Antiviral Research, 11 (1989) 119–126
Elsevier

AVR 00342

Inhibition of HIV replication by liposomal encapsulated amphotericin B

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(Received 19 August 1988; accepted 17 January 1989)

Summary

This report shows the potential of using a liposomal encapsulated preparation of amphotericin B (a polyene macrolide antibiotic) for the in vitro inhibition of HIV. There was no significant difference between the effective doses of the free form of drug when compared to the liposomal encapsulated preparation in inhibiting the growth of HIV. Virus expression was suppressed at a concentration of 5–10 μg/ml of the drugs. The liposomal preparation showed greatly reduced cytotoxicity in experiments using cultures of murine leukocytes. These results show the potential usefulness of liposomal encapsulated drugs in the treatment of patients with AIDS or AIDS related complex.

Amphotericin B; Inhibition of HIV replication; Liposomal encapsulated drug

Introduction

The involvement of human immunodeficiency virus (HIV-1) as the etiologic agent of acquired immunodeficiency syndrome (AIDS) (Barré-Sinoussi et al., 1983; Gallo et al., 1984; Sarin and Gallo, 1986; Gallo, 1987) has provided an opportunity for the development of anti-viral drugs for the treatment of the disease (Sarin, 1988).

The polyene macrolide antibiotic amphotericin B has been shown to inhibit cell death and virus expression in cultures of T cells infected with HIV-1 (Schaffner et

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al., 1986). Amphotericin B binds to cholesterol in the cell membrane and lipid enveloped viruses changing their permeability and function (Bolard, 1986), and probably inhibits HIV replication by binding to cholesterol present in the HIV-envelope, which has a high cholesterol/phospholipid ratio (Aloia et al., 1988; Crews et al., 1988).

Although the levels of drug used in vitro are below cytotoxic levels, amphotericin B is known to cause severe nephrotoxicity upon prolonged use during antifungal chemotherapy (Butler, 1966). Due to the problems associated with toxicity in humans, new formulations are being developed for improved efficacy in treatment (Lopez-Berestein et al., 1983). Recent animal studies indicate that liposomal encapsulation of drugs results in reduced toxicity compared to the free drug (Lopez-Berestein, personal communication). We have compared the inhibitory activity of amphotericin B with and without liposomal encapsulation against the AIDS virus, and the potentiation of the in vitro response of primed spleen cells to antigen.

Materials and Methods

Drugs

Amphotericin B was obtained from Squibb (Princeton, N.J.) as the commercial preparation Fungizone. The liposome encapsulated amphotericin B was supplied by Dr. George Strauss, Rutgers University and Dr. Lopez-Berestein, M.D. Anderson Hospital. Liposomal encapsulation of amphotericin B was carried out as described (Lopez-Berestein et al., 1983). Briefly, the drugs in methanol and the phospholipids (dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) dissolved in chloroform (DMPC:DMPG, 7:3)) were mixed in a ratio of 1:10 and the organic solvents were evaporated (Lopez-Berenstein et al., 1983). Both compounds were diluted using sterile water to give final concentrations in culture of 0.5 to 50 μg/ml.

Virus

HIV-1 (HTLV-III/B) was prepared from HIV infected H9 cells in culture. Cells were harvested and the virus isolated from the culture medium by banding on sucrose gradients. The virus band sediments with a density of 1.16 to 1.19 g/ml, and was collected by pelleting. The pellet was resuspended in 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

These cells are a transformed line of human lymphocytes permissive for HIV-1. The cultures were maintained using RPMI supplemented with 20% fetal bovine serum, 2% glutamine and 1% gentamicin. Cells were harvested in log phase of growth for testing and seeded at 5×10^5 cells/test culture.

Culture conditions

HIV-1 at 5×10^8 particles/ml was added to the test cultures (5×10^5 cells/ml) simultaneously with compounds to be tested. The cultures were incubated for 4 days at 37°C in an atmosphere containing 5% CO_2. Cells were then harvested and assayed for virus growth as described below.

Immunofluorescence assays

The cells were collected by centrifugation, washed and resuspended in 0.01 M phosphate buffered saline (PBS), to approximately 1×10^6 cells/ml. An aliquot was used for determining cell viability by the exclusion of trypan blue dye. The remaining cells were added to wells on a toxoplasmosis slide, allowed to dry for 1 h and fixed in acetone methanol (1:1, v/v) for 15 minutes, as described (Sarin et al., 1985; Schaffner et al., 1986). After fixation, the slides were pretreated with 10% normal goat serum at room temperature for 30 minutes. The slides were rinsed four times with PBS, 15 min each wash. Mouse monoclonal antibody to HIV-1 p24 antigen was added to each slide and incubated for 30 min at 37°C, in a humidified chamber. The cells were then rinsed with PBS, and washed 4 times as above. The cells were then stained with FITC labeled goat anti-mouse IgG by incubating for 30 minutes as above. The slides were rinsed with PBS, washed 4 times and given a final wash overnight with fresh PBS. The slides were counterstained with 0.02% Evans blue for 1 min followed by rinsing with distilled water. After air-drying the slides were mounted with 50% glycerol, and fluorescence measurements were done with a Zeiss fluorescence microscope. The results are expressed as the percent of fluorescent positive cells derived from the average of several microscopic fields.

Reverse transcriptase activity

The supernatant fraction of the test cultures was assayed for reverse transcriptase activity as described previously (Sarin et al., 1985; Sarin and Gallo, 1986). This was measured by the incorporation of ^{3}H-deoxythymidine triphosphate into trichloroacetic acid-insoluble DNA using $(dT)_{15}:(A)_{n'}$ as a template primer.

Antibody induction assay

C57BL/6 mice were primed with 1×10^7 sheep red blood cells (SRBCs) ip 10 days prior to the experiment. The mice were sacrificed, spleens removed and prepared as a homogeneous cell population using a tissue homogenizer. Cultures were set up with 1×10^7 primed spleen cells, 1×10^5 SRBCs and 0.1 ml of the test compound. The cultures were incubated for 6 days in Click's medium (Click et al., 1972) under the same conditions as the H9 cultures. After incubation the supernatant fluids were collected by centrifugation and assayed for the presence of antibody to sheep red blood cells by the complement dependent immune hemolysis assay.

Complement dependent immune hemolysis

Various dilutions (1:10, 1:100, 1:1000) of the culture supernatants were added to 1×10^8 SRBC's for 30 minutes at room temperature. The cells were collected

by centrifugation, washed with veronal buffer, and mixed with an excess of complement (Gibco) determined by hemolysin titration. The suspensions were incubated for 1 h in a 37°C water bath. After incubation the supernatants were collected by centrifugation and read spectrophotometrically at 541 nm for the presence of hemoglobin from the lysed SRBCs. This direct test measured the amount of IgM in the cultures. To measure IgG, total antibody was measured by an indirect technique using rabbit anti-mouse IgG. The antibody was added to the suspensions before the addition of complement to facilitate complement binding. The value obtained from the indirect technique is subtracted from the total antibody and the result is the amount of IgG in the culture. The unit of antibody is defined as the amount of antibody which can sensitize 50% of the SRBCs lysed in the presence of complement.

Results and Discussion

A comparison of the inhibitory activity of free and liposomal encapsulated amphotericin B on the HIV-1 infection of H9 cells is shown in Figs. 1 and 2. Both drugs showed optimum inhibition on HIV-1 replication at a dose of 5 and 10 μg/ml, as seen by the inhibition of reverse transcriptase activity and the expression of HIV-1 p24 on infected cells. Significant HIV-1 inhibitory activity was also observed at a concentration of 1 μg/ml with amphotericin B, whereas the liposome preparation was less effective at this low dose showing reduction in virus markers only as low as 2.5 μg/ml. The ED_{50} of amphotericin B was approximately 1 μg/ml while the liposomal preparation of amphotericin B has an ED_{50} of 2.5 μg/ml. The cryoprotective effect of both drugs is shown in Fig. 3. These experiments show that both preparations are almost equally non-toxic and that the effective dose of each of these compounds is well below their toxic levels, consistent with the earlier results for amphotericin B in H9 cultures (Schaffner et al., 1986).

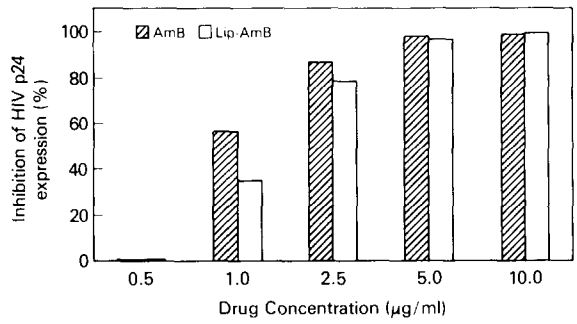

Fig. 1. Inhibition of HIV expression in infected cells by amphotericin B and liposomal encapsulated amphotericin B. HIV (HTLV-III/B) infected H9 target cells were incubated in the presence or absence of drugs for 4 days. The expression of HIV p24 antigen was measured by immunofluorescence with monoclonal antibody to p24. In H9 target cells without drugs 25% of the cells expressed viral p24 antigen.

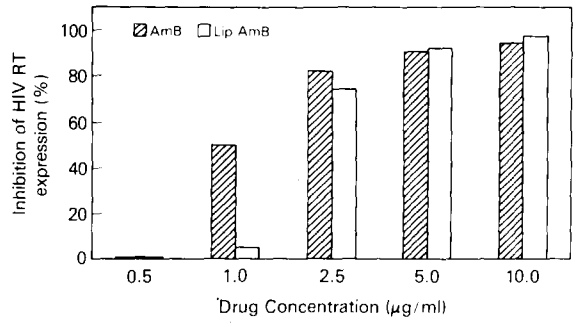

Fig. 2. Inhibition of reverse transcriptase by amphotericin B and liposomal encapsulated amphotericin B. The culture supernatants were collected from cultures of HIV-infected H9 cells after 4 days of incubation and assayed for the presence of reverse transcriptase as described in the text.

Comparison of the effects of amphotericin B and the liposomal encapsulated amphotericin B at a concentration of 50 μg/ml for thirty minutes on cultures of freshly prepared murine leukocytes (Fig. 4), showed virtually no cytotoxicity with the liposome preparation, whereas amphotericin B at this concentration killed almost 97% of the cells. The human plasma bound drug, like the liposomal preparation, was also not toxic to the murine leukocytes. It may be that the protein bound drug is released slowly, and hence the cytotoxic concentration is not reached under the test conditions. The toxicity of each compound is also measured using uninfected cultures with drug alone, and these results show no cytotoxicity of either drug at all levels tested for anti-viral activity, control cultures had 1.5×10^6 cells/ml after 4 days, while amphotericin B had 1.4×10^5 at 10 μg/ml and the liposomal preparation 1.55×10^6 at 10 μg/ml concentration.

Polyenes have been shown to potentiate the immune response to various antigens (Schaffner et al., 1984). To compare the activity of liposome encapsulated amphotericin B to the free drug on the production of IgG antibody, the two drugs were added to cultures of primed spleen cells. The liposomal preparation is much

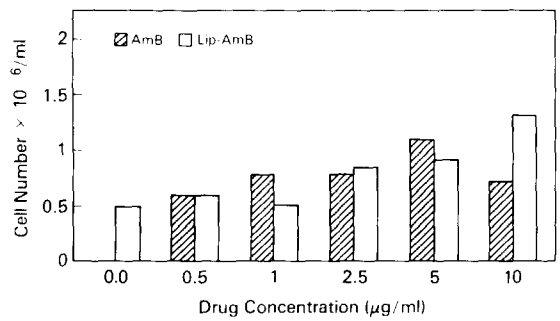

Fig. 3. Cytotoxicity of amphotericin B and liposomal encapsulated amphotericin B in H9 cells. H9 target cells uninfected or infected with HIV were incubated in the presence of drugs for 4 days. Cell viability was determined by the exclusion of trypan blue dye.

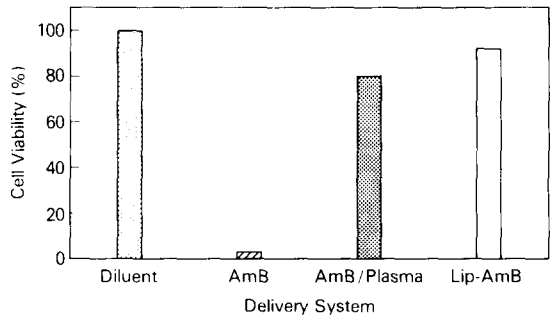

Fig. 4. Effect of delivery systems on the cytotoxicity of amphotericin B. Murine leukocytes were incubated for 30 min in the presence of 50 μg/ml amphotericin B, liposomal encapsulated amphotericin B, or amphotericin B adsorbed with 50% human plasma. Cell viability was determined by the exclusion of trypan blue dye.

more effective as an enhancer of immune activity, at 0.1 μg/ml showing a 50% increase in the amount of IgG antibody produced, whereas the free drug has no effect at this concentration. With increasing concentrations both drugs show similar activity. At 1.0 μg/ml there is a 28% increase in antibody production, and at the highest level tested, 10 μg/ml, both drugs show a suppressive effect (Fig. 5).

The present results clearly show that the liposomal preparation of amphotericin B has similar anti-HIV activity to the free drug and at non-cytotoxic levels both drugs can protect cell cultures from the cytopathic effect of HIV. The liposomal encapsulated amphotericin B is less toxic and has increased immuno-enhancing properties which may prove to be useful in the treatment of patients with AIDS. It will be of interest to examine the effect of liposomal encapsulation on nephrotoxicity. These studies show that liposomal encapsulated amphotericin B is very effective in inhibiting HIV-1 replication and due to its lowered cell toxicity may be more useful than the free amphotericin B in the treatment of patients with AIDS, AIDS related complex (ARC), lymphadenopathy syndrome (LAS) and

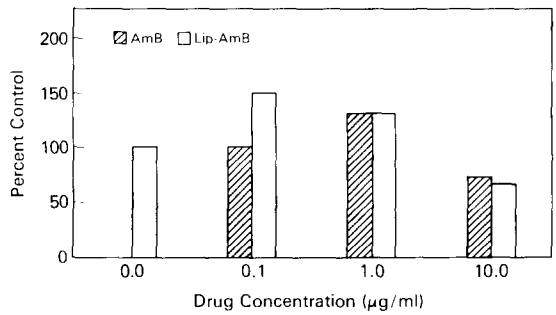

Fig. 5. The immuno-enhancing activity of amphotericin B and liposomal encapsulated amphotericin B. Murine splenocytes, primed with sheep red blood cells (SRBCs) in vivo were incubated for 6 days in the presence of SRBCs and drugs. The culture supernatants were collected and assayed for antibody to SRBCs by measurement of hemoglobin released due to the complement dependent lysis of SRBCs.

seropositive asymptomatic individuals, either alone or in combination with other drugs and/or immune modulators.

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