol. Indeed, according to one theory, advanced to explain the mechanism of AMP-B activity, sterol is an integral part of the AMP-B sterol–membrane pore [1,2]. Other workers have shown that amphotericin has higher affinity for ergosterol than for cholesterol, both in solution and natural membranes [24].

Data from our experiments do not indicate whether ergosterol, relative to cholesterol (1) was more efficiently transferred from growth medium to cell membrane, (2) more effectively prepared the membrane for association with AMP-B, or (3) interacted with the AMP-B in a manner to cause cell membrane damage. Differences in sterol transport systems, membrane–sterol concentrations and structural distinctions between ergosterol and cholesterol could account for any of these possibilities.

Shinitzky and Inbar [18] have reported that plasma membranes of malignant murine and human lymphocytes were cholesterol poor and had one-half the microviscosity of corresponding normal forms. Both the cholesterol level and microviscosity of the surface membrane lipid layer of mouse lymphoma cells could be raised to levels characteristic of normal lymphocytes by incubation with lecithin-cholesterol liposomes. Such treatment significantly reduced ascites tumor development when the cells were inoculated into mice [25]. The membrane modification could be effected by a 2-hr incubation at 4°, and was the result of an equilibrium between cholesterol of cell membranes and liposomes, rather than cholesterol incorporation during cell growth and membrane formation. The optimization of ergosterol incorporation into cell membranes under conditions approximating those *in vivo* and the possibility that such incorporation may be more dependent on membrane structure and sterol availability than on membrane growth and development require further study.

Of possible major significance has been the observation that plant sterols, previously thought to be confined to the plant kingdom, have been found in certain human malignant tumors [26, 27]. The sterols presumably originated from dietary sources, and were absorbed from the blood stream.

Because some tumor cell membranes are sterol deficient, or have affinity for certain forms of sterol, it may be possible to preferentially sequester ergosterol in membranes of such tumor cells after oral or parenteral administration and thereby confer AMP-B sensitivity. Even if this two-step schedule were not cytocidal, by selectively altering such tumor cell membranes it may be possible to potentiate the activity of certain chemotherapeutic agents [28], which when used alone are poorly transported into the cell.

REFERENCES

1. T. E. Andreoli, *Ann. N.Y. Acad. Sci.* **235,** 448 (1974).
2. R. W. Holz, *Ann. N.Y. Acad. Sci.* **235,** 469 (1974).
3. J. M. T. Hamilton-Miller, *Bact. Rev.* **37,** 166 (1973).
4. M. J. Rubissow, *Lancet* **1,** 565 (1970).
5. F. Colardyn and G. Verdonk, *Lancet* **2,** 974 (1973).
6. A. Cass, A. Finkelstein and V. Krespi, *J. gen. Physiol.* **56,** 100 (1970).
7. R. Bittman, W. C. Chen and O. R. Anderson, *Biochemistry* **13,** 1364 (1974).
8. C. C. HsuChen and D. S. Feingold, *Antimicrob. Agents Chemother.* **4,** 309 (1973).
9. R. A. Woods, M. Bard, I. E. Jackson and D. J. Drutz, *J. Infect. Dis* **129,** 53 (1974).
10. R. L. Conner, F. B. Mallory, J. R. Landrey, K. A. Ferguson, E. S. Kaneshiro and E. Ray, *Biochem. biophys. Res. Commun.* **44,** 995 (1971).
11. C. C. HsuChen and D. S. Feingold, *Antimicrob. Agents Chemother.* **4,** 316 (1973).
12. D. Perlman, N. A. Giuffre and S. A. Brindle, *Proc. Soc. exp. Biol. Med.* **106,** 880 (1961).
13. G. A. Tomkins and J. Ferguson, *Aust. J. exp. Biol. med. Sci.* **43,** 743 (1965).
14. B. Mondovi, R. Strom, A. Agro, P. Caiafa, P. DeSole, A. Bozzi, G. Rotilio and A. R. Fanelli, *Cancer Res.* **31,** 505 (1971).
15. P. Amati and C. Lago, *Nature, Lond.* **247,** 466 (1974).
16. G. Medoff, D. Schlessinger and G. S. Kobayashi, *Cancer Res.* **34,** 823 (1974).
17. P. B. Fisher, N. I. Goldstein, D. P. Bonner, W. Mechlinski, V. Bryson and C. P. Schaffner, *Cancer Res.* **35,** 1996 (1975).
18. M. Shinitzky and M. Inbar, *J. molec. Biol.* **85,** 603 (1974).
19. M. Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **17,** 753 (1968).
20. C. C. HsuChen and D. S. Feingold, *Biochem. biophys. Res. Commun.* **51,** 972 (1973).
21. R. A. Demel, W. S. M. Guerts Van Kessel and L. L. M. Van Deenen, *Biochim. biophys. Acta* **266,** 26 (1972).
22. K. W. Butler, I. C. P. Smith and H. Schneider, *Biochim. biophys. Acta* **219,** 514 (1970).
23. D. Ghosh and J. Tinoco, *Biochim. biophys. Acta* **266,** 41 (1972).
24. D. B. Archer and E. F. Gale, *J. gen. Microbiol.* **90,** 187 (1976).
25. M. Inbar and M. Shinitzky, *Proc. natn. Acad. Sci. U.S.A.* **71,** 2128 (1974).
26. G. S. Gordan, M. E. Fitzpatrick and W. P. Lubich, *Trans. Ass. Am. Physns* **80,** 183 (1967).
27. E. A. Gay, G. T. Malcom and M. F. Beeler, *Metabolism* **18,** 646 (1969).
28. G. Medoff, D. S. Schlessinger and G. S. Kobayashi, *J. natn. Cancer Inst.* **50,** 1047 (1973).