Biochemical Pharmacology, Vol. 35, No. 22, pp. 4110–4113, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.0
Pergamon Journals Ltd

Anti-viral activity of amphotericin B methyl ester: inhibition of HTLV-III replication in cell culture

(*Received* 14 *March* 1986; *accepted* 12 *June* 1986)

Recent reports [1, 2] of the involvement of a human T lymphotropic retrovirus, designated HTLV-III/LAV, in the etiology of acquired immunodeficiency syndrome (AIDS) have stimulated an active search for effective chemotherapeutic antiviral drugs.

Amphotericin B methyl ester (AME), a water-soluble derivative of amphotericin B [3] which is a polyene macrolide antifungal antibiotic, is known to be active against a variety of lipid-enveloped RNA and DNA viruses*, several oncogenic retroviruses† and different strains of herpes viruses [4]. This antiviral property of AME prompted us to examine its activity against HTLV/III/LAV, which is also a lipid-enveloped retrovirus.

AME, being a member of the polyene macrolide group of antifungal antibiotics, inherently interacts with sterols and binds to them irreversibly [5]. The binding of AME to cholesterol in the membrane of cells causes changes in cell permeability and function, and its binding to sterols of lipid-enveloped viruses causes loss of their infectivity.

It was not unexpected, therefore, that AME, in tests for activity in the infection of H9 cells by HTLV-III in culture, proved active in inhibiting cell death due to virus infection, and inhibiting the expression of virus protein antigens p24 and p15. Amphotericin B was also active, whereas candicidin, which is another polyene macrolide that binds to sterols, was too cytotoxic at levels needed to inhibit HTLV-III replication.

Materials and methods

Test drugs. Amphotericin B and candicidin were obtained from Xechem, Inc., Melrose Park, IL. They were dissolved in 0.01% dimethylformamide (DMF) to prepare solutions ranging in concentration from 0.1 to 100 μg/ml. The amphotericin B methyl ester (AME), as the ascorbic acid salt, was prepared as described by Mechlinksi and Schaffner [6]. AME salts are water-soluble, in contrast to amphotericin B, so that AME ascorbate was added to cultures of virus-infected H9 cells as an aqueous solution.

HTLV-III virus. Stock virus was prepared from HTLV-III infected H9 cells in culture. Cells were harvested and the virus was isolated from the conditioned medium by twice banding on sucrose gradients. The virus band sedimenting at a density of 1.16 to 1.19 g/ml was collected, pelleted by centrifugation, and resuspended by TNE (10 mM Tris·HCl, pH 7.5, 0.1 M NaCl, 0.001 M EDTA) at 2×10^{11} virus particles/ml.

H9 cells. This is a transformed human lymphocyte cell line that is permissive for HTLV-III [1]. It is maintained by serial passage in culture, using medium containing RPMI-1640 supplemented with 20% fetal bovine serum, 2% glutamine and 1% gentamicin. For test purposes, cells were harvested in log phase, and 5×10^5 cells were added per ml of test culture.

HTLV-III infection of H9 cells. In some experiments the cells were pretreated with polybrene (2 μg/ml) to facilitate their infection by virus. Polybrene (1,5-dimethyl-1,5 diazaundecamethylene polymethobromide) was purchased from Sigma. In all cases infection was initiated by adding 5×10^8 virus particles/ml culture. Cultures were incubated for 4 days at 37° in a humidified atmosphere containing 5% CO_2. Afterwards, the cells in each culture were dispersed by trituration, and an aliquot was analyzed for cell viability using the trypan blue dye exclusion technique. Cells that excluded due were considered viable. The remaining portion of each culture was used to determine virus reverse transcriptase activity and expression of HTLV-III antigens p24 and p15 [7].

Immunofluorescence assays. After removal of the aliquot of cell culture for determination of ceil number and viability, 5 ml of the remaining culture were centrifuged to separate the cells. The supernatant fraction was saved to determine reverse transcriptase activity. The cells were resuspended in phosphate-buffered saline (PBS) to about 1×10^6/ml, and 10–50 μl was added to each well of an 8-well toxoplasmosis slide for immunofluorescence studies with HTLV-III p15 and p24, as described [7]. Briefly, the slides were air-dried for 1 hr, fixed in acetone–methanol (1:1, v/v) for 15 min at room temperature, and then air dried for 10 min. The fixed cells were pretreated with 10% normal goat serum for 30–45 min at 37° in a moist chamber, to preclude the non-specific adsorption of FITC-goat anti-mouse IgG to be added later, after which the slides were rinsed three to four times in PBS, 15 min each time. Mouse monoclonal antibodies to HTLV-III p24 and p15 were added to duplicate wells, incubated at room temperature in a humid chamber for 1 hr, and washed with PBS containing 0.25% Triton X-100 for 2 hr. The cells were then exposed to fluorescein (FITC)-labeled goat anti-mouse IgG (Cappel Laboratories) for 1 hr, and washed overnight with PBS containing 0.25% Triton X-100. The slides were mounted with 50% glycerol, and cell fluorescence was observed under a Zeiss fluorescence microscope. The number of fluorescence-positive cells in several fields were counted, and reported as percent of total cells in those fields.

Reverse transcriptase activity. The supernatant fraction of 4-day cultures of HTLV-III-infected H9 cells was assayed for reverse transcriptase activity, as described previously [7]. It was measured in terms of the incorporation of [^{3}H]deoxythymidine triphosphate into trichloroacetic acid-insoluble polynucleotide whose synthesis was directed by reverse transcriptase using $(dT)_{15}:(A)_n$ as a template primer.

Results and discussion

AME ascorbate, the water-soluble methyl ester salt of amphotericin B, was tested for activity against HTLV-III by adding it to cultures of H9 cells infected with this virus. It was added in amounts so that its final concentration in culture varied from 10^{-2} to 10 μg/ml, and it was added simultaneously with virus or it was pre-incubated with virus for 45 min. Figure 1 shows the results of a representative experiment in which AME and virus had been preincubated. AME was not cytotoxic even at the highest concentration, and it showed significant anti-HTLV-III activity beginning at 1 μg/ml. It apparently inhibited the expression of the virus protein antigens p24 and p15, as measured by immunofluorescence with monoclonal antibodies specific for these antigens. The percent of cells expressing HTLV-III antigens, after 4 days of culture in the presence of AME-treated virus, was less than 20% of control. Both antigens,

* J. E. Dahlberg, R. W. Simpson, D. P. Bonner, W. Mechlinski and C. P. Schaffner, 13th Interscience Conf. Antimicrob. Ag. Chemother., Sept. 19–21, 1973, p. 190.

† M. A. Chirigos, J. W. Pearson, G. J. Spahn, D. P. Bonner, W. Mechlinski and C. P. Schaffner, 13th Interscience Cong. Antimcrob. Ag. Chemother., Sept. 19–21, 1973, p. 250

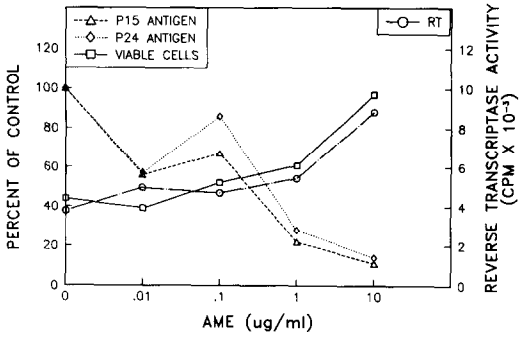

Fig. 1. Effect of AME on HTLV-III replication in H9 cells. AME was preincubated with HTLV-III prior to its addition to cultures of H9 cells that had been treated with polybrene. Control cultures with only virus and no drug, and without virus or drug, were included. After 4 days in culture, surviving viable cells were harvested, counted (□), and examined for expression of virus protein antigens p24 (◇) and p15 (△). The supernatant fractions were assayed for reverse transcriptase activity (○). The results (average of duplicates) are expressed as percent of control values. The percent of control virus-infected cells that expressed p24 and p15 antigens was 7 and 9 respectively. The number of viable cells recovered from cultures of non-infected H9 cells was 1.27×10^6.

p15 and p24, were apparently inhibited to the same degree. In contrast, reverse transcriptase activity increased with concentration of AME. This increase in reverse transcriptase activity correlated instead with the increase in the number of viable cells recovered from these cultures. These results suggest that AME prevents replication of the virus, reflected by the apparent inhibition of the expression of p24 and p15 virus antigens, but it does not prevent penetration of the target cells by the virus nor does it inhibit its reverse transcriptase in viable cells. Protection of H9 cells by AME against the cytopathic effect of HTLV-III (44% survival of cells in virus-infected cultures without drug) resulted in increased survival of cells (97% survival with AME at 10 μg/ml) with reverse transcriptase being expressed. A possible interpretation of the reduction in fluorescence-positive cells is that the expression of virus antigens is not inhibited, but the antigens are inhibited by AME from binding to the monoclonal antibodies. This interpretation is unlikely in view of the pronounced effect of AME in protecting target H9 cells against the cytopathic action of HTLV-III.

Surviving cells from this first set of cultures (depicted in Fig. 1) were washed free of any extracellular virus and AME, and recultured for an additional 4 days, without any added drug, to assess the long-term effect of virus and drug on the cells in terms of their capacity to proliferate, and to express virus protein antigens and reverse transcriptase. The results are shown in Fig. 2. Only the virus-infected cells treated with 10 μg/ml of AME, that showed no cytopathic effect of the virus after the first culture period of 4 days, continued to proliferate when recultured. Equally important, there was little, if any, evidence of virus replication. Expression of virus antigens, low at the start (13% of control), decreased further (2% of control), and the initial high level of reverse transcriptase activity during the first culture period was essentially reduced to zero. This latter finding is considered significant. It was noted earlier that the expression of reverse transcriptase correlates with cell survival (cf. Fig. 1). It would be expected to increase in virus-infected viable cells if virus continues to replicate, but to decrease to background level in the absence of viral

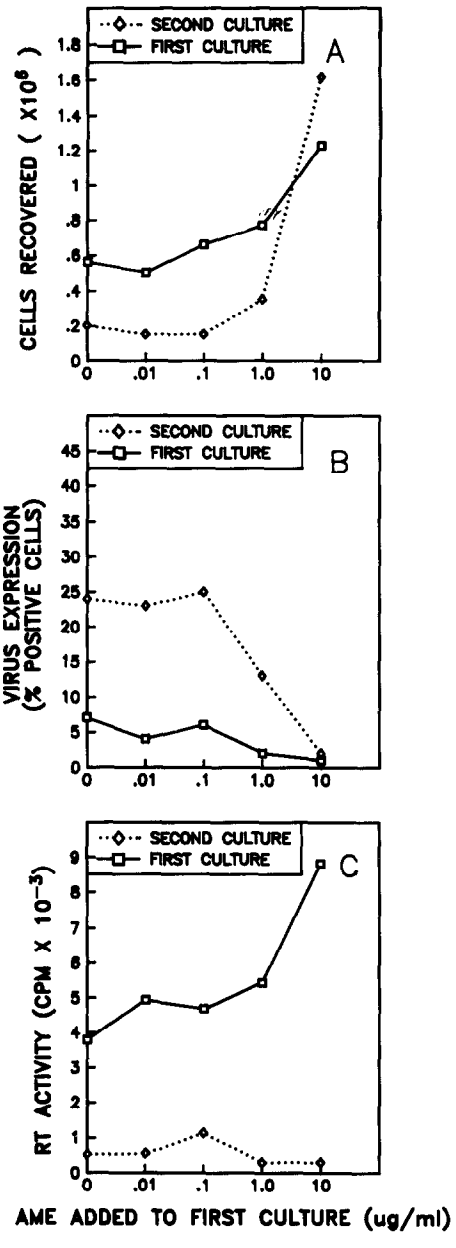

Fig. 2. Long-term effect of AME on HTLV-III-infected H9 cells in culture. AME was preincubated with HTLV-III for 45 min prior to its addition to cultures of H9 cells that had been treated with polybrene. Control cultures were inoculated with virus alone. After 4 days in culture, surviving viable cells were counted in aliquots taken from each culture, as well as the percent of fluorescence-positive cells and reverse transcriptase activity. The remaining surviving cells from each culture were washed free of any extracellular AME and virus present in the first culture, and were recultured for 4 days. Again the surviving cells were counted (A) and examined for virus protein antigens (B) and reverse transcriptase activity (C). The results (average of duplicates) of the first culture are denoted by □, and ◇ for the second culture. Both p24 and p15 antigens were assessed; only the results for p24 are shown, since the expression of p15 was essentially the same. The percent of control virus-infected cells that expressed virus protein antigens were: first set of cultures, 7 and 9% for p24 and p15 respectively; second set of cultures, 24 and 30%.

replication. The actual results (cf. Fig. 2) support this prediction, and they provide additional evidence that AME inhibits replication of HTLV-III in H9 cells.

The action of these drugs is due generally to their binding to sterols in cell membranes, and at high concentration they cause changes in membrane permeability and disruption. We used sheep red blood cells to assess the cytotoxicity of AME and amphotericin B because disruption of their membrane can be quantitated by the release of hemoglobin into the fluid phase, measured spectrophotometrically. Sheep red blood cells were incubated with AME and amphotericin B (0.01 to 100 μg/ml) at room temperature for 1 hr. Neither drug caused lysis of the red blood cells at 10 μg/ml, the concentration at which they are active in inhibiting the replication of HTLV-III in cultures of H9 cells. AME was not cytotoxic even at 100 μg/ml, whereas amphotericin B caused about 50% of the red blood cells to lyse (data not shown).

Cytotoxicity of the drugs was also determined directly against H9 cells used in this study. Drugs were added to cultures of H9 cells in log phase, varying their concentration and the time of culture. Cultures were set up in quadruplicate. At the end of the culture, cells were harvested and the number of viable cells (impermeable to trypan blue) was determined. The results are shown in Fig. 3. Neither drug was cytotoxic at 25 μg/ml, in excess of the concentration (1–10 μg/ml) needed to protect H9 cells against infection by HTLV-III.

It is evident from these results that AME, at a non-cytotoxic dose, can protect target cells against the cytopathic effect of HTLV-III. This protection is associated with its apparent inhibition of virus expression. This is not surprising since AME is known to act directly against herpes virus, which is an enveloped virus with lipid membrane that contains cholesterol to which AME binds [4]. HTLV-III is also an enveloped virus and has been shown to be inactivated by AL721 which works by extracting cholesterol from the virus envelope [8]. Hence, HTLV-III should also be sensitive to AME. The inactivation of HTLV-III is probably dependent on the ratio of AME concentration to virus particles. At the concentration of AME used in our experiments, it would seem that the antiviral activity of AME may also be due in part to its action on the target cells whose membrane also contains sterols to which AME can bind. This is consistent with the effect of polyene macrolides on cells of the immune system [9]. In particular, they modulate the activity of lymphocytes. In addition, the anti-viral activity of AME was essentially the same whether or not it was preincubated with HTLV-III (data not shown), providing further support of the notion that AME can protect cells against HTLV-III by its direct action on the cells as well as its action on the virus. In antiherpetic studies [4] employing monolayer cultures of HeLa cells, preincubation of the viruses with AME revealed a time and concentration dependency for loss of viral infectivity. Preincubation of the cells with the drug followed by washing also resulted in a significant resistance to viral challenge.

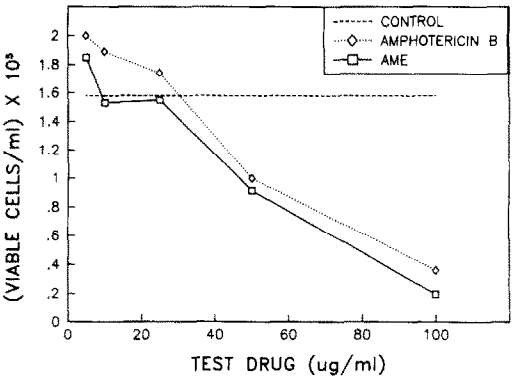

Fig. 3. Cytotoxicity of amphotericin B and AME against H9 cells in culture. Drugs were added to cultures of H9 cells in log phase, varying their concentration, and the time of culture (24 to 72 hr). Shown are the results for 72 hr. Cultures were set up in quadruplicate for each concentration, including controls without drug. Cells, harvested at the end of the culture, were pooled into two groups of two cultures for each set of four cultures. Two aliquots of each pool were examined for viable cells. Each point is the average of four determinations.

AME was compared with amphotericin B, its parent compound, for anti-HTLV-III activity. They were essentially equal in protecting against cell death, and in their inhibition of expression of virus protein antigens (Table 1). The advantages of AME are that it is a water-soluble compound, and is relatively non-cytotoxic against normal cells compared with amphotericin B [10]. Against transformed dividing cells they are essentially equal (cf. Fig. 3).

Table 1. Comparison of polyene macrolide antibiotics for anti-HTLV-III activity

Test drug* (μg/ml)	Cell survival (% control)	Virus protein antigens (% control)	
		p24	p15
Control (no drug, no virus)	100		
Control (virus only)	57	100	100
AME (10)	80	57	28
AME (1)	48	57	43
Amphotericin B (10)	72	28	7
Amphotericin B (1)	51	28	14
Amphotericin B (0.1)	57	71	57
Amphotericin B (0.01)	44	71	100
Candicidin (10)	13	43	57
Candicidin (1)	10	114	128
Candicidin (0.1)	45	86	86
Candicidin (0.01)	64	100	86

* Added to cultures of H9 cells (without polybrene) simultaneously with HTLV-III virus, no precincubation. After 4 days in culture, surviving viable cells were harvested, counted, and examined for expression of virus proteins p24 and p15. The number of viable cells recovered from control cultures was 2.76×10^6. The percent of fluorescent-positive cells in control virus-infected cells was 7 for both p24 and p15 antigens. Values represent averages of duplicate cultures.

Candicidin, like amphotericin B, is a polyene macrolide antifungal antibiotic [11] that binds sterols. However, it is more cytotoxic. This is evident in its action against virus-infected H9 cells (Table 1). At noncytotoxic doses it did not protect cells against HTLV-III infection.

AME has been tested several times, and it has consistently been effective in inhibiting the replication of HTLV-III in H9 cells. This effect of AME is therefore reproducible and, equally important, it is consistent with its demonstrated activity against other retroviruses, and lipid-enveloped viruses.

Binding of sterols by AME and amphotericin B may well be the key mechanism in their protection of target cells against HTLV-III infection, but clearly not all polyene macrolides that share this property may be therapeutically useful. Amphotericin B and its derivative, AME, are significantly effective in blocking HTLV-III infection and replication in T cells, and hence they deserve further evaluation in the potential treatment of patients with AIDS or AIDS-related complex.

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